**Distribution Agreement**

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Charles Smoller April 12, 2022

# Impact of the Maternal Microbiome on ENS Development and GI Motility of Offspring

by

Charles Smoller

Andrew Neish

Adviser

Department of Biology

Andrew Neish

Adviser

Rheinallt Jones

Committee Member

Roger deal

Committee Member

2022

# Impact of the Maternal Microbiome on ENS Development and GI Motility of Offspring

By

Charles Smoller

Andrew Neish

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Department of Biology

2022

Abstract

Impact of the Maternal Microbiome on ENS Development and GI Motility of Offspring

By Charles Smoller

The gut microbiome has become a growing field of research in biology due to its connection with multiple facets of human health including juvenile development, homeostasis, and metabolic function. Recently, research has found that the maternal gut microbiome plays a role in the development of the nervous system. However, studies have neglected to focus on the enteric nervous system (ENS), which is an autonomous component of the nervous system that regulates GI function and physiology. Here we show that the depletion of the maternal mouse microbiome through antibiotic administration during pregnancy causes the offspring to have structural deficits in the ENS and motility issues in the GI tract. By utilizing functional motility assays, we saw abnormalities in gut motility such as reduced transit through the gastrointestinal (GI) tract and signs of constipation in the 3-week-old offspring of antibiotic-treated mothers. Moreover, by utilizing immunohistochemistry techniques such as CLARITY to image the gut, we found deficits in the number of developing neuronal cells as well as lower myenteric plexus intensity and epithelial innervation in the ENS in both postnatal day 2 and 3-week-old mice. These data suggest that prescribing antibiotics to a mother during pregnancy could cause possible harm to offspring. It is also the first to find that the depletion of the maternal gut microbiome can cause deficits in the development of the ENS of offspring.

# Impact of the Maternal Microbiome on ENS Development and GI Motility of Offspring

By

Charles Smoller

Andrew Neish

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Department of Biology

2022

Acknowledgements

I would first like to thank my advisor, Dr. Andrew Neish, for his willingness to allow me to join his lab as well as his support and guidance during my tenure in the lab. I would especially like to thank Dr. Bindu Chandrasekharan for training and overseeing my research. I cannot thank her enough for all the time and energy she spent guiding me. I could not have asked for a more patient and helpful supporter during this time and this project could not have been done without her help. I would also like to thank Dr. Dr. Sammi Kim for supporting my work in the lab. Lastly, I would like to thank Dr. Rheinallt Jones and Dr. Roger Deal for taking the time out of their day to serve on my committee.

# Table of Contents

*Introduction* ..... *1*

*Methods* ..... *6*

**Ethical approval** ..... **6**

**Animal Studies** ..... **6**

**Antibiotic-Treated (Abx) Mice Experimental Plan** ..... **6**

**DNA Extraction and qPCR to Validate the Maternal Antibiotic Model** ..... **7**

**GI Motility Assays** ..... **7**

**CLARITY Immunostaining** ..... **9**

**Confocal Imaging for CLARITY** ..... **10**

**Immunohistochemistry on Frozen Ileal Sections** ..... **10**

**Arbitrary Fluorescence Quantification** ..... **12**

**Statistical Analysis** ..... **12**

*Results* ..... *12*

*Discussion* ..... *20*

**Overall Findings and Importance** ..... **20**

**Validation of the Antibiotic Model** ..... **21**

**Functional GI Issues of Offspring Born from Pregnant Mothers Treated with Antibiotics** ..... **21**

**Structural Issues of Offspring Born from Pregnant Mothers Treated with Antibiotics** ..... **22**

**Limitations** ..... **24**

*Conclusion* ..... *25*

*References* ..... *27*

## Tables and Figures

*Table 1*……...*11*

*Figure 1*……..*13*

*Figure 2*……..*16*

*Figure 3*……..*18*

*Figure 4*……..*20*

## Introduction

The microbiome is a multi-species community of microorganisms consisting of bacteria, archaea, and fungi that interact with each other. In many cases, these communities reside on or inside of a host organism. Communities thrive on the skin, mouth, and intestinal tract of virtually all metazoan organisms, including humans. Historically microorganisms were portrayed as primarily detrimental to host organisms due to the association with bacterial pathogens and human diseases. However, in recent times, research has shown that there are also benefits to these communities. These microbes have developed symbiotic relationships with their host organisms that can benefit both species. Specifically, in humans, most microbes reside in the gut. The gut includes both the small and large intestine which is part of the gastrointestinal (GI) tract. In every healthy adult's gut, there are over 1000 species of bacteria that play a role in immune health, digestion, and metabolic functions (Ley et al., 2006). For example, gut microbiota communities are essential for digestion and growth. Many enzymes such as xyloglucan found in vegetables cannot be processed and broken down without the help of specific gut *Bacteroidetes* (Larsbrink et al., 2014).

Recent research has shown that the microbiome not only impacts the GI tract but also juvenile development and brain functioning. Research from teams such as ones led by microbiologist, Dr. Jeffrey Gordon, at Washington University in Saint Louis has found that children suffering from malnutrition have developmental delays compared to their healthy counterparts. However, when supplemented with foods that supported the growth of their microbiome, previously malnourished children showed significant improvements in their development, far more than those children fed a nutritionally rich diet that had less of a positive effect on their gut microbiome (Subramanian et al., 2014). This data proved the importance of

the gut microbiome on overall human health. Another breakthrough related to the gut microbiome was the discovery of the gut-brain axis and its effect on maintaining homeostasis. The gut-brain axis (GBA) is a network of bidirectional communication between the central nervous system (CNS), the autonomic nervous system (ANS), the hypothalamic-pituitary-adrenal (HPA) axis, and the enteric nervous system (ENS). The GBA has been found to impact diverse processes from hormone secretion to memory and emotion as well as play a role in neurogenesis and myelination (Carabotti et al., 2015). Moreover, there is increasing evidence that the GBA is influenced by the gut microbiome. Probiotics administered to rat pups after maternal separation countered abnormally high corticosterone levels and better regulated colonic function (Gareau et al., 2007).

With all these new data revealing that the gut microbiome is a significant factor in sustaining a healthy life, it is equally as important to study ecological disturbances of the gut microbiome, termed gut dysbiosis, that can impede healthy development and functioning. One of the major reasons why gut dysbiosis arises is due to antibiotic administration. When patients are prescribed antibiotics to suppress infections of pathogenic bacteria in the body, these drugs suppress multiple species of bacteria in the gut microbiome including beneficial bacteria that humans need. In adults, antibiotics are said to only cause short-term disturbances in community composition. In most cases, the community of a healthy young adult will return to a composition like that of the pre-antibiotic state in a week or so (Palleja et al., 2018). However, infants and young children are not as resilient. The gut microbiome is dynamic and constantly developing for about 3 years after birth before it forms a stable adult-like composition of bacteria (Yatsunenko et al., 2012). Once infants reach this time point, the species present will form the basis of their gut microbiome for the rest of their life. As a result, perturbations of the gut

microbiome before this time point can impact these individuals over the long term. Studies have already shown the detrimental effects of a depleted gut microbiome during this critical period of gut microbial community growth. Antibiotic exposure before the age of 2 was correlated with an increase in obesity and body mass index (BMI) compared to children who were not exposed to antibiotics (Saari et al., 2012). Common disorders such as asthma, allergies, and atopic disease development were also found to be associated with early-life gut dysbiosis (Stiemsma & Michels, 2018).

One area of gut dysbiosis research that has been understudied has been how disrupting the maternal microbiome affects fetal development. Up until at least the start of the 21st century, researchers concluded that the fetus during pregnancy was sterile, and the infant does not become colonized with microbes until the start of the birthing process (Mackie et al., 1999). This has led physicians to assume that non-teratogenic antibiotics are safe to use during pregnancy as they will not affect fetal gut microbiome development. As a result, high rates of antibiotic use during pregnancy have become common. Between 1994 and 2009, for 18,873 pregnancies studied, antibiotics were prescribed to approximately 20% of all mothers during their pregnancy (De Jonge et al., 2013). Moreover, in the past 15 years, some researchers have challenged this paradigm of a sterile womb. Some researchers have believed to have discovered bacterial growth in the placenta, amniotic fluid, and meconium of humans and mice (Satokari et al., 2008) (Jiménez et al., 2008). However, further research on this theory has disproven and shown this data to be a result of erroneous methods and microbial contamination (Theis et al., 2020) (Lauder et al., 2016).

