

Handling stress may confound murine gut microbiota studies

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There is accumulating evidence of interactions between human milk composition, particularly sugars (human milk oligosaccharides or HMO), the gut microbiota of human infants, and behavioral effects. Some HMO secreted in human milk is unable to be endogenously digested by the human infant but are able to be metabolized by certain species of gut microbiota, including *Bifidobacterium longum* subsp. *infantis* (*B. infantis*), a species sensitive to host stress (Bailey and Coe 2004). Exposure to gut bacteria like *B. infantis* during critical neurodevelopment windows in early life appears to have behavioral consequences; however, environmental, physical, and social stress during this period can also have behavioral and microbial consequences. While rodent models are a useful method for determining causal relationships between HMO, gut microbiota, and behavior, murine studies of gut microbiota usually employ oral gavage, a technique both stressful and potentially injurious to the mouse. Our aim was to develop a less-invasive technique for HMO administration to remove the potential confound of gavage stress.

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ABSTRACT:

There is accumulating evidence of interactions between human milk composition, particularly sugars (human milk oligosaccharides or HMO), the gut microbiota of human infants, and behavioral effects. Some HMO secreted in human milk is unable to be endogenously digested by the human infant but are able to be metabolized by certain species of gut microbiota, including *Bifidobacterium longum* subsp. *infantis* (*B. infantis*), a species sensitive to host stress (Bailey and Coe 2004). Exposure to gut bacteria like *B. infantis* during critical neurodevelopment windows in early life appears to have behavioral consequences; however, environmental, physical, and social stress during this period can also have behavioral and microbial consequences. While rodent models are a useful method for determining causal relationships between HMO, gut microbiota, and behavior, murine studies of gut microbiota usually employ oral gavage, a technique both stressful and potentially injurious to the mouse. Our aim was to develop a less-invasive technique for HMO administration to remove the potential confound of gavage stress.

This study was designed to test two methods, active and passive, of solution administration to mice and the effects on their gut microbiome. We provided increasing doses of fructooligosaccharide (FOS) solution or deionized, distilled water to neonatal C57/BL6 mice housed in a specific-pathogen free facility. Gastrointestinal (GI) tracts were collected from 41 pups, five dams, and six sires. GI tracts were collected at post-natal day (PND) 0, 7, 14, and 21. Seven fecal pellets from unhandled pups and two pellets from unhandled dams were also

collected. Qualitative real-time polymerase chain reaction (qRT-PCR) was used to quantify and compare the amount of *Bifidobacterium*, *Bacteroides*, Bacteroidetes, and Firmicutes in feces collected from GI tracts and fecal pellets collected from non-handled mouse pups. Our results demonstrate significant differences in fecal microbiota between handled and non-handled mouse pups. Thus, even handling for dosing without gavage may induce enough stress to alter the murine gut microbiota profile. To adequately assess the true effects of HMO on gut microbiota, synbiotic (a combination of the HMO and the beneficial bacteria of interest) administration should be considered.

INTRODUCTION:

The gut microbiota has major physiological and potentially biopsychological implications for human health (Lyte 2010, Walter and Ley 2011, Oh et al. 2010, Allen-Blevins et al. 2015). The collection of hundreds of bacterial species in the human gut exerts strong influences on immune function, nutrition, and neurodevelopment through maintaining gut barrier function, fermenting dietary fiber to short-chain fatty acids, and producing neurotransmitters (Dinan et al. 2015, Grenham et al. 2011). Processes such as programming the immune system likely begin with microbial exposure at birth and perturbations in the gut microbiota early in development have been implicated in chronic disease, including psychological conditions (Backhed et al 2015, Douglas-Escobar et al. 2013, Rook and Lowry 2013).

Recent research in mice suggests early gut microbiota affects neurodevelopment and behavior (Borre et al. 2014, Sudo et al. 2004, Diaz Heijtz et al. 2011). Mice reared without microbiota ("germ-free") exhibit increased corticosterone response to restraint stress and reduced expression levels of brain-derived neurotrophic factor in the hippocampus (Sudo et al. 2004). The increased corticosterone response is partially reversed with exposure to *Bifidobacterium longum* subsp. *infantis*, a species dominating the human infant gut (Sudo et al. 2004, Yatsuenko

et al.) Notably, colonization with *B. infantis* only partly normalizes the corticosterone response in 6 week old mice, but not 14 week old mice (Sudo et al, 2004). Slightly contrary to this research, germ-free mice showed reduced anxiety behavior in light-dark and elevated maze plus tests (Diaz Heijtz et al 2011). The differences in stress response may be due to differences in the types of stressors. Regardless, Diaz Heijtz and colleagues also demonstrated only early life colonization of germ-free mice, not colonization in mature mice, could normalize the behavior of germ-free mice (Diaz Heijtz et al 2011). These studies suggest critical neurodevelopmental windows exist in early life during which gut microbiota are crucial to shaping behavior (Borre et al. 2014, Allen-Blevins et al. 2015).

