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Walter Moises Avila                          April 10, 2023

A Taxonomic Approach to Understanding Diet-Induced Changes in Mouse Fecal and Ileal
Immunoglobulin A (IgA) Concentrations

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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The gut microbiome influences immunity and mucosal barrier maintenance and processes in the small intestine. Changes in diet alter the gut microbiota composition, impacting the gut immune system and its constituents. Immunoglobulin A (IgA), an antibody secreted by plasma cells in the intestine that helps defend against harmful pathogens, is also impacted by dietary change. Our preliminary research has shown that mice weaned to a low-fermentable-fiber only (LFF) diet have significantly lower concentrations of ileal and fecal IgA than mice born and maintained on a standard chow diet (SCD). However, the mechanisms underlying this change remain largely unclear. One explanation could be that B cells in the guts of LFF mice produce less IgA in response to the LFF diet. It is also possible that the LFF diet significantly alters the gut microbiome composition, decreasing the repertoire of clonal plasma cells.

To test these hypotheses, we incubated LFF and SCD mouse ileal and fecal contents with IgA-targeting antibodies. These were bound to IgA attached to ileal bacteria, identifying them with a fluorescent signal and allowing them to be sorted. Sorted groups will undergo 16s rRNA sequencing to identify the attached bacteria. If no significant differences are found in the bacteria bound by IgA in LFF or SCD ileum and feces contents, this will indicate possible overall IgA reduction because of dietary change. Conversely, if differences are found, this will imply that the B cell repertoire is affected by dietary modification-induced microbiota changes.

To uncover which bacteria orders may impact IgA production, we produced predictive LASSO models that showed that the following bacterial orders had the highest explanatory power in predicting Lamina propria (LP) IgA plasma and B cell frequencies out of total lymphocytes in SCD and LFF mice: *CyanobacteriaMLE1-12*, *EW055*, *Clostridiales*, *Enterobacteriales*, and *Xanthomonadales*. Our modeling results indicate that these bacteria orders significantly explain IgA-producing immune cell frequencies and their roles will be investigated further when analyzing the 16s sequencing data from our experiment. Ultimately, this experiment will provide insight into how the absence of dietary fiber affects IgA expression and influences its role in defending against gut pathogens.

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# A TAXONOMIC APPROACH TO

# UNDERSTANDING DIET-INDUCED CHANGES IN MOUSE FECAL AND ILEAL IMMUNOGLOBULIN A

# CONCENTRATIONS

Worked on by Walter Moises Avila[1], Charlotte Royer M.S[2], Jarreth Caldwell[2], Dormarie Rivera-Rodriguez[2], Naomi Rodriguez-Marino[2], and Luisa Cervantes-Barragan, Ph.D.[2,#]

Experimentation Timeline: March 7th, 2022, to March 10th, 2023


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**SUMMARY**

The gut microbiome influences immunity and mucosal barrier maintenance and processes in the small intestine. Changes in diet alter the gut microbiota composition, impacting the gut immune system and its constituents. Immunoglobulin A (IgA), an antibody secreted by plasma cells in the intestine that helps defend against harmful pathogens, is also impacted by dietary change. Our preliminary research has shown that mice weaned to a low-fermentable-fiber only (LFF) diet have significantly lower concentrations of ileal and fecal IgA than mice born and maintained on a standard chow diet (SCD). However, the mechanisms underlying this change remain largely unclear. One explanation could be that B cells in the guts of LFF mice produce less IgA in response to the LFF diet. It is also possible that the LFF diet significantly alters the gut microbiome composition, decreasing the repertoire of clonal plasma cells.

To test these hypotheses, we incubated LFF and SCD mouse ileal and fecal contents with IgA-targeting antibodies. These were bound to IgA attached to ileal bacteria, identifying them with a fluorescent signal and allowing them to be sorted. Sorted groups will undergo 16s rRNA sequencing to identify the attached bacteria. If no significant differences are found in the bacteria bound by IgA in LFF or SCD ileum and feces contents, this will indicate possible overall IgA reduction because of dietary change. Conversely, if differences are found, this will imply that the B cell repertoire is primarily affected by changes in the microbiota induced by dietary modifications.

To uncover which bacteria orders may impact IgA production, we produced predictive LASSO models that showed that the following bacterial orders had the highest explanatory power in predicting Lamina propria (LP) IgA plasma and B cell frequencies out of total lymphocytes in SCD and LFF mice: CyanobacteriaMLE1-12, EW055, Clostridiales, Enterobacteriales, and Xanthomonadales. Our modeling results indicate that these bacteria orders significantly explain IgA-producing immune cell frequencies and their roles will be investigated further when analyzing the 16s sequencing data from our experiment. Ultimately, this experiment will provide insight into how the absence of dietary fiber affects IgA expression and influences its role in defending against gut pathogens.