The evidence for the lack of the existence of the fetal microbiome in utero is a result of the introduction of germ-free animal models into microbiome research. In this field of

gnotobiology, germ-free animals are born from normally colonized mothers through C-section and placed in sterile isolation. Germ-free animals are created when the pregnant uteruses are aseptically harvested, introduced into a germicidal bath, and transferred into a sterile isolator to develop into germ-free offspring (Qv et al., 2020). This has allowed researchers to study the interaction of the microbiome with the host. If the fetus acquired a gut microbiome in utero from the non-germ-free mother, then researchers would see evidence of microbial growth in the sterile environment. However, this is not the case as no microbes are found in the offspring (Perez-Muñoz et al., 2017). This helps validate the idea that fetuses do not have a gut microbiome in utero; the uterus is sterile.

While the possibility of a fetal microbiome developing in utero has been controversial and most likely untrue, data has shown that the maternal microbiome still plays a prominent role in fetal development. Recent studies have shown microbes and bioactive metabolites stemming from the mother's gut microbiome impact the fetus. The metabolite butyrate, which is a short-chain fatty acid created as a fermentation product of the microbiome, has been shown to have protective effects on the colon of the offspring. Specifically, butyrate supplementation in pregnant mice was associated with increased microbiome taxonomic diversity, protection against colitis, and downregulation of colonic genes associated with inflammation in offspring (Barbian et al., 2021). Moreover, depleting the microbiome in pregnant mothers can be detrimental. Using RNA sequencing to evaluate gene expression in the brain, image analysis of brain tissues, and behavioral assays, a team at the University of California Los Angeles (UCLA) found that depleting the maternal microbiome during pregnancy in gnotobiotic and antibiotic-induced models produced adult offspring with altered tactile sensitivity, impaired fetal gene expression and impaired thalamocortical axonogenesis in the brain (Vuong et al., 2020). To prove that the

deficits in neurodevelopment were indeed a result of a depleted maternal microbiome, the researchers inoculated germ-free pregnant mothers with specific bacteria such as spore-forming bacteria from the *Clostridium* species. After inoculation, abnormalities in brain abnormalities in the offspring were prevented. This research was some of the first published to connect healthy fetal development to microbial interactions in the maternal environment. Despite this evidence of deficiencies in fetal development due to a depleted microbiome, little research has focused on the effect of a depleted maternal microbiome on another part of the gut-brain axis, the ENS.

The ENS is a subdivision of the nervous system that works autonomously of the central nervous system to regulate gut function and physiology. This includes processes such as contraction and relaxation of the intestinal wall, gastrointestinal blood flow, and epithelial cell function (Nezami & Srinivasan, 2010). Disruption of ENS growth has been linked to GI abnormalities and neurological issues (Rao & Gershon, 2016). Studies of the mechanisms underlying gut dysbiosis have already shown that the gut microbiome is significantly associated with the development and regulation of the ENS as germ-free mice have an atrophic ENS (Collins et al., 2014). Infant antibiotic use has also been connected to gut dysbiosis and impaired neuromuscular function of the ENS. This research showed that antibiotic administration to juvenile mice for 14 days until 3 weeks of age resulted in impaired GI transit and bowel architecture, with a reduction in glial cell numbers and myenteric neurons (Caputi et al., 2017). Thus, it has become important to study the ENS as it can have detrimental effects on both digestive and neurological pathology.

As a result of a lack of research on the importance of the maternal microbial environment on the ENS development and GI motility of the offspring, this study will focus on tracking the development of the ENS of offspring from mice that were administered antibiotics during

gestation. The study will aim to analyze functional changes of the GI tract in 3-week-old offspring by measuring GI motility. Secondly, the study will analyze structural changes in the ENS of the offspring during their early postnatal life as well at 3 weeks of age by utilizing immunofluorescent imaging techniques to evaluate changes in the enteric neurons of the system. I have hypothesized that the disruption of the maternal gut microbiome during pregnancy due to antibiotic administration will cause GI motility issues as well as ENS developmental deficits in offspring in comparison to untreated wild-type mice offspring.

## Methods

### *Ethical approval*

All murine experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University and were performed according to the Emory guidelines for the ethical treatment of animals.

### *Animal Studies*

All experiments were performed using wild-type (WT) C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME). The mice were bred in the Whitehead Biomedical Research Building animal facility at Emory University. Mice between postnatal day two through eight weeks of age were used during these experiments. After experimental procedures were complete, mice were euthanized with carbon dioxide (CO2).

### *Antibiotic-Treated (Abx) Mice Experimental Plan*

One, six-week-old WT male and two, six-week-old WT female mice were placed in a breeding cage and administered an antibiotic cocktail (1g/ L each of Ampicillin, Streptomycin, Vancomycin, Metronidazole; Sigma-Aldrich, St. Louis, MO) in drinking water to eliminate the

microbial flora. Every day the females were checked for a vaginal plug and once found, the pregnant female was isolated from the males and placed in their own cage. The plugged females were continued on antibiotics throughout the gestation period up until the offspring were no longer weaning. The pups with maternal antibiotic exposure were constituted as the antibiotic (Abx) group while the control group was formed by the offspring of breeding pairs without maternal antibiotic exposure. Abx and control group mice were assessed for myenteric density and epithelial innervation of the ENS as well as GI motility deficits at postnatal day 2 (P2) and at 3 weeks of age.

*DNA Extraction and qPCR to Validate the Maternal Antibiotic Model*

Stool samples from the pregnant mothers of the Abx and control group at the 18$^{th}$ day of gestation were taken, and fecal DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Subsequent, quantitative PCR (qPCR) using forward and reverse primers for 16s DNA as well as SYBR green reaction master mix (Bio-Rad, Hercules, CA) were added to the DNA sample to create the PCR amplification mix. β-actin was used as the housekeeping gene to compare and quantify the amount of 16s DNA in the fecal samples and the CFX Connect Real-Time PCR System (Bio-Rad, Hercules, Ca) was used for the real-time PCR. Relative fold change of 16S DNA was found using the Comparative CT Method for relative quantification.

*GI Motility Assays*

a. *Pellet Frequency and Fecal Water Content Analysis*

Fecal pellet frequency was examined in control and Abx mice for 1-hour intervals. Randomized mice were individually placed in a clean cage and observed for 60 minutes at a time. The number of pellets per mouse was recorded and weighed

to determine the wet weight. Pellets were subsequently dried overnight at 65°C and reweighed to determine the dry weight. The water content was then calculated by finding the difference between the wet weight and dry weight.

*b.* *Red Carmine Dye GI Transit Assay*

Red carmine dye, which cannot be absorbed and bypass the lumen of the gut, was used to study total GI transit time in 3-week-old mice (Koester et al., 2021) A solution of carmine red (300 µl; 6%; Sigma-Aldrich, St. Louis, MO) suspended in 0.5% Sodium carboxymethyl cellulose (Sigma-Aldrich, St. Louis) was administered by gavage through a feeding needle. After oral gavage administration, fecal pellets were monitored at 10 min intervals for the presence of the red carmine solution. Total GI transit time was measured as the interval between the initial time point and the time of the first observance of red coloring in the stool due to the red carmine solution.

*c.* *Gastrointestinal Transit using FITC-Labeled Dextran*

GI transit was assessed by evaluating the distribution of orally gavaged fluorescein isothiocyanate (FITC)-labeled dextran (FITC-dextran, 70 kDa; 25 mg/mL in 0.9% saline solution, Sigma-Aldrich, St. Louis, MO) through the GI tract of 3-week-old mice. After 4 hours, mice were euthanized with CO2, and the GI tract from stomach to distal colon was collected and placed into a Krebs solution. The stomach and caecum were isolated and analyzed separately while the small intestine and the colon were divided into 10 and 4 segments of equal length respectively. The FITC-labeled dextran intensity of each segment was quantified using a spectrofluorometer at 492/521 nm.

*CLARITY Immunostaining*

Mice at P2 as well as at 3 weeks of age were euthanized, and their colons were prepared for imaging with CLARITY (Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging compatible Tissue hYdrogel) to look for structural differences between Abx and the control groups. CLARITY is a 6-day process involving acrylamide embedding to delipidate tissue without sectioning. This allows preservation of the tissue for immunostaining the 3-dimensional architecture (Chung et al., 2013).