Evolutionary Context

If early life gut microbiota are critical for normal neurodevelopment, mothers necessarily play an essential role in programming neurodevelopment through transmitting and supporting the microbiota (Backhed et al 2015, Allen-Blevins et al 2015, Sela and Mills 2010). Initial microbial colonization is vertically transmitted from mother to offspring during delivery (Backhed et al 2015, Dominguez-Bello et al. 2010, Mueller et al 2015; Hinde and Lewis 2015). The newly colonized infant gut is then exposed to mother's milk, which in humans contains glycans such as the human milk oligosaccharides (HMO) that are not digested by the infant (Marcobal and Sonnenburg 2012, Sela and Mills 2010). While the infant does not possess endogenous enzymes to cleave HMO, certain species of gut microbiota can metabolize HMO, including *B. infantis* (Sela and Mills 2010, Sela et al. 2008, Sela et al. 2011, Sela et al. 2012). *B. infantis* is capable of metabolizing HMO as a sole carbon source, and *B. infantis*, *Bifidobacterium longum* and *Bifidobacterium breve* affect stress and anxiety behaviors (Yatsuenko et al. 2012, Sudo et al. 2004, Savignac et al. 2014, Sela and Mills 2010, Sela et al. 2008). *Bifidobacterium* can also produce γ -aminobutyric acid (GABA), a major inhibitory neurotransmitter (Barrett et al 2012).

The resulting interactions create a milk-microbiota-brain-behavior (M2B2) system, which may allow mothers to influence infant behavior through their milk (Allen-Blevins et al. 2015).

Experimental Rationale

Studying the M2B2 system in model organisms presents unique challenges because experimental techniques can induce stress in animals that as a byproduct affect microbiota and behavior (Hoggatt et al 2010, Bailey and Coe 1999, Bailey et al. 2004). Stress is a challenge to homeostasis which may be caused by environmental, physiological, social, or psychological stimuli (Bailey 2014, Mendoza in press). Early life stress, including neonatal handling and maternal separation, can have long-term developmental consequences and disrupt the regulation of crucial biopsychological pathways, such as the hypothalamus-pituitary-adrenal axis (Dalmaz et al. 2015). Gut microbiome experiments frequently involve oral gavage of rodents with known bacterial strains, fecal matter, or other compounds (Turnbaugh et al. 2006, Fujimura et al. 2014, Ji et al. 2012.). This technique induces stress responses and can be injurious or fatal to mice, particularly when they are very young (Hoggatt et al 2010, Flamm 2013). Alterations in gut microbiota in response to host stress have been demonstrated in mice and rhesus macaques (Tarr et al 2015, Bailey et al. 2004, Bailey and Coe 1999). If gut microbiota are sensitive to stress, invasive techniques like gavage introduce a potential confounding factor. Changes in microbial profiles over the course of an experiment could be due to the treatment or simply the challenges of experimental techniques. Since the M2B2 system must be studied early in life prior to weaning, methods such as dosing water or standard chow with HMO are not effective. Therefore, a non-invasive technique for administering prebiotic solutions directly to very young mice is necessary.

The purpose of this experiment was to determine a less stressful method for administering experimental prebiotic liquids to conventional mouse pups. Our aims were to develop a method

of studying particular diet-microbe interactions in non-gnotobiotic mice. Reducing psychological perturbation was a main goal because bifidobacteria that dominate the infant gut microbiome are reduced after stress exposure (De Loez et al 2014, Bailey et al 2004). We tested two methods, active and passive, of administering fructooligosaccharide (FOS), a previously demonstrated bifidogenic prebiotic (Howard et al 1995), to mouse pups from post-natal day 1 to post-natal day 21 (PND1-PND21). *Bifidobacterium* and *Bacteroides* counts were analyzed because of their potential roles in neurodevelopment (Allen-Blevins et al. 2015, Hsiao et al. 2013, O'Sullivan et al. 2011), while Bacteroidetes and Firmicutes were analyzed due to these phyla being dominant within human gut microbiomes.

MATERIALS & METHODS:

Subjects: We conducted our methodological study (Figure 1) in captive-bred laboratory mice (*Mus musculus*). Six timed-pregnant C57Bl/6J females and six C57Bl/6J males were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) at six weeks old. Animals were housed in the Harvard University Biological Research Infrastructure, a specific-pathogen free facility, under standard Institutional Animal Care and Usage Committee (IACUC) murine environmental conditions. Water and PicoLab commercial chow from were available *ad libitum*. In consideration of the greater risk of reduced maternal care and increased pup mortality among C57Bl/6J primiparae (Brown et al 1999), initial litters were culled and dams placed in single pair mating cages for one week to conduct experiments with a dam's second litter. One dam may have still been nulliparous, as she exhibited signs of pregnancy but no litter was observed prior to being placed in a mating cage. Alternately, the littler may have been delivered and cannibalized prior to observation. This is consistent with other first litters from these dams being cannibalized or found dead. Due to the unexpected death of one male, M1 was mated to F1 and then F6. The other matings were as follows: M2-F2, M3-F4, M4-F3, and M5-F5. Animals were maintained in breeding cages for 7 days before females were removed to individual cages for

experimental manipulations. Males remained singly in their home cages, undisturbed except for cage changes, until euthanasia approximately one week after the birth of their sired litter. All cages were clear plastic, 10.5 inches by 6.5 inches by 5.0 inches.

Experimental Manipulations: Dams were randomly assigned to the following experimental groups: passive water, passive fructooligosaccharide (FOS), active water, active FOS, buccal water, and buccal FOS. The litters for each group were reduced to six on PND0, with the exception of the active water litter, which only contained five pups at birth. The pregnancy of the buccal water dam failed, leaving no experimental litter for the condition. To administer each condition, the home cage was placed next to a clean cage with fresh bedding. The dam was removed from the home cage and placed into the clean cage for the duration of daily pup manipulation. Pups were immobilized by grasping the skin at the nape and along the spine, and rotating their bodies to reveal the ventrum. Starting at PND10, pups were lifted by the tails prior to grasping the nape. For active FOS and water conditions, a micropipette tip was placed in the pup's mouth and the dosage injected. In passive conditions, a micropipette tip was used to transfer the dosage to a Crematocrit tube that was placed near the pup's mouth with the intent to induce the suckling response (Szczytko et al 1999). As the pups in the passive litters developed and the dosages became larger, only micropipette tips were used to place the dosage near the pups' mouths. The switch to only micropipette tips occurred on PND12 for the passive water group and PND15 for the passive FOS group. All tips and tubes were autoclaved prior to use. For buccal conditions, the daily dosage (Figure 2) was micropipetted onto a sterile cotton swab which was inserted into the pup's mouth. Distilled, deionized water was used in all conditions. After receiving the daily dosage, pups were returned directly to the home cage and the next pup was removed for dosing. Once pups began to open their eyes, they were placed into the clean cage with the dam after dosing. When all pups had received their daily treatment, the dam and pups were returned to the home cage.