**INTRODUCTION**

Immunoglobulin A (IgA) are a type of antibody made by plasma cells in the gut and other mucosal tissues and are the most abundantly produced antibodies in humans.[1] These antibodies bind to bacteria near the epithelium and prevent their direct interaction with the host via immune exclusion; IgA helps remove antigens and pathogens from the intestinal lumen by blocking their access to epithelial cells and promote entrapping them in mucus for eventual removal.[2] Furthermore, IgA has homeostatic properties stemming from their interactions with microbiota populations. These properties are determined largely by antibody specificity against microbial consortia. Overall, IgA is a crucial antibody that protects the host's mucosal surfaces against harmful pathogens and mediates host-microbiota homeostasis.[3]

IgA's production depends on the integrity of the gut microbiota composition. Intestinal commensal bacteria residing within this diverse community are crucial in maintaining immune homeostasis and protecting against pathogens by providing signals that can improve the host's immune response.[4] Segmented filamentous bacteria (SFB) are one example of a commensal, bacteria that modulates the intestinal immune system. SFB are auxtotrophic, autochthonous, anaerobic, gram-positive bacteria present in the intestines of many vertebrates, including humans. SFB has been shown to increase the number of IgA-secreting cells in the intestinal mucosa of formerly germ-free (GF) mice inoculated with SFB[5] and stimulate natural IgA production in gut tissue.[6]

The microbiota's impact on immune system development has been documented extensively, but diet's influence on the microbiota and immune system is less understood. Specifically, changes in dietary fiber content can alter the microbiome and mucosal immune system function significantly as it serves as a major gut microbiota food source.[7] Low-fiber diets are associated with a low diversity and proinflammatory microbiota and have thus been associated with the pathogenesis of many inflammatory diseases and infections.[8]

To investigate if fiber content in the diet influences intestinal IgA production, we previously analyzed fecal and ileal IgA concentrations in mice fed diets with standard or low fiber content. In this analysis, mice were reared and maintained on a standard chow diet (SCD; PicoLab rodent diet 5053) (SCD/SCD) consisting of 20% protein and 4.5% fat, reared on an SCD

diet and then weaned to a low-fermentable fiber diet (LFF; Research Diet AIN-93G) (SCD/LFF) where fermentable fiber was substituted with microcrystalline cellulose, and reared and maintained on an LFF diet (LFF/LFF). These analyses showed that the latter two groups had significantly lower IgA concentrations than SCD/SCD mice (**Figure 1**). Therefore, these studies have provided evidence of the immunological impact of low-fiber diets; mice fed an LFF diet experience a deficiency in fecal and ileal IgA relative to mice fed standard chow.

Our observations present the possibility that the LFF diet impacts immune functioning via alteration of the gut microbiota and its crucial inhabitants, such as SFB. This finding is important because IgA deficiency is the most widespread immunodeficiency in humans and is associated with a predisposition for several diseases, such as metabolic syndrome.[9] However, there is no single underlying cause of human IgA deficiency. Familial primary IgA deficiency lacks a common Mendelian inheritance pattern, and secondary IgA deficiency can result from countless factors, such as the use of drugs like penicillamine and antiepileptics.[10] Therefore, all causes of IgA deficiency are not yet fully understood.[11]

Furthermore, little is known about the identities of the bacterial taxa targeted by IgA.[12] Therefore, a taxonomic approach may reveal what bacterial taxa are impacted by the LFF diet and how their absence contributes to lower IgA concentrations. So, we seek to uncover what biological mechanisms lead to lower fecal and ileal IgA concentrations in mice. We will investigate whether these changes are caused directly by perturbations in the small intestinal microbiome by identifying the taxonomic profiles of ileal bacteria interacting with IgA in LFF and SCD mice. We will achieve this by studying bacteria attached to or lacking IgA, referred to here as IgA+ and IgA- fractions, respectively, via immunostaining and fluorescence-activated cell-sorting (FACS).