On day 1 of the CLARITY process, the intestine of P2 and 3-week-old Abx and control mice were removed, the fecal contents were flushed out with ice-cold phosphate-buffered saline (PBS) (Bio-Rad, Hercules, CA), and the colon were placed into a 15 mL conical tube filled with 4% Paraformaldehyde (PFA) (Affymetrix, Cleveland, OH) at 4 °C overnight for fixation. On day 2, each intestinal segment was transferred into a new tube to be washed three times with PBS for five minutes each time on a shaker at 200 rpm to remove any residual PFA. The intestinal segment was subsequently transferred into a 4% hydrogel solution created from a 30% gel solution (Protogel, Natural Diagnostics, Atlanta, GA) diluted with PBS to be incubated at 4 °C overnight. On the 3$^{rd}$ day, to allow for hydrogel polymerization of the sample, the tissue is transferred into a 37 °C water bath and incubated for one hour then washed with PBS to remove the excess hydrogel. The tissue was delipidated by transferring it to a 50 mL conical tube and incubated in sodium dodecyl sulfate (8% SDS, Thermo-Fischer Scientific, Waltham, MA) in PBS on a 37 °C shaker at 200 rpm at room temperature for 2 days, which would complete the delipidation process. After 2 days, the tissue is removed from the SDS solution and washed with PBS shaken at 200 rpm in a new 50 mL conical tube. This process was repeated 10 times throughout an 8-hour period to make sure all the SDS is gone. Finally, the tissue was stained

with the immunofluorescence labeled primary antibody for peripherin to explore neuronal marker changes in the colonic tissue. This was accomplished by transferring the tissue to a 2 mL tube and incubated in a 500 uL solution of primary antibody for peripherin (1:500) diluted in 0.5% normal donkey serum/NDS in PBS containing 0.3% Triton X-100 (Sigma-Aldrich, St, Louis, MO) overnight at 4 °C. After this overnight incubation, the tissue was then washed with PBS for 5 minutes 3 times to remove any unbound remaining primary antibody. Then the tissue was combined with a 500 ul solution of the secondary donkey anti-mouse 488nm Alexa Fluor antibody (Table 1) for 1 hour at room temperature in the dark. After 1 hour, the tissue was washed again with PBS then incubated for 5 minutes at room temperature with DAPI (1:500) to stain the nuclei of the tissue. Lastly, the tissue was then transferred onto a clean glass Colorfrost Plus slide (Fischer Scientific, Hampton, NH), mounted with 120 μL of VectaShield (Vector Laboratories, San Francisco, CA), and protected with a coverslip to prepare for confocal imaging. The slides were then placed in the dark at room temperature for 30 minutes to dry. After drying, the slides could then be imaged or stored at -20 °C until further notice.

*Confocal Imaging for CLARITY*

The images were analyzed using an Olympus FV1000 confocal microscope. Z-Stack transformations were taken across 50 μm thickness of the gut tissue to visualize the gut lumen toward the serosal layer. The cell bodies in the ENS were traced and counted based on whether they were positive for peripherin using the software program, Image J. The nuclei were stained with DAPI to visualize and identify the nuclei.

*Immunohistochemistry on Frozen Ileal Sections*

Mice at P2 were euthanized and the terminal ileum and colonic segments were resected and embedded in optimal cutting temperature (OCT) mounting medium and sectioned at 5 μm

thickness with a cryostat. The sections were prepared for immunofluorescence microscopy by mounting them onto Colorfrost Plus slides. In this two-day antibody staining process, ileal sections from mice from the antibiotic and control groups were thawed for 1 minute before being fixed with 4% PFA at room temperature for 15 minutes. Sections were then washed with PBS for 5 minutes before being permeabilized in PBS containing Triton-X-100 (PBS-T) for 15 minutes at room temperature. The sections were then blocked with 5% bovine serum albumin (5% BSA in PBS-T) for 1 hour before incubating the sections with the primary antibodies for the peripherin (1 in 500 dilution), glial fibrillary acidic protein (GFAP) (1:500), and SOX-10 (1:250) overnight at 4 °C. On day 2, sections were washed with PBS then incubated with their respective secondary antibody at a 1 in 500 dilution for 1 hour in the dark (Table 1). Along with the addition of the secondary antibody, the nuclear stain DAPI (1 in 500 dilution) was added to the BSA solution. After 1 hour, slides were mounted with 120 mL of Vectashield mounting medium and covered with a coverslip to preserve the fluorescence before imaging. Images were then taken using an Olympus FV1000 Confocal microscope at 40x magnification.

**Table 1: Primary and secondary antibodies used for CLARITY and immunohistochemistry on frozen Ileal sections (GFAP: Glial fibrillary acidic protein)**

| Primary Antibody | Host Species | Dilution | Source |
|---|---|---|---|
| SOX-10 | Rabbit | 1:250 | Abcam (Milan, Italy) |
| GFAP | Rat | 1:500 | Abcam (Milan, Italy) |
| Peripherin | Mouse, Rabbit | 1:500 | Abcam (Milan, Italy) |
| **Secondary Antibody** | | | |
| Donkey anti-rabbit IgG Alexa Fluor 488 | - | 1:500 | Abcam (Milan, Italy) |

| | | | |
|---|---|---|---|
| Goat anti-rat IgG Alexa Fluor 555 | - | 1:500 | Invitrogen (Eugene, Or) |
| Donkey anti-mouse IgG Alexa Fluor 488 | - | 1:500 | Invitrogen (Eugene, Or) |
| Donkey anti-rabbit IgG Alexa Fluor 555 | - | 1:500 | Invitrogen (Eugene, Or) |

*Arbitrary Fluorescence Quantification*

Staining intensity in at least 8 to 10 myenteric ganglia per mouse was assessed in a blinded fashion using Image J software after the composite image was split into respective color channels (blue, DAPI; green, SOX-10 or peripherin), and the intensity of green fluorescence in the myenteric ganglia was calculated and plotted as relative arbitrary intensity units. The intensity from n = 3 to 5 mice per group was averaged to plot the relative arbitrary intensity unit graphs for the respective experiments. Due to the use of arbitrary fluorescence units, the fluorescence intensity was calculated as a fraction of the control group data where the control group was valued at 100% fluorescence.

*Statistical Analysis*

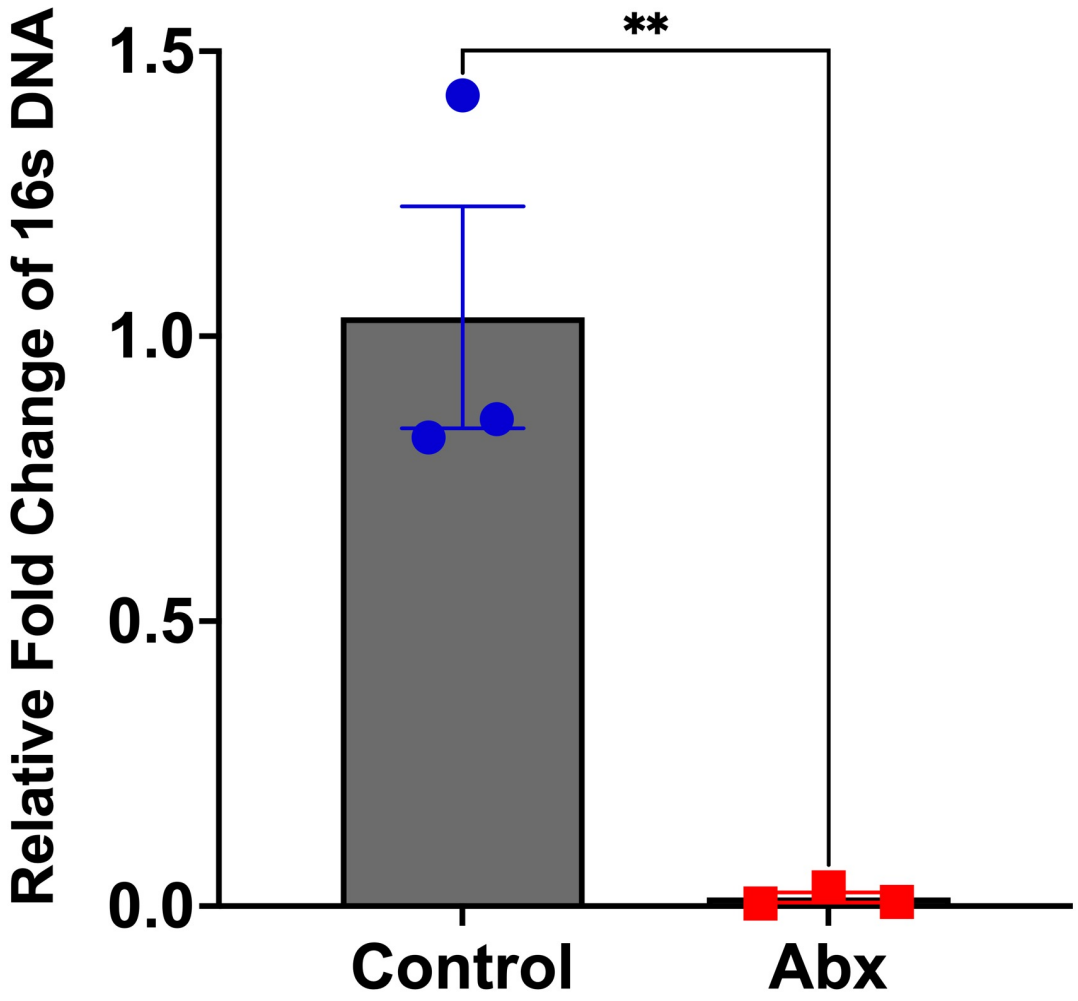
The data was analyzed using Graph Pad Prism 9 software (GraphPad, La Jolla, Ca). The data was represented as Mean ± SEM in all figures. Two-tailed *t*-tests were used to compare the data between the two treatment groups. Differences were considered significant at $p < .05$.