159

160 FOS was purchased from Sigma- Co. and administered in a 2.5mM solution for PND1-
161 PND7 and a 25mM solution for PND8-PND21. 25mM was the concentration of HMO producing
162 results when given to mice from birth to weaning in Kurakevich et al. (2013). The 2.5mM dosage
163 was used to determine whether the 25mM could be further reduced, to decrease future HMO
164 cost.

165

166 Sample Collection: Gastrointestinal tracts (GI) were collected from experimental groups on
167 PND0, PND7, PND14, and PND21, while control fecal samples from non-handled, non-dosed
168 mice were collected on PND14 and PND20. On PND0 as many pups as necessary to reduce
169 litter size to six were anesthetized with carbon dioxide, decapitated with sharp scissors, and
170 their GI tracts were collected. Since the active water litter had only five pups at birth, one pup
171 was euthanized on PND0. On PND 7, one pup from each litter was euthanized in the same
172 manner and their GI tract collected. At PND14, one pup from each litter was euthanized with
173 carbon dioxide and their GI tract collected. On PND21, all remaining pups in the litter were
174 euthanized with carbon dioxide and their GI tracts collected. Dams were also euthanized with
175 carbon dioxide on PND21 and GI tracts were collected. Sires were euthanized with carbon
176 dioxide and GI tracts collected approximately one week after the birth of their litter. GI tracts
177 were snap-frozen in dry ice and stored at -80C until analysis. Fecal pellet samples were also
178 collected on PND14 and PND20 from non-handled C57Bl/6J pups and two non-handled dams
179 housed in the same facility, matched for living conditions and diet, which served as controls to
180 the treatment groups. Animal use was approved by the Harvard University Institutional Animal
181 Care and Use Committee under protocol 14-08-217.

182

183 qRT-PCR Analysis:

184 Fecal pellet and GI tract material was transferred from Harvard University, Cambridge,
 185 Massachusetts on dry ice to University of Massachusetts-Amherst, Amherst, Massachusetts for
 186 quantitative polymerase chain reaction analysis. GI tracts were thawed on ice and fecal material
 187 was scraped into sterile sample tubes. Samples were unattainable from PND0 GI tracts (N=9),
 188 due to lack of fecal matter. Additionally, samples from the buccal FOS group (F6, N=10) were
 189 not analyzed because there was no litter from F3 (buccal water). DNA was extracted using a
 190 bead beating protocol (FastPrep-24™ 5G MP Biomedicals Inc, US) and standard protocol for
 191 the QIAmp DNA stool kit (Qiagen, Valencia, CA, US).. DNA quality was determined via
 192 nanodrop (Thermo Scientific, Waltham, MA, US). Extracted DNA was diluted to 4 ng/μL.
 193 Custom TaqMan gene expression assays (Thermo Scientific) for *Bifidobacterium*, *Firmicutes*,
 194 *Bacteroides*, and *Bacteroidetes* were designed using the following sequences:
 195 *Bifidobacterium* (Penders et al. 2005):
 196 Forward primer: GCGTGCTTAACACATGCAAGTC
 197 Reverse primer: CACCCGTTTCCAGGAGCTATT
 198 Probe: TCACGCATTACTCACCCGTTTCGCC
 199 *Firmicutes* (Lecerf et al. 2012):
 200 Forward primer: GAATCTTCCACAATGGACGAAAG
 201 Reverse primer: AATACCGTCAATACCTGAACAGTTACTC
 202 Probe: CTGATGGAGCAACGCCGCGT
 203 *Bacteroides* (Layton et al. 2006):
 204 Forward primer: GAGAGGAAGGTCCCCCAC
 205 Reverse primer: CGCTACTTGGCTGGTTCAG
 206 Probe: CCATTGACCAATATTCCTCACTGCTGCCT
 207 *Bacteroidetes* (Dick et al. 2004):
 208 Forward primer: AACGCTAGCTACAGGCTTAACA
 209 Reverse primer: ACGCTACTTGGCTGGTTCA

Probe: CAATATTCCTCACTGCTGCCTCCCGTA

Samples were run in triplicate with negative control blanks of RNase free water and a standard curve included on each 96-well plate using Applied Biosystems 7500 Fast Real-Time PCR system (Thermo Scientific). Wells included 1µL of Taqman gene expression assay, 10µL of Taqman master mix (Thermo Scientific), 5µL of RNase free water, and 4µL of DNA sample for a total of 20µL in each well. Plates were run at 50C for 2 minutes, 95C for 10 minutes, and then 45 cycles of 95C for 15 seconds and 60C for 1 minute.

