**METHODS**

**Animals**

CD-1 IGS mice ages 6-8 weeks were obtained from Charles Rivers Laboratories and housed three per cage under alternating 12:12 hours light dark cycle. Mice had an acclamation period of 6 days prior to starting the experiment. Mice had *ad libitum* access to water and standard chow. Each cage housed either male or female mice and all mice in the cage received the same intervention. CD-1 IGS outbred mice were chosen for use in this experiment because they are more genetically, and behaviorally, diverse than inbred mouse strains and because we have experience running behavioral tests on outbred mice. All study procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Nationwide Children’s Hospital.

**Drugs**

Polyethylene Glycol **(**PEG) 3350 (Perrigo Company) was used at a dosage of 4g/kg (designated as Hi PEG) or 1g/kg (designated as Lo PEG). PEG3350 powder was dissolved in water and each mouse received 0.25 mL via oral gavage.

Magnesium citrate (Spectrum Chemical MFG. Corp.) was used to determine if any potential behavioral changes were specific to PEG3350 or to laxative use in general. Two dosages were selected for use, 600 mg/kg (designated as Hi Mag) and 450 mg/kg (designated as Lo Mag). Magnesium citrate powder was dissolved in water and each mouse received 0.25 mL via oral gavage.

The two different doses used for PEG 3350 and magnesium citrate were based on a pilot study that evaluated effects of different dosages on stool consistency. Based on pilot study results, two doses from each laxative that were able to change stool consistency were selected.

**Behavioral Tests**

Behavior testing included light/dark exploration, open field exploration and elevated plus maze. All tests were performed between 0900 and 1300 on each day to maintain consistency. Behavioral tests were performed in isolated behavioral test rooms to minimize noise. Mice had at least a 60 minute acclamation to the behavioral test room before the start of the test. Behavior testing occurred over two consecutive days, with the light/dark test followed by the open field test on day 1 and the elevated plus maze on day 2.

**Light/Dark Exploration**

The light/dark exploration apparatus consisted of a box made of Plexiglas measuring L 60 x W 45 x H 30 cm and divided into two compartments by a black plexiglass wall with an opening that allowed mice to move freely between the two compartments. The light compartment occupied two thirds of the box and was illuminated by a 13watt white bulb with illuminance at the center of the chamber being ~ 500 lux; the dark compartment was enclosed by black plexiglass. Mice were placed in the center of the illuminated compartment and allowed to explore for 300 seconds. Animal movement was recorded using a video camera mounted above the box. Behavior was scored from videos. Latency to the first entrance from the light to the dark compartment (defined as all 4 paws inside the compartment), the amount of time spent in the dark and in the light portions of the box, and the number of transitions between the light and the dark portions of the box were recorded. More time spent in the dark reflects more anxiety like behavior while more time spent in the light reflects more exploratory behavior. Similarly, shorter latency period to enter the dark chamber and less number of transitions between light and dark chambers reflects more anxiety like behavior while longer latency and more transitions reflect more exploratory behavior.

**Open Field Exploration**

The open field apparatus, manufactured by San Diego Instruments, was composed of clear acrylic enclosures measuring L 40 x W 40 x H 38 cm. Illumination at the center of the enclosure was ~ 40 lux. Mice were placed in the front of the left corner and allowed to explore for 300 seconds. Behavior was scored using 16 x16 photo-beam configuration analyzed by the software San Diego Instrument’s Photo-beam Activity System version 2 to track the animal path within the enclosure. The amount of time the mice spent in the center of the open field (defined as the inner 30 x 30 cm) or periphery (defined as the outer 10 cm x 10 cm) was recorded, along with the number of transitions between the center and periphery of the open field, the total distance, average speed traveled, and the amount of time resting with no detected movement for 2 seconds. More time spent in the periphery reflects more anxiety like behavior. Similarly, less distance and more resting time reflects more anxiety like behavior and the opposite reflects more exploratory behavior.

**Elevated Plus Maze**

The elevated plus maze (EPM) apparatus was manufactured by MED Associates INC and consisted of a plus shaped maze with two closed and two open arms 35 x 7.5 cm. The closed arm wall height was 20 cm. The arms intersected at 90 degrees and the junction square measured 6 x 6 cm. The maze was elevated 73 cm above ground. Illuminance in the room was ~ 940 lux. Mice were placed in the junction with their nose facing one of the open arms and allowed to explore for 300 seconds. Behavior was scored using infrared I/R photo-beam activity tracker and MED PC IV software. The number of full entrances to closed arms and open arms was tallied, as well as the amount of time spent in each arm. A full arm entrance was scored when all 4 paws entered an arm. When only 2 paws entered an arm, it was scored as an exploration. More time spent in the closed arm reflects more anxiety like behavior while more time in the open arm reflects more exploratory behavior.

**16S rRNA gene sequencing**

Stool samples from experiment 2 collected on days 0, 14 and 28, were selected randomly from both male and female mice in four groups (Hi PEG, Lo PEG, Hi Mag, and vehicle). The QIAamp Fast DNA Stool mini kit (Qiagen, Valencia, CA) was used for DNA extraction. Illumina MiSeq 2x250 paired end sequencing using NEXTERA unique dual index primers was used to sequence the V4-V5 16s rRNA variable region. Demultiplexing, quality filtering, calling of amplicon sequence variants (ASVs) by DADA2, and taxonomic assignment (SILVA database) of 16S rRNA gene sequencing data was conducted using the open-source, community-supported software program Quantitative Insights Into Microbial Ecology 2 (QIIME2).

 Differences in bacterial alpha diversity (Faith’s phylogenetic diversity and Pielou’s evenness) were determined using the Kruskal-Wallis non parametric ANOVA. Unweighted and weighted UniFrac distances and permutational multivariate analysis of variance (PerMANOVA) were used to monitor differences in microbial community beta-diversity. Differences in bacterial taxa were determined using Kruskal-Wallis non-parametric tests and p values were corrected for multiple comparisons using the Bonferroni correction. For diversity and differential abundance analyses, samples were rarefied to 12,100 sequences (the lowest number of reads among the samples).