## Results

*Antibiotic Exposure Sufficiently Suppresses the Bacteria Found in the Gut of Pregnant Mothers*

The qPCR analysis of DNA extracted from stool samples from the antibiotic-exposed (Abx) and control group pregnant mothers on the 18$^{th}$ day of gestation measuring the levels of

16s DNA showed statistically significant decreases in 16s DNA bacterial load of the Abx mothers (n=3) compared to the control group pregnant mothers (n=3) ($p$= 0.0064) (Figure 1).




*Gastrointestinal Motility is Impaired in Antibiotic Treated Offspring*

Juvenile mice raised from mothers administered with antibiotics showed overall reduced GI motility compared to the convectional mice group. Abx mice showed a combination of reduced stool frequency (pellets/hour), slower GI transit, as well as an increased total GI transit time.

a. *FITC-Labeled Dextran GI Transit Assay*

The GI transit assay using FITC Labeled dextran to evaluate the distribution of the orally gavaged labeled dextran through the GI tract showed that in the Abx group, 3-week-old offspring had limited transit of the FITC-labeled dextran. In the 3-week-old Abx offspring (n=3), most of the dextran was found to be scattered throughout the small intestine without entering the colon. We found that only a small quantity of the dextran was able to clear the small intestine and move into the caecum and colon. Comparatively, the dextran measured in the control group mice (n=3) was able to completely empty out of the small intestine and end up in the caecum and colon during the 4-hour experiment (Figure 2D).

b. *Total GI Transit Time Using a Red Carmine Dyed Solution*

The second test used to look for signs of GI dysbiosis, motility issues, and possible constipation was the utilization of a red carmine dyed solution to track total GI transit time. By orally gavaging the mice with a solution containing a red carmine dye, we could measure the time between the administration of the solution and the appearance of the first red fecal pellet. This assay showed statistically significant increases in total GI transit time in 3-week-old Abx offspring illustrating gut motility deficits ($p$ value= 0.0024) (Figure 2A).

c. *Stool Water Content*

By measuring the difference between the wet and dry weight of the fecal pellets of 3-week-old offspring, we measured the average water weight of the fecal pellets. The Abx 3-week-old offspring (n=7) did not show any significant differences in stool water content ($p$ value= 0.269) (Figure 2B) compared to the control group (n=3) (Figure 2B).

*d.* *Stool Frequency Analysis to Measure Symptoms of Constipation*

Lastly, to test for GI motility as well as look at signs of altered rates of excretion such as constipation, a stool frequency assay was completed for 3-week-old offspring by recording the number of pellets excrete per mice in 60 minutes. This assay showed a reduction in stool frequency through a reduction in pellets excreted per hour by the Abx mice group (n=7) versus the control group (n=7) (*p* value= 0.006) (Figure 2C).

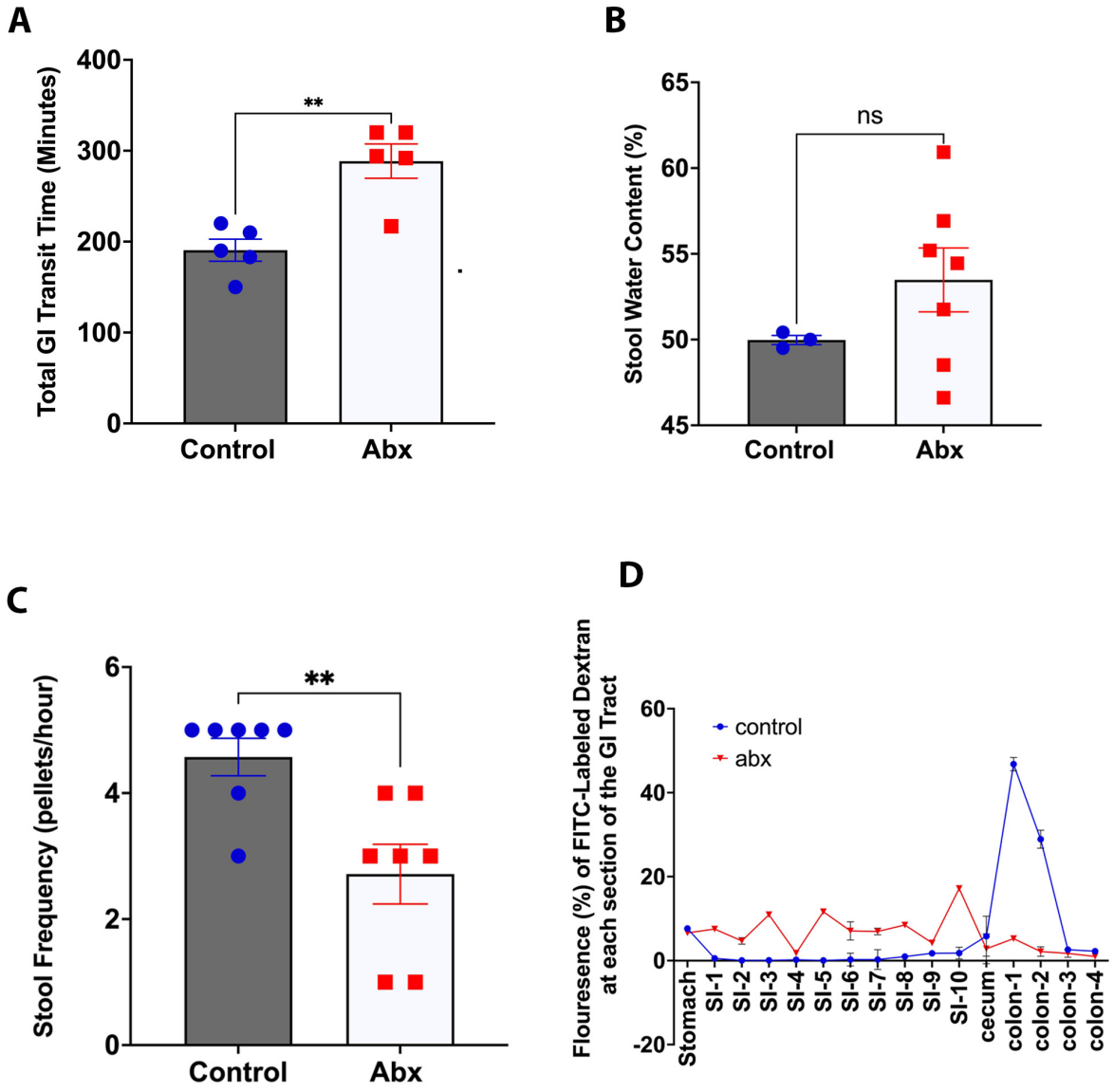



**Figure 2: Gastrointestinal motility is impaired in the offspring of antibiotic-treated pregnant mothers: (A)** Red carmine dye assay tracking the total GI transit time in 3-week-old mice. The antibiotic (Abx) group where the offspring's mothers were given antibiotics during pregnancy and control group offspring were orally gavaged with carmine red (300 µl; 6%; Sigma-Aldrich, St. Louis) suspended in 0.5% Sodium carboxymethyl cellulose (MW 90.00) (Sigma-Aldrich, St. Louis). **(B)** Stool water content analysis for 3-week-old Abx and control group offspring measuring the amount of water per fecal pellet. Water content was calculated by dividing water weight by dry weight. **(C)** Stool frequency assay measuring the number of fecal pellets excreted per hour in 3-week-old mice over multiple 1-hour increments. (**D**) FITC-labeled dextran GI transit time assay in 3-week-old Abx and control mice by evaluating the distribution of orally gavaged fluorescein isothiocyanate (FITC)-labeled dextran (FITC-dextran, 70 kDa; 25 mg/mL in 0.9% saline solution) through the GI tract using a spectrofluorometer to measure the fluorescence of the stomach, 10 sections of the small intestine, the caecum, and 4 sections of the large intestine 4 hours after dextran was orally gavaged into mice. Unpaired two-tailed t-tests were used for statistical significance with $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. n= 5 for both groups in (A), n= 3 and n= 7 for control group and Abx group respectively in (B), and n= 7 and n= 8 for control and Abx group respectively in (C)