Statistical Analysis:

ANOVA was performed in Excel (Microsoft) to compare variation in *Bifidobacterium*, Firmicutes, *Bacteroides*, and Bacteroidetes quantity between treatment groups: active fructooligosaccharide (FOS), passive FOS, active water, and passive water. Wilcoxon rank sum tests were performed in RStudio (Version 0.98.1103) to compare variation in *Bifidobacterium*, Firmicutes, *Bacteroides*, and Bacteroidetes quantity in the combined totality of treatment groups active FOS, passive FOS, active water, and passive water versus the fecal pellet samples from non-handled mice housed in the same facility, for both pup and dam samples. Since the sample sizes were quite skewed for non-handled control (N=7) versus treatment (N=22) pups, Wilcoxon tests were also performed to compare the quantity of *Bifidobacterium*, Firmicutes, *Bacteroides*, and Bacteroidetes in combined treatment groups active FOS, passive FOS, active water, and passive water against the fecal pellet samples from pups on PND14 and PND21. For PND14, sample sizes were equal at four control and four treatment pups. As no fecal pellet samples were collected on PND21 and no GI tracts were collected on PND20, the fecal pellet samples from PND20 were compared to GI tract samples from PND21. Significance for ANOVA and Wilcoxon tests was established as $p < 0.05$. Due to the small sample size, permutation tests with 999 Monte Carlo simulations were also performed in RStudio in an effort to increase power.

Monte Carlo simulations were performed with 99% confidence intervals and significance at the level of $p < 0.01$.

RESULTS:

Gastrointestinal (GI) Tract Samples

Exponential cell counts were measured for *Bifidobacterium*, *Bacteroides*, Bacteroidetes, and Firmicutes for each pup treatment group (N=22, Figure 3). All treatment group samples demonstrated some amount of Bacteroidetes, *Bacteroides*, and Firmicutes, with the exception that no Firmicutes were identified in a PND7 sample from the passive FOS administration pup. Maximum, minimum, and mean counts for each taxa are listed in Table 1. One data point was excluded from the Firmicutes counts due to lack of agreement among triplicate samples (passive FOS, PND 7). One data point was also excluded for the same reasons from the *Bifidobacterium* counts (active water, PND7). From a total of 21 treatment samples, qRT-PCR revealed the majority (19) to have $<10^1$ quantity of bifidobacteria. ANOVA determined no significant difference in bacterial counts across treatment groups.

Bacterial counts were also quantified for samples from the GI tracts of the litter sires (N=4, Figure 4) and the dams of each treatment group and the buccal water dam with no litter (N=5, Figure 5). Maximum, minimum and mean counts are in Table 2. The maximum *Bifidobacterium* count for the sires came from the sire mated to the buccal water dam without issue. All samples for the dams contained $<10^1$ *Bifidobacterium*.

Fecal Pellet Samples

Fecal pellet samples from non-handled pups and dams were measured for *Bifidobacterium*, *Bacteroides*, Bacteroidetes, and Firmicutes exponential cell counts as a control (Figure 3 for pups, Figure 5 for dams). Maximum, minimum, and mean counts for the pups are in Table 1,

while the same counts for the dams can be found in Table 2. The maximum *Bifidobacterium* count of 10^4 was found in four pup samples. For the dams, one sample contained 10^2 *Bifidobacterium*, while one contained 10^3 *Bifidobacterium*.

Gastrointestinal Tract versus Fecal Samples

Despite no significant difference in bacterial counts across treatment groups, Wilcoxon rank sum test did reveal significant differences between counts for all bacterial taxa (p value <0.05) in the treatment groups and bacterial counts for the fecal pellets collected from pups (Figure 2). Exact permutation tests with 999 Monte Carlo simulations demonstrated significant p-values (p>0.01) for *Bacteroides*, *Bifidobacterium*, and Firmicutes. However, the p-values fell within the 99% confidence intervals, making them non-significant.

Comparisons by post-natal day also revealed significant differences between non-handled and handled pups. Wilcoxon rank sum test demonstrated significant differences in the means of the tested bacterial taxa contained in the control and treatment samples for *Bacteroides*, Firmicutes, and *Bifidobacterium* (p-value <0.05) on PND14, but an insignificant difference for Bacteroidetes (p-value=0.057, Figure 6). These significant differences for *Bacteroides*, Firmicutes, and *Bifidobacterium* counts were also evident at PND21, despite a large skew in sample sizes (control N=3, treatment N=14).

Wilcoxon rank sum test found no significant difference between samples from the two control dams and samples from the treatment dams (N=4). This lack of significance was maintained when the F3 dam, who had no litter and received no treatment, was included (Figure 5).

DISCUSSION:

The major finding of this paper is the significant decrease in *Bifidobacterium*, Firmicutes, and Bacteroidetes, phyla frequently studied in animal models due to their importance in the human gut, present in the gut microbiota of handled animals provided either water or FOS. Cognizant of stress effects on bifidobacteria (Bailey et al 2004), our experiment was designed to determine a less stressful technique to administer bifidogenic compounds to neonatal mice. Thus, we expected to find the most bifidobacteria in the group passively fed FOS, since this was expected to be the least stressful technique and included supplementation of a previously shown bifidogenic compound (Howard et al 1995). However, there were no significant differences in Bacteroidetes, *Bacteroides*, Firmicutes, or *Bifidobacterium* across our treatment groups, falsifying our prediction. The significant differences in quantities of *Bacteroides*, Firmicutes, and *Bifidobacterium* between the control and treatment groups suggest a factor common to all four treatment groups may have affected their gut microbiota.