This approach will reveal if binding of IgA to certain bacterial taxa is impacted by the LFF diet or results in general lower IgA concentrations, but no changes in which bacterial taxa are bound by IgA. The findings from this research will further our understanding of how diet alters the immune system and may inform future studies delineating the possible causes of human IgA deficiency and developing appropriate treatments and therapies.
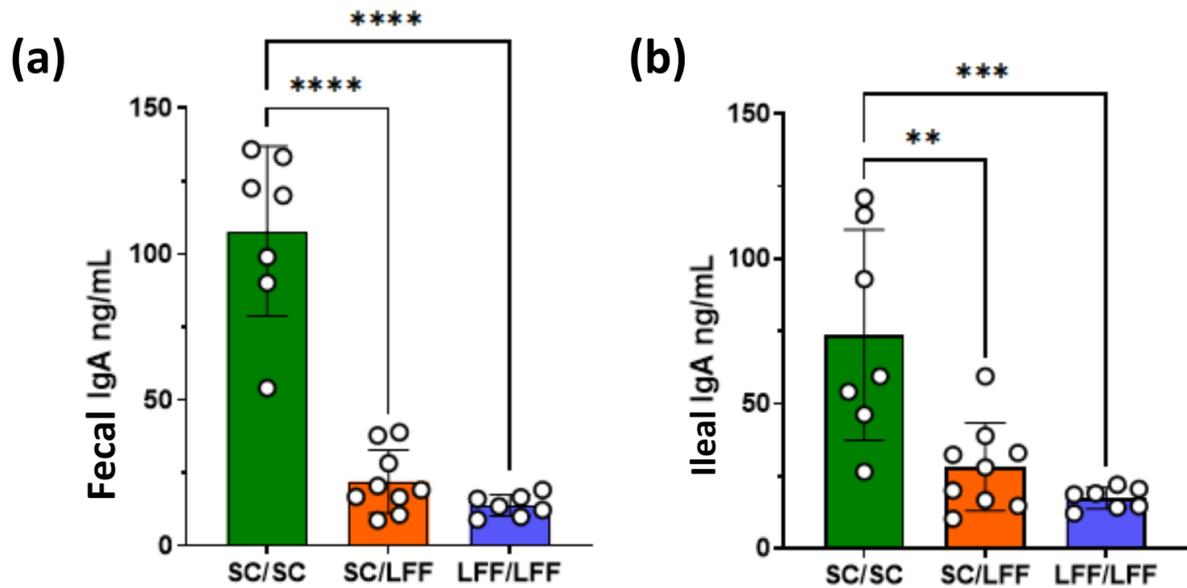
**Figure 1:** Fecal and Ileal IgA concentrations in the intestinal content of mice fed a standard chow (SC) or a low-fiber diet (LFF). Littermate C57BL/6 (B6) mice were bred from females in an SC or LFF diet, weaned, and further fed an SC or LFF diet for 4 weeks. **(a)** Small intestinal contents and **(b)** fecal pellets were collected, and IgA concentrations were measured by ELISA. Bars represent means, symbols represent single mice. Data are representative of 2 independent experiments (n=3-4). Statistical analysis was performed using a Student's *t*-test (**, p<0.01;***, p<0.001; ****, p<0.0001).

## RESEARCH QUESTION AND HYPOTHESES

**Question:** What mechanisms underlie lower fecal and ileal IgA concentrations in LFF mice relative to SCD mice? Will a taxonomic approach provide insights into these mechanisms?

**Hypotheses:**

**1. Bacteria-Induced IgA Loss Hypothesis***:* B cells in the guts of LFF mice produce less IgA on average in response to the LFF diet. By influencing interactions between the gut immune system and gut microbiota, the LFF diet decreases the concentration of IgA antibodies secreted by gut B cells, thereby decreasing the total fecal and ileal IgA concentrations in LFF mice relative to SCD (**Figure 2**).

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**2. Plasma B cell Death or Deletion Hypothesis***: The LFF diet significantly decreases the repertoire of clonal plasma B cells (**Figure 3**).
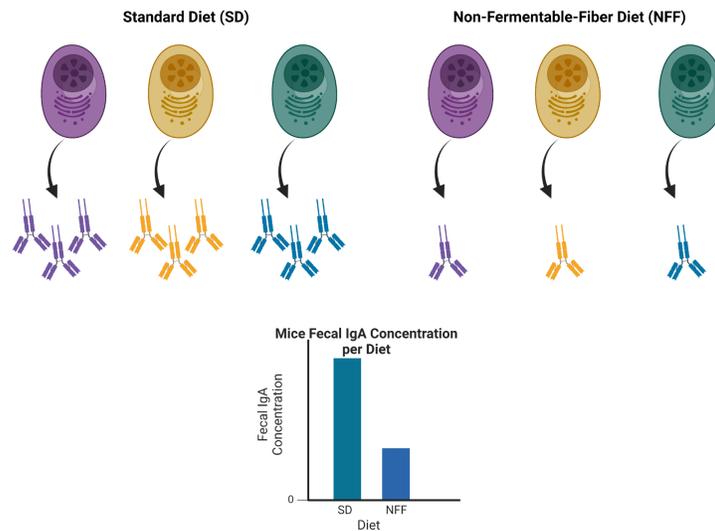