*Maternal Antibiotic Treatment Causes Morphological Abnormalities in the Architecture of the Myenteric Plexus of Offspring*

To quantify and evaluate the structure of tissue samples prepared with CLARITY and stained with the marker for peripherin and DAPI, we analyzed the longitudinal muscle myenteric plexus (LMMP) in colon samples of postnatal day 2 (P2) and 3-week-old offspring using Image J software. This analysis showed a significant decrease in the intensity of the myenteric plexus in both the P2 (n=3) and the 3-week-old (n=5) Abx groups compared to the P2 (n=3) ($p$ value <0.0001) (Figure 3E) and 3-week-old (n=4) ($p$ value= 0.0024) (Figure 3E) control groups.

*Maternal Antibiotic Treatment Causes Morphological Abnormalities in the Architecture of Epithelial Innervation of Offspring*

Similarly, to the analysis of the myenteric plexus, epithelial innervation intensity was measured by quantifying CLARITY-prepared images of the colon stained with the markers for peripherin and DAPI on Image J software. In both P2 (n=3) and 3-week-old mice (n=5) in the Abx groups, epithelial innervation decreased significantly compared to the control group (n=3 for P2 control, n=4 for Week-3 control) (p value= <0.0001 for P2, $p$ value= 0.0002 for Week-3) (Figure 3F).

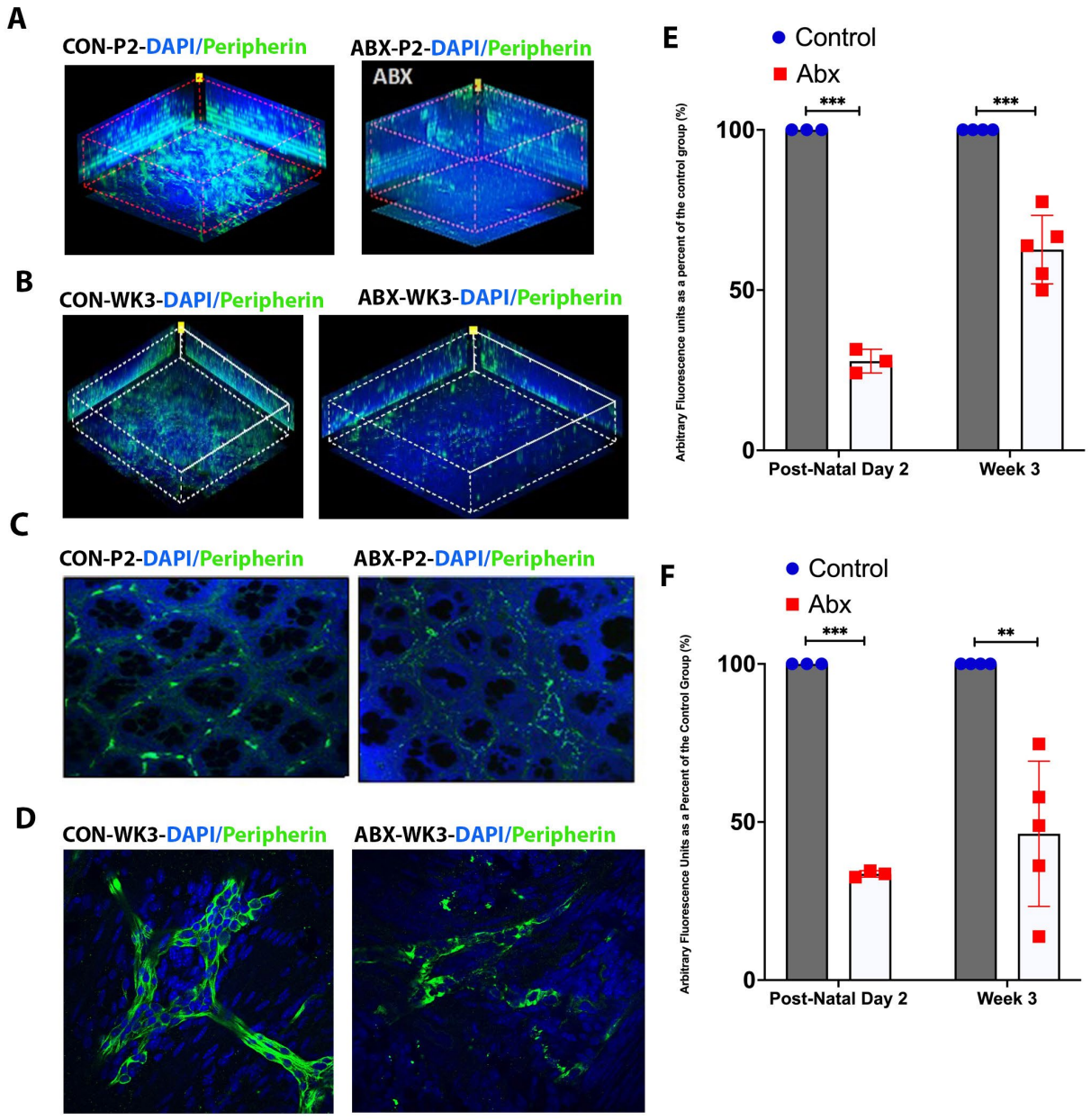



**Figure 3: Maternal antibiotic treatment causes morphological abnormalities in the architecture of epithelial innervation and the myenteric plexus of offspring. (A)** CLARITY Z-stack images taken of the colon of postnatal day 2 (P2) mice from the control group and the antibiotic (Abx) group where the offspring's' mothers were given antibiotics during pregnancy. Tissue samples were stained with the fluorescently labeled antibody for the neuronal marker peripherin and nuclear stain DAPI to visualize changes in epithelial innervation of the offspring due to maternal antibiotic use. **(B)** CLARITY Z-stack images taken of colon of 3-week-old mice from the control group and the Abx group. Tissue samples were stained with the fluorescently labeled antibody for the neuronal marker peripherin and nuclear stain DAPI to visualize changes in epithelial innervation and myenteric plexus of the offspring due to maternal antibiotic use. **(C)** Z-slice images prepared with CLARITY taken of the colon of P2 mice to visualize changes in epithelial innervation of the Abx group versus the control group. Tissue samples were stained with the fluorescently labeled antibody for the neuronal marker peripherin as well as the nuclear stain DAPI. **(D)** CLARITY images taken of the longitudinal muscle myenteric plexus (LMMP) of the colon of 3-week-old mice to visualize changes in myenteric plexus density of the Abx group versus the control group. Tissue

samples were stained with the labeled antibody for the neuronal marker peripherin as well as the nuclear stain DAPI. **(E)** Quantification of fluorescence intensity measuring epithelial innervation using Image J software. Fluorescence from the Abx group was calculated as a percentage of the total average fluorescence found in the control group, with the control group fluorescence set at 100%. To calculate this, every Abx group image quantified was divided by the average control group intensity. **(F)** Quantification of fluorescence intensity to measure myenteric plexus density using Image J software. Fluorescence from the Abx group was calculated as a percentage of the total average fluorescence found in the control group, with the control group fluorescence set at 100%. To calculate this, every Abx group image quantified was divided by the average control group intensity. Unpaired two-tailed t-tests were used for statistical significance with $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. n= 3/4 and n= 3/5 for control and Abx groups in (E) respectively, n= 3/4 and n= 3/5 for control and Abx groups in (F) respectively.