Lack of initial exposure to bacteria and differences between passed stool and GI tract samples may explain the differences in bacterial quantities between our handled and non-handled animals, however we think these are unlikely explanations. The absence of bifidobacteria in the handled dams (with the exception of less than 10^1 in the passive water dam) presents the possibility of the pups lacking bifidobacteria because they were never exposed to it. However, the counts of *Bifidobacterium* in the sires indicate the dams were exposed to bifidobacteria for at least seven days. The sires were cohoused with the dams for one week and since mice are coprophagic (Heinrichs 2001), the dams were likely exposed to bifidobacteria during cohousing, even if they had no prior exposure. The potential exception is the active FOS dam, since the sample from her consort indicated $>10^1$ bifidobacteria. However, one of her pups had the highest count of bifidobacteria in the treatment groups (10^2), so non-exposure is also unlikely for her. Non-exposure also cannot explain the differences in *Bacteroides* and Firmicutes, as all of the treated dams contained these taxa. Additionally, the differences in the use of passed fecal

samples from the control animals (collected from cages) and internal fecal samples from the treatment animals (collected from the distal colon of GI tracts) are also unlikely to explain the magnitude of difference in bifidobacteria. Fecal samples in both mice and humans have demonstrated much higher bifidobacterial counts than gut lumen samples (Marcotte and Lavoie 1996, Ouwehand et al 2004). However, our treatment samples were of intact feces collected from the distal colon and not samples of the mucosa or luminal fluid. Also, the majority of the sires in this study had counts of *Bifidobacterium* in stool from their intestinal tract that was similar to counts in the passed stool of the control dams (with the exception of the active FOS sire). Therefore, while some differences may be expected, it is unlikely to be on the magnitude of 10^3 , which is the difference between the mean bifidobacteria of the control pup samples and the treatment pup samples.

While differential exposure is an unlikely explanation, the stress of handling, common to all treatment groups, seems likely to have contributed to the changes across the treatment group gut microbiota. *Bifidobacterium*, are known to be susceptible to host stress (Bailey et al 2004), was decreased to the point of $>10^1$ in the majority of our experimental mice, the largest decrease of any of the bacterial taxa in our study. Since all of the control animals and the majority of our minimally handled sires largely maintained *Bifidobacterium* in their gut, and all animals were of the same genetic background, in the same facility on the same diet, the sharp decrease in this taxa known to be stress-sensitive, is likely due to a stressor. Additionally, *Lactobacillus*, a genus within the Firmicutes phylum is also stress-sensitive (Bailey 2014), suggesting stress could be driving the decrease in the other bacterial taxa as well. Both passive and active handling techniques appear to negatively influence the amount of *Bifidobacterium*, Firmicutes, and Bacteroidetes in the guts of our subjects, with the quantity of *Bifidobacterium* being the most severely affected.

If even non-invasive handling stress is correlated with significant changes to the gut microbiota and the loss of an entire taxon, this creates a particular challenge for gut microbiota research, especially in young animals. Early life stress, such as maternal separation, is correlated with significant changes gut microbiota (Bailey and Coe 1999). If non-invasive handling is provoking significant enough stress response in laboratory animals to affect gut microbiota, then handling young animals during microbiota experiments may confound the results. For example, handling all of the animals, including controls and those receiving vehicles, may lead a researcher to conclude there are none or reduced levels of a bacterium that may actually be diminished due to handling stress. Such a reduction may mask potential reactions to an experimental compound if it were to be administered without stress.

Synbiotics, a combination of prebiotics and the bacteria of interest (Schrezenmeir and de Vrese 2001), and communal use of non-handled control animals may mitigate the challenges of handling stress affecting gut microbiota. Since synbiotics provide both the substrate for bacterial growth and the bacteria (Schrezenmeir and de Vrese 2001), they may provide a method for studying the most stress sensitive bacteria. For both human and animal studies, exposing the subject to the desired bacteria daily may continually replace the diminished strains and mimic the effects of permanent colonization for the duration of exposure. Microbial and other changes can then be compared in these animals to non-handled control animals. As a guiding principle of the American Association for Laboratory Animal Science (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011) is understandably to reduce the number of animals used in experiments, there may be a reluctance to include negative control animals that are not handled at all. The reduction principle can still be achieved for gut microbiota studies if multiple labs share feces and potentially other data from non-handled animals housed in the same facilities, on the same diets, and from the same genetic backgrounds.

365

366 It is important to note the small, skewed sample sizes in our study and the lack of bifidobacteria
 367 in the active FOS sire and the buccal water dam without a litter. The small number of control
 368 samples compared to the treatment groups may have impacted our results. To determine if
 369 handling is truly causing such a large difference in gut microbiota, replicating this study with a
 370 greater number of control samples would be of great value. Additionally, the stress of handling
 371 does not explain the lack of bifidobacteria in the active FOS sire or the buccal water dam. Both
 372 animals contained counts of Bacteroidetes, *Bacteroides*, and Firmicutes that were on par with
 373 their sex and age-matched conspecifics. After removal from mating cages, all dams were
 374 housed separately until parturition. While isolation of pregnant females to prevent cannibalism of
 375 pups is an accepted practice (Committee for the Update of the Guide for the Care and Use of
 376 Laboratory Animals, 2011), since this female never had a litter, the social stress of isolation may
 377 have affected her differently. The active FOS sire may have lacked bifidobacteria due to an
 378 unnoticed illness or a difference in genotype (Bevins and Salzman 2011, Wacklin et al 2011),
 379 but this is purely conjecture. Therefore, since these two animals present a conundrum and it is
 380 highly important to determine the extent of handling effects on gut microbiota, this research
 381 should be repeated in larger studies. Prior to that, researchers should remain cognizant of
 382 potential handling effects on their data.

383

384 **CONCLUSION:**

385 Although more research is clearly necessary, the stress of handling, or even the social stress of
 386 isolation, may affect murine gut microbiota. Care should be taken during gut microbiota
 387 experiments to ensure necessary controls and accurate data collection. Sharing fecal samples
 388 from control animals of the same genetic background, housed in the same facility, and fed the
 389 same diet would met standards of animal reduction, while enabling comparison of treatment

animals to unhandled animals. Synbiotics, a mixture of substrate and the bacteria of interest, may also be necessary for experiments studying stress-susceptible bacteria.