*Maternal Antibiotic Treatment Causes Deficits in the Number of SOX-10 Positive Cells per Ganglion in Offspring*

The number of SOX-10 positive cells per ganglion were measured by quantifying the confocal images of frozen whole ileal sections stained with markers for SOX-10 and DAPI on Image J software. In P2 Abx offspring, the amount of SOX-10 positive cells significantly decreased per ganglion (n=3) compared to the number of positive SOX-10 cells identified per ganglion in the control group (n=3) ($p$ value= <0.0001 for P2, $p$ value= 0.0002 for Wk3) (Figure 4B). Glial fibrillary acidic protein (GFAP) was co-stained with SOX-10 but was not used for quantification.

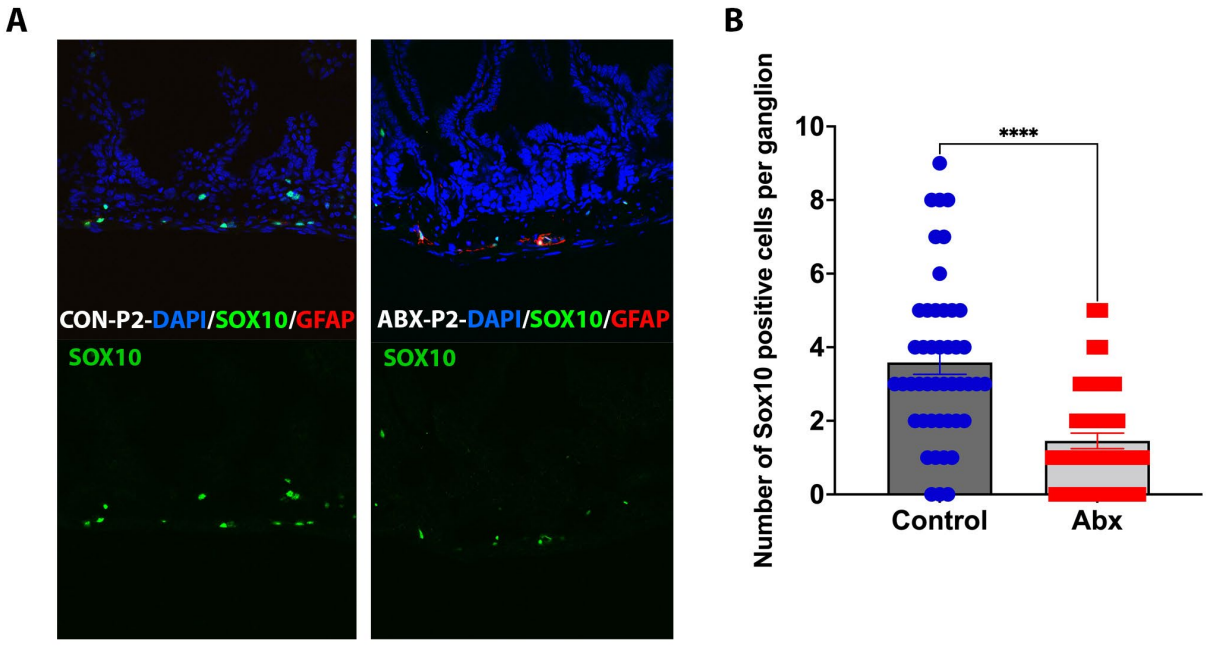



**Figure 4: Maternal antibiotic treatment causes deficits in the number of SOX-10 positive cells per ganglion in offspring. (A)** 5 µm Ileal sectioned tissue samples were stained for with the labeled antibodies for the markers SOX-10 (green) and GFAP (red) as well as the nuclear stain DAPI (blue). The green channel isolated for SOX-10 was completed with Image J software. Photos were taken with the Olympus FV1000 confocal microscope at 40x magnification. **(B)** Confocal images were analyzed for the number of SOX-10 positive cells per ganglia using image J software. Unpaired two-tailed t-tests were used for statistical significance with *$p$< 0.05, **$p$< 0.01, ***p< 0.001, and ****$p$< 0.0001. n= 3 and n= 3 for control and Abx groups in (B) respectively.

## Discussion

*Overall Findings and Importance*

Throughout the research process, we aimed to determine if there were detrimental effects on juvenile gut motility and ENS development when the maternal microbiome was suppressed during gestation. Research has already shown the importance of the gut microbiome on the modulation of the GI tract where the microbiota has a bi-directional relationship with gut motor patterns as well as gut sensorimotor functions (Quiqley et al., 2011). In this study, we showed initial data pointing to the maternal microbial environment having a larger influence on ENS growth than previously hypothesized. Overall, the results of this study help clearly point to the connection between a depleted maternal microbial environment and abnormalities in gut motility

and ENS development of offspring. Both the GI motility assays and imaging quantification data point to a combination of functional and structural deficits of the GI tract and ENS of offspring due to maternal antibiotic use during gestation.

*Validation of the Antibiotic Model*

By conducting a qPCR analysis, we were able to validate the model that the antibiotic cocktail used greatly suppresses the gut flora found in the gut of pregnant mothers. These data support the claim that antibiotics will suppress the gut flora and mimics that of a germ-free model. This allowed us to confidently conduct our experiment knowing that our model of antibiotic use was implemented correctly and depleted the maternal microbiome.

*Functional GI Issues of Offspring Born from Pregnant Mothers Treated with Antibiotics*

All the functional assays completed to study GI transit and motility showed signs of deficits through slower and decreased movement through the intestinal tract. Our data demonstrates similarities to results found in previous experiments that showed that antibiotics administered directly to 3-week-old mice would induce gut dysbiosis and slow transit time through the GI tract (Caputi et al., 2017). Not only was there a statistically significant increase in total GI transit time found through tracking red carmine dye movement (Figure 2A), but also the FITC-labeled dextran assay showed that motility in the Abx group was much slower than the control group (Figure 2D). While conventional mice empty their small intestine almost fully in 4 hours, the Abx mice in this study showed signs of constipation as the FITC-labeled dextran was present along the small intestine and was rarely found in any section of the colon past the cecum at the 30-minute mark (Figure 2D). Along with a reduced stool frequency seen in 3-week-old Abx mice, we observed that the abnormalities included constipation (Figure 2C). This data can further prove a connection between a depleted maternal microbiome and functional deficits in

the GI tract. The only inconsistent result found was that there was not a statistically significant difference in stool water weight in 3-week-old Abx offspring (Figure 2B). This assay, which tests for diarrhea-like stool conditions, only highlights that we cannot confirm that our experimental methods increase the possibility of diarrhea-like stool.

*Structural Issues of Offspring Born from Pregnant Mothers Treated with Antibiotics*

The combination of CLARITY, as well as imaging data from whole ileal frozen sections, has provided evidence that there is a relationship between maternal antibiotic use and deficits found in the GI tract structure of the offspring. We utilized CLARITY as it is an important tool discovered recently to image fully intact tissue samples. In 2013, this method was named one of the most notable breakthroughs by *Science* as it made tissue samples durable enough to stain and image unlike previous methods developed ("2013 Runners-Up. CLARITY makes it perfectly clear", 2013). This technique also allows us to perform confocal z-stack images and recreate 3-dimensional models of the tissue, which creates the opportunity to measure the densities, microarchitecture, and cell-cell interaction among various cell types more authentically than from 2-dimensional ileal section images (Chandrasekharan & Neish, 2021). In this study, markers for peripherin and SOX-10 were utilized due to their ability to accurately track neuronal cell development. Peripherin, which is an intermediate filament protein, has become a widely used marker for developing peripheral neurons including ganglion cells in the ENS. In a previous study by Szabolcs and his team, the researchers found that using the marker for peripherin allowed them to quantify abnormal ganglion cell numbers which have become important for identifying developmental issues in the ENS. They also discovered that peripherin was the most accurate immunofluorescent marker for enteric ganglion cell quantification. As a result, it has become one of the most utilized markers to study deficits and abnormalities in the ENS

(Szabolcs et al., 1996). Secondly, we used a marker for SOX-10, which is a transcription factor involved in the formation of migratory multipotent neural crest (NC) progenitors as well as the differentiation of glial cells. Recently, SOX-10 has become commonly studied due to its connection to ENS abnormalities such as Waardenburg-Hirschsprung disease. Hirschsprung's disease is a congenital bowel disorder characterized by decreased intestinal innervation as well as reductions in the number of ganglion cells in the myenteric and submucosal plexus of the distal colon (Szylberg & Marszalek, 2014). Moreover, SOX-10 gene deficits such as haploinsufficiency have been recognized as factors that cause intestinal obstruction and chronic constipation (Paratore et al., 2002).

The significant structural deficits found in the ENS of the offspring of the antibiotic-exposed mice support the data found in the functional GI motility assays. By utilizing CLARITY, we were able to prove a deficit in the development of the myenteric plexus and the epithelial innervations in both P2 and 3-week-old Abx mice by staining the tissue for peripherin (Figure3E, F). Imaging of frozen ileal sections of the gut of P2 mice also showed that there were deficits in neuronal cell growth in the Abx mice. There were decreased levels of cell bodies positive for SOX-10 per ganglion in Abx mice compared to control group mice (Figure 4B).

In the experiment, both markers help support the hypothesis that a depleted maternal microbiome during gestation will cause deficits in the ENS development of offspring. The reductions in the number of SOX-10 positive cells and the reduction in peripherin in the cells correlate well with the functional results found in the GI motility assays. The slower transit times as well as the decreased ability to move through the colon mirror the symptoms reported in

previous studies regarding both Hirschsprung's disease and a reduction in SOX-10 positive cells (Szylberg & Marszalek, 2014).

*Limitations*

While this study can help to push the discussion forward towards restricting antibiotic use in humans, there are still some limitations regarding the generalizability of the study. To begin, this study was limited to mice. As a result, ENS deficits in humans still need to be investigated. Secondly, humans do not continually use antibiotics for long periods of time such as during the whole period of pregnancy. It is also unrealistic to assume that human patients will be prescribed multiple antibiotics at once. As a result, researchers must be careful with the full generalizability of this study. There is the possibility that short-term antibiotic use during fetal development will not show this same effect as the gut flora could recolonize and support development again. Also, there is the possibility that instead of the lack of a gut microbiome limiting ENS development and gut motility, the antibiotics themselves may be toxic to the body and limit juvenile development directly**.** Previous studies have reported that prolonged antibiotic use in humans can lead to detrimental neurological issues, especially to the CNS (Grill & Maganti, 2011). Lastly, another limitation of the study, due to time and resource restrictions, was the inability to track the effects of maternal antibiotic use on offspring at maturity. While we were able to track maternal antibiotics use on juvenile mice, we were not able to track the effects past 3 weeks. This study can only help support the evidence that maternal antibiotic use during pregnancy is detrimental to offspring up to 3 weeks of age.

## Conclusion

This study was undertaken to continue the work done previously to investigate antibiotic use and nervous system development. Following in the footsteps of studies such as Barbian et al., 2020 as well as a Vuong et al., 2020, we further studied the effects of the maternal gut microbiome and its ability to modulate juvenile development. This study was able to add valuable information to this field of human gut microbiome research showing both structural deficits in the ENS and functional issues of the GI tract due to the depletion of the maternal microbiome during pregnancy. One can also conclude that there must be communication between the maternal microbiome, the uterus, and the fetus through signaling factors such as metabolites and other signaling molecules due to this deficit in development. In the end, it is important to note that the depletion of the microbiome during pregnancy can be detrimental and should lead to more discussions on restricting the use of antibiotics during human pregnancy.

While impactful implications discussed here are a promising start to the discussion of the connection between the maternal microbial environment and ENS development, the ability to firmly show casual relationships between the maternal microbiome and development is highly contingent on the production of several key findings in the future. In the future, it will be important to dissect the mechanism of communication in these signaling pathways between the mother and fetus. longitudinal studies focusing on long-term antibiotic use during the fetal period and early on postnatally could also be an important first step to supporting the generalizability of this research. The use of short term as well as narrow-spectrum antibiotics would also help us understand the relationship between a depleted gut flora in pregnant mothers and fetal development of the ENS. Another important step would be to conduct the same experiment but with germ-free mice. This would allow researchers to confirm that the disruption of the maternal

gut microbiome is the cause of the developmental issues of the ENS and not antibiotic toxicity. Lastly, an important forward path would be to investigate the ability to reverse the effects of gut dysbiosis. This could be evaluated by determining the benefits of probiotics after maternal antibiotic use. Dr. Andrew Neish and Dr. Rheinallt Jones at Emory University found that probiotics (Lactobacillus rhamnosus GG/LGG) can improve GI motility by activating pathways such as the formyl peptide receptor 1 (FPR1) signaling pathway as well as stimulate gut epithelial proliferation (Chandrasekharan et al., Gastroenterology, 2019) (Jones et al., 2013). As a result, it would be beneficial to see if there is a similar effect with probiotics restricting the ability of antibiotics to cause.

## References

Barbian, M.E., Owens, J.A., Naudin, C.R. *et al.* Butyrate supplementation to pregnant mice elicits cytoprotection against colonic injury in the offspring. *Pediatr Res* (2021). https://doi.org/10.1038/s41390-021-01767-1

Buffington, S. A., Di Prisco, G. V., Auchtung, T. A., Ajami, N. J., Petrosino, J. F., & Costa-Mattioli, M. (2016). Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. *Cell*, *165*(7), 1762–1775. https://doi.org/10.1016/j.cell.2016.06.001

Caputi, V., Marsilio, I., Filpa, V., Cerantola, S., Orso, G., Bistoletti, M., Paccagnella, N., De Martin, S., Montopoli, M., Dall'Acqua, S., Crema, F., Di Gangi, I. M., Galuppini, F., Lante, I., Bogialli, S., Rugge, M., Debetto, P., Giaroni, C., & Giron, M. C. (2017). Antibiotic-induced dysbiosis of the microbiota impairs gut neuromuscular function in juvenile mice. *British journal of pharmacology*, *174*(20), 3623–3639. https://doi.org/10.1111/bph.13965)

Carabotti, M., Scirocco, A., Maselli, M. A., & Severi, C. (2015). The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. *Annals of gastroenterology*, *28*(2), 203–209

Chandrasekharan B, Saeedi BJ, Alam A, Houser M, Srinivasan S, Tansey M, Jones R, Nusrat A, Neish AS. Interactions Between Commensal Bacteria and Enteric Neurons, via FPR1 Induction of ROS, Increase Gastrointestinal Motility in Mice. Gastroenterology. 2019 Jul;157(1):179-192.e2. doi: 10.1053/j.gastro.2019.03.045. Epub 2019 Mar 28. PMID: 30930024.

Chung, K., Wallace, J., Kim, S. Y., Kalyanasundaram, S., Andalman, A. S., Davidson, T. J., Mirzabekov, J. J., Zalocusky, K. A., Mattis, J., Denisin, A. K., Pak, S., Bernstein, H., Ramakrishnan, C., Grosenick, L., Gradinaru, V., & Deisseroth, K. (2013). Structural and molecular interrogation of intact biological systems. *Nature*, *497*(7449), 332–337. https://doi.org/10.1038/nature12107

Codagnone, Martin G., et al. "Microbiota and Neurodevelopmental Trajectories: Role of Maternal and Early-Life Nutrition." *Annals of Nutrition and Metabolism*, vol. 74, no. Suppl. 2, 2019, pp. 16–27., https://doi.org/10.1159/000499144.

Collins J, Borojevic R, Verdu EF, Huizinga JD, Ratcliffe EM. Intestinal microbiota influence the early postnatal development of the enteric nervous system. Neurogastroenterol Motil. 2014 Jan;26(1):98-107. doi: 10.1111/nmo.12236. Epub 2013 Oct 8. PMID: 24329946.

Costa M, Brookes SJH, Hennig GW. Anatomy and physiology of the enteric nervous system *Gut* 2000;47:iv15-iv19.

dDe Jonge, L., Bos, H. J., van Langen, I. M., de Jong-van den Berg, L. T., & Bakker, M. K. (2013). Antibiotics prescribed before, during and after pregnancy in the Netherlands: A drug utilization study. Pharmacoepidemiology and Drug Safety, 23(1), 60–68. https://doi.org/10.1002/pds.3492

Gareau, M. G., Jury, J., MacQueen, G., Sherman, P. M., & Perdue, M. H. (2007). Probiotic treatment of rat pups normalises corticosterone release and ameliorates colonic dysfunction induced by maternal separation. *Gut*, *56*(11), 1522–1528. https://doi.org/10.1136/gut.2006.117176

Grill, M. F., & Maganti, R. K. (2011). Neurotoxic effects associated with antibiotic use: management considerations. *British journal of clinical pharmacology*, *72*(3), 381–393. https://doi.org/10.1111/j.1365-2125.2011.03991

Gur, Tamar L., et al. "Prenatal stress affects placental cytokines and Nnurotrophins, commensal microbes, and anxiety-like behavior in adult female offspring." *Brain, Behavior, and Immunity*, vol. 64, 2017, pp. 50–58., https://doi.org/10.1016/j.bbi.2016.12.021.