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Timeline for Treatment Groups

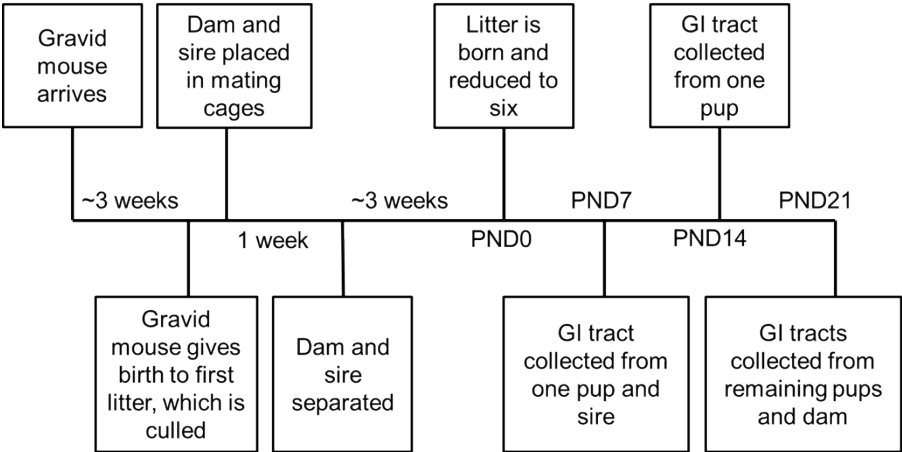


Figure 1. Timeline for each treatment group. Samples were collected at time points post-natal day (PND)0, PND7, PND14, and PND21.

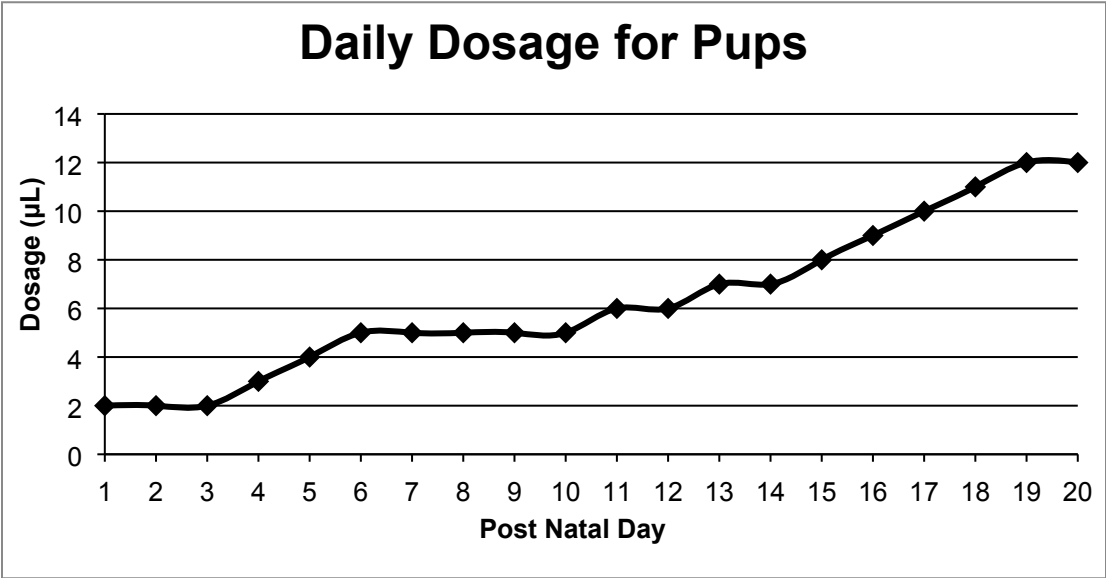


Figure 2. Daily dosage of FOS or water in microliters.

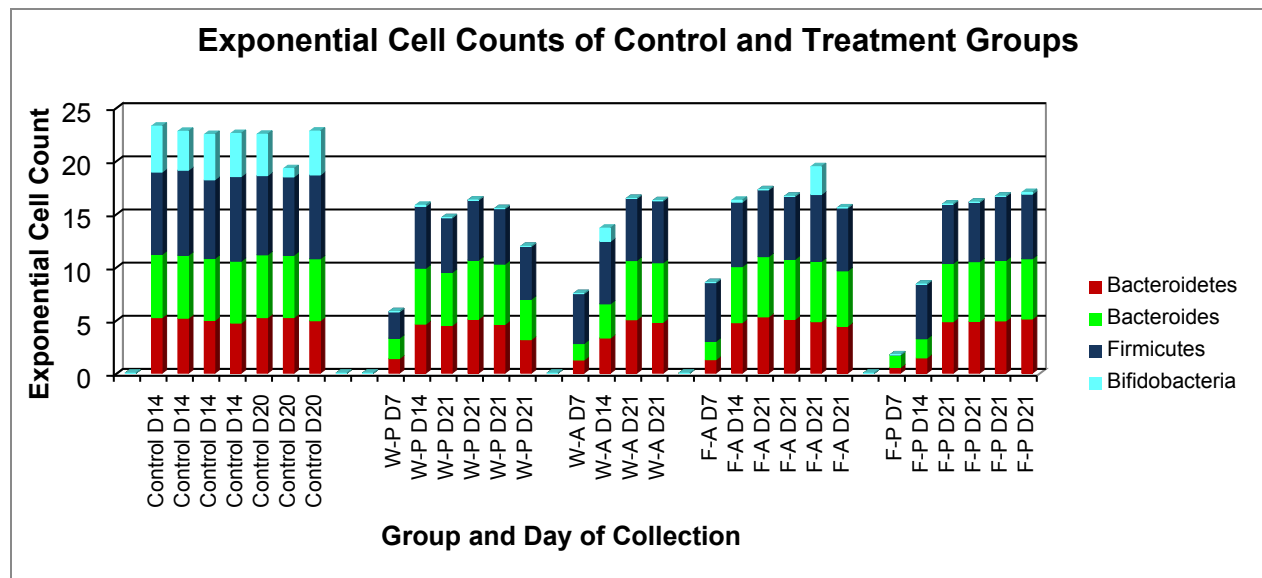


Figure 3 Exponential cell counts of control and treatment groups. Wilcoxon rank sum test demonstrated significant differences between the control and treatment samples for all bacterial taxa ($p < 0.05$).

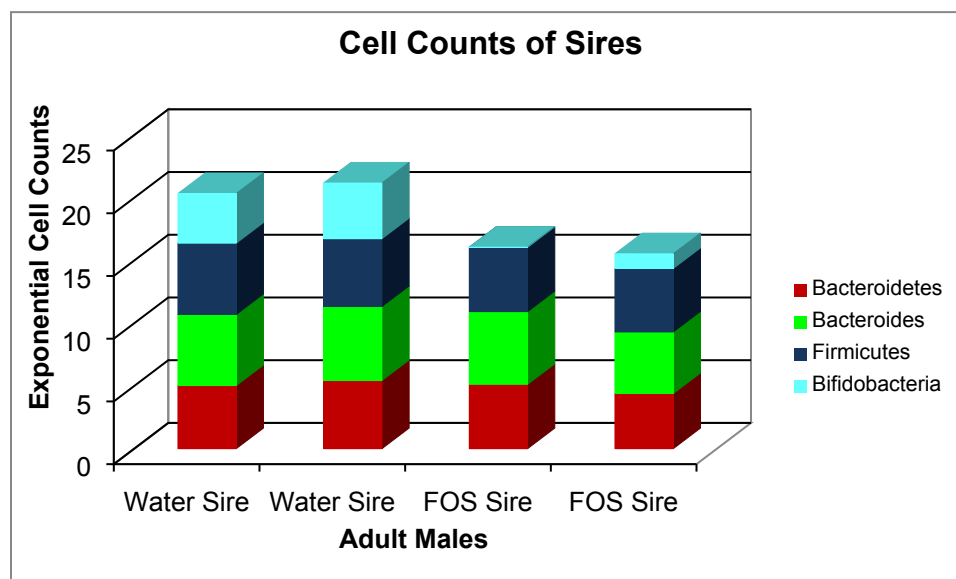


Figure 4. Exponential cell counts of feces collected from the GI tracts of treatment litter sires.

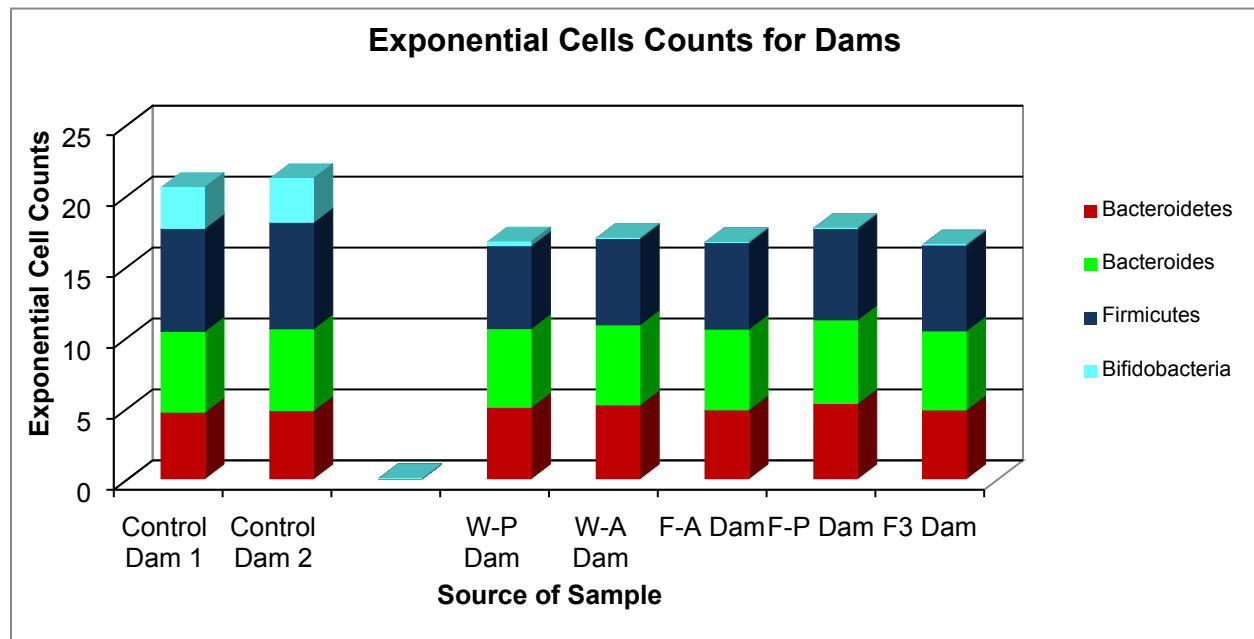


Figure 5. Exponential cell counts from samples of control and experimental dams. There was no significant difference between the samples, despite four of the treatment dams having no bifidobacteria. The F3 dam was the buccal water dam that did not deliver a litter. W-P= passive water, W-A= active water, F-A= active FOS, F-P= passive FOS.