Jiménez E, Marín ML, Martín R, Odriozola JM, Olivares M, Xaus J, Fernández L, Rodríguez JM. Is meconium from healthy newborns actually sterile? Res Microbiol. 2008 Apr;159(3):187-93. doi: 10.1016/j.resmic.2007.12.007. Epub 2008 Jan 11. PMID: 18281199.

Jones, R.M., Luo, L., Ardita, C.S., Richardson, A.N., Kwon, Y.M., Mercante, J.W., Alam, A., Gates, C.L., Wu, H., Swanson, P.A., Lambeth, J.D., Denning, P.W. and Neish, A.S. (2013), Symbiotic lactobacilli stimulate gut epithelial proliferation *via* Nox-mediated generation of reactive oxygen species. The EMBO Journal, 32: 3017-3028. https://doi.org/10.1038/emboj.2013.224

Koester, Sean T., et al. "Marker-Based Assays for Studying Gut Transit in Gnotobiotic and Conventional Mouse Models." *STAR Protocols*, vol. 2, no. 4, 2021, p. 100938., https://doi.org/10.1016/j.xpro.2021.100938.

Larsbrink, J., Rogers, T. E., Hemsworth, G. R., McKee, L. S., Tauzin, A. S., Spadiut, O., Klinter, S., Pudlo, N. A., Urs, K., Koropatkin, N. M., Creagh, A. L., Haynes, C. A., Kelly, A. G., Cederholm, S. N., Davies, G. J., Martens, E. C., & Brumer, H. (2014). A discrete genetic locus confers xyloglucan metabolism in select human gut Bacteroidetes. Nature, 506(7489), 498–502. https://doi.org/10.1038/nature12907

Lauder AP, Roche AM, Sherrill-Mix S, Bailey A, Laughlin AL, Bittinger K, Leite R, Elovitz MA, Parry S, Bushman FD. Comparison of placenta samples with contamination controls does not provide evidence for a distinct placenta microbiota. Microbiome. 2016 Jun 23;4(1):29. doi: 10.1186/s40168-016-0172-3. PMID: 27338728; PMCID: PMC4917942.

Ley, R. E., Peterson, D. A., & Gordon, J. I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*, *124*(4), 837–848. https://doi.org/10.1016/j.cell.2006.02.017

Longstreth, G. F., Thompson, W. G., Chey, W. D., Houghton, L. A., Mearin, F., & Spiller, R. C. (2006). Functional bowel disorders. *Gastroenterology*, *130*(5), 1480–1491. https://doi.org/10.1053/j.gastro.2005.11.061

Mackie, R. I., Sghir, A., & Gaskins, H. R. (1999). Developmental microbial ecology of the neonatal gastrointestinal tract. *The American Journal of Clinical Nutrition*, *69*(5). https://doi.org/10.1093/ajcn/69.5.1035s

Nezami, B. G., & Srinivasan, S. (2010). Enteric nervous system in the small intestine: pathophysiology and clinical implications. *Current gastroenterology reports*, *12*(5), 358–365. https://doi.org/10.1007/s11894-010-0129-9

Palleja, A., Mikkelsen, K.H., Forslund, S.K. *et al.* Recovery of gut microbiota of healthy adults following antibiotic exposure. *Nat Microbiol* 3, 1255–1265 (2018). https://doi.org/10.1038/s41564-018-0257-9

Paratore, C. (2002). SOX-10 haploinsufficiency affects maintenance of progenitor cells in a mouse model of Hirschsprung Disease. *Human Molecular Genetics*, *11*(24), 3075–3085. https://doi.org/10.1093/hmg/11.24.3075

Perez-Muñoz, M. E., Arrieta, M. C., Ramer-Tait, A. E., & Walter, J. (2017). A critical assessment of the "sterile womb" and "in utero colonization" hypotheses: implications for research on the pioneer infant microbiome. *Microbiome*, *5*(1), 48. https://doi.org/10.1186/s40168-017-0268-4

Qv, L., Yang, Z., Yao, M., Mao, S., Li, Y., Zhang, J., & Li, L. (2020). Methods for establishment and maintenance of germ-free rat models. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.01148

Rao, M., Gershon, M. The bowel and beyond: the enteric nervous system in neurological disorders. *Nat Rev Gastroenterol Hepatol* 13, 517–528 (2016). https://doi.org/10.1038/nrgastro.2016.107

Saari, A., Virta, L. J., Sankilampi, U., Dunkel, L., & Saxen, H. (2015). Antibiotic exposure in infancy and risk of being overweight in the first 24 months of life. *Pediatrics*, *135*(4), 617–626. https://doi.org/10.1542/peds.2014-3407

Satokari R, Grönroos T, Laitinen K, Salminen S, Isolauri E. Bifidobacterium and Lactobacillus DNA in the human placenta. Lett Appl Microbiol. 2009 Jan;48(1):8-12. doi: 10.1111/j.1472-765X.2008.02475.x. Epub 2008 Oct 17. PMID: 19018955.

Schwarzer, M. (2018). Gut microbiota. *Current Opinion in Clinical Nutrition & Metabolic Care*, *21*(3), 179–183. https://doi.org/10.1097/mco.0000000000000463

Stiemsma LT, Michels KB. The Role of the Microbiome in the Developmental Origins of Health and Disease. Pediatrics. 2018 Apr;141(4):e20172437. doi: 10.1542/peds.2017-2437. Epub 2018 Mar 8. PMID: 29519955; PMCID: PMC5869344.

Subramanian, S., Huq, S., Yatsunenko, T., Haque, R., Mahfuz, M., Alam, M. A., Benezra, A., DeStefano, J., Meier, M. F., Muegge, B. D., Barratt, M. J., VanArendonk, L. G., Zhang, Q., Province, M. A., Petri, W. A., Jr, Ahmed, T., & Gordon, J. I. (2014). Persistent gut microbiota immaturity in malnourished Bangladeshi children. Nature, 510(7505), 417–421. https://doi.org/10.1038/nature13421

Szabolcs, M., Visser, J., Shelanski, M., O'Toole, K., & Schullinger, J. (1996). Peripherin: A novel marker for the immunohistochemical study of malformations of the Enteric Nervous System. *Fetal and Pediatric Pathology*, *16*(1), 51–70. https://doi.org/10.3109/15513819609168661

Szylberg, L., & Marszałek, A. (2014). Diagnosis of Hirschsprung's disease with particular emphasis on histopathology. A systematic review of current literature. *Przeglad gastroenterologiczny*, *9*(5), 264–269. https://doi.org/10.5114/pg.2014.46160

Tapiainen, T., Koivusaari, P., Brinkac, L. *et al.* Impact of intrapartum and postnatal antibiotics on the gut microbiome and emergence of antimicrobial resistance in infants. *Sci Rep* 9, 10635 (2019). https://doi.org/10.1038/s41598-019-46964-5

Theis, K. R., Romero, R., Greenberg, J. M., Winters, A. D., Garcia-Flores, V., Motomura, K., Ahmad, M. M., Galaz, J., Arenas-Hernandez, M., & Gomez-Lopez, N. (2020). No Consistent Evidence for Microbiota in Murine Placental and Fetal Tissues. *mSphere*, *5*(1), e00933-19. https://doi.org/10.1128/mSphere.00933-19

Uzan-Yulzari, A., Turta, O., Belogolovski, A. *et al.* Neonatal antibiotic exposure impairs child growth during the first six years of life by perturbing intestinal microbial colonization. *Nat Commun* 12, 443 (2021). https://doi.org/10.1038/s41467-020-20495-4

Vandeputte D, Falony G, Vieira-Silva S, *et al* Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates. *Gut* 2016;65:57-62.

Vuong, H.E., Pronovost, G.N., Williams, D.W. et al. The maternal microbiome modulates fetal neurodevelopment in mice. Nature 586, 281–286 (2020). https://doi.org/10.1038/s41586-020-2745-3

Yatsunenko, T., Rey, F., Manary, M. *et al.* Human gut microbiome viewed across age and geography. *Nature* 486, 222–227 (2012). https://doi.org/10.1038/nature11053

2013 Runners-Up. CLARITY makes it perfectly clear. Science. 2013 Dec 20;342(6165):1434-5. doi: 10.1126/science.342.6165.1434-b. PMID: 24357286.