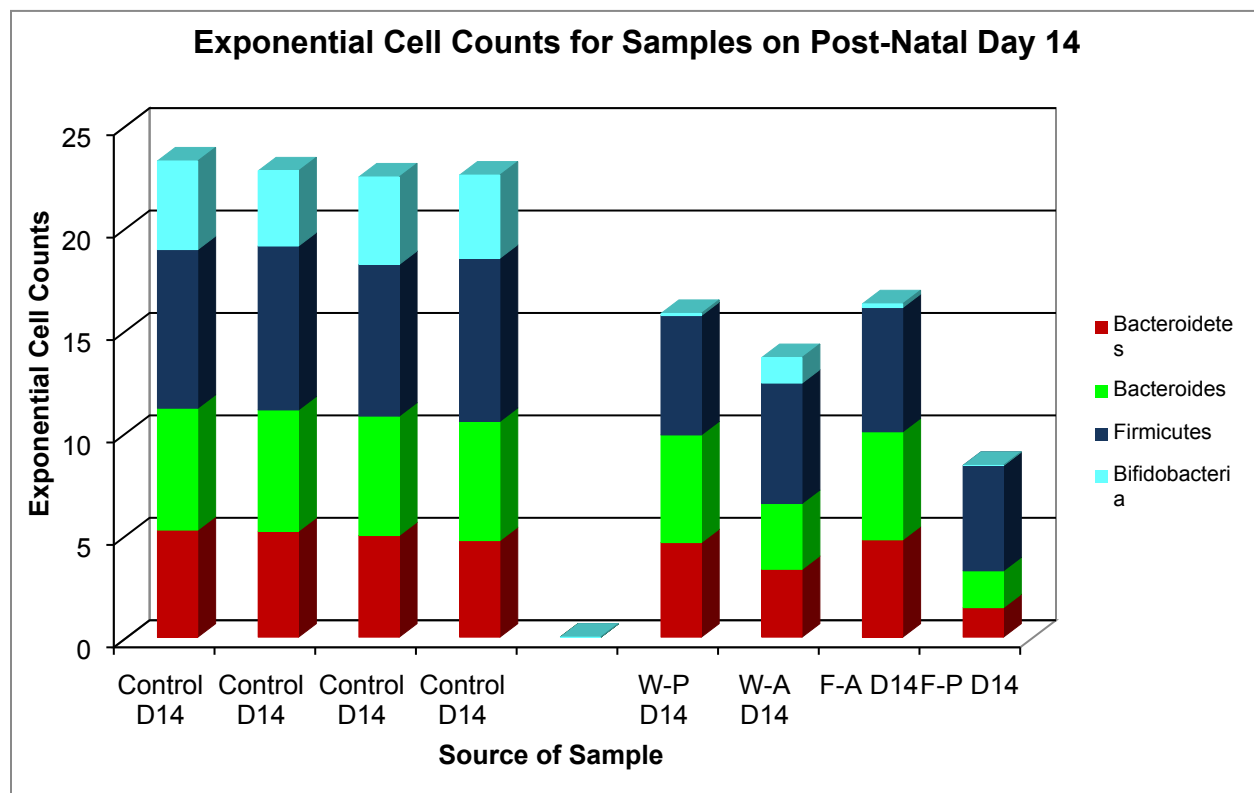


Figure 6. Exponential cell counts for control and treatment samples from post-natal day 14. The mean *Bifidobacterium*, Firmicutes, and *Bacteroides* counts in the control samples were significantly different from the treatment samples (p-value<0.05) as tested by Wilcoxon rank sum test. The means of Bacteroidetes counts were not significantly different between groups (p-value=0.057). W-P= passive water, W-A= active water, F-A= active FOS, F-P= passive FOS.

Group/Taxa	Maximum	Minimum	Mean
GI Tract—Pups (N=22)			
Bacteroidetes	$>10^5$	$<10^1$	10^3
Bacteroides	10^5	10^1	10^4
Bifidobacterium	10^2	$<10^1$	$<10^1$
Firmicutes	10^6	10^2	10^5
Fecal Pellets—Pups (N=7)			
Bacteroidetes	10^5	10^4	10^5
Bacteroides	10^5	10^5	10^5
Bifidobacterium	10^4	$>10^1$	10^3
Firmicutes	10^8	10^7	10^7

Table 1. Bacterial cell counts for pups. Maximum, minimum, and mean exponential cell counts for Bacteroidetes, *Bacteroides*, *Bifidobacterium*, and Firmicutes in treatment and control pups.

Group/Taxa	Maximum	Minimum	Mean
GI Tract—Dams (N=5)			
Bacteroidetes	10^5	10^4	10^5
Bacteroides	10^5	10^5	10^5
Bifidobacterium	$<10^1$	$<10^1$	$<10^1$
Firmicutes	10^6	10^5	10^6
GI Tract—Sires (N=4)			
Bacteroidetes	10^5	10^4	10^5
Bacteroides	10^5	10^4	10^5
Bifidobacterium	10^4	$<10^1$	10^3
Firmicutes	10^5	10^5	10^5
Fecal Pellets—Dams (N=2)			
Bacteroidetes	10^4	10^4	10^4
Bacteroides	10^5	10^5	10^5
Bifidobacterium	10^3	10^2	10^2
Firmicutes	10^7	10^7	10^7

Table 2. Bacterial cell counts for dams and sires. Maximum, minimum, and mean exponential cell counts for Bacteroidetes, *Bacteroides*, *Bifidobacterium*, and Firmicutes in sires, and treatment and control dams.