**Figure 2:** Simplified diagram showing three hypothetical B cell populations in the gut immune system that each produce molecularly distinct IgA antibodies that interact with a specific bacterial taxon; the purple cell produces IgA specific to taxon X, the yellow cell produces IgA specific to taxon Y, and the green cell produces IgA specific to taxon Z.
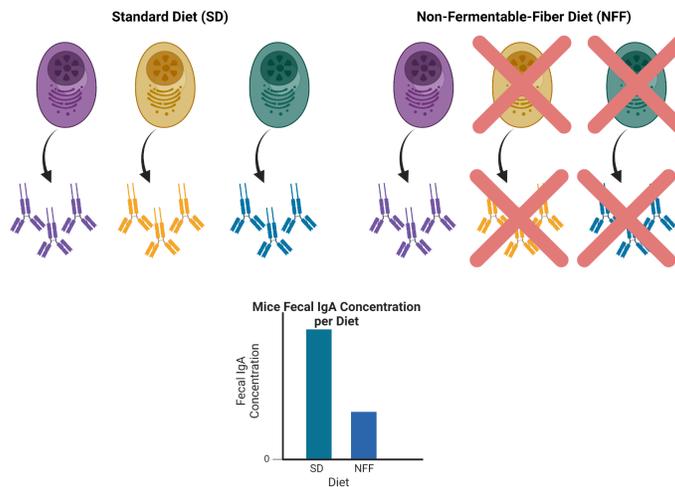


**Figure 3:** The same simplified hypothetical system where the LFF diet reduces the plasma B cell repertoire but not the IgA concentration. The LFF diet eliminates or significantly reduces the

yellow and green B cell populations but not the purple cell population, thereby reducing the total IgA produced.

## BACTERIAL SORT-EXPERIMENTAL DESIGN

To infer the source of fecal and ileal IgA concentration reduction, we took a taxonomic approach comparing bacterial populations among littermate Charles River C57BL/6 (CR B6) mice born on an SCD and weaned to an SCD or LFF diet. We sought to perform the following experiment to make these comparisons: isolate ileal content from mice. Obtain the ileal contents by scraping the ileum, and process those samples to isolate sufficient amounts of intestinal bacteria. Stain fecal samples with fluorescent anti-mouse secondary IgA antibodies that attach to mouse IgA bound to intestinal bacteria. Identify cells bound by secondary IgA antibodies via flow cytometry. Cell-sort the samples, via flow cytometry, into the following four groups: **1**. IgA+ Bacterial Taxa from LFF mice, **2.** IgA- Bacterial Taxa from LFF mice, **3.** IgA+ Bacterial Taxa from SCD mice, **4.** IgA+ Bacterial Taxa from SCD mice. Send sorted samples to 16s rRNA gene sequencing (**Figure 4**).
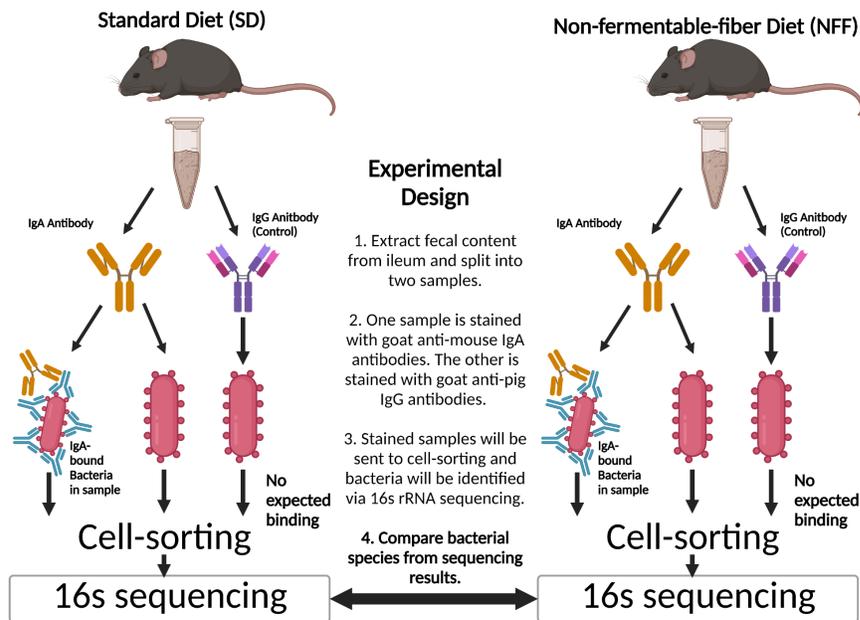
**Figure 4:** Bacterial sort experimental overview design. Ileal content bacteria from mice on fed a SCD/SCD or SCD/LFF diet will be stained with anti-mouse IgA. IgA-bound and non-IgA-bound bacteria (IgA+ and IgA-, respectively) will be cell-sorted and analyzed via 16s RNA sequencing. Anti-pig IgG antibody will be used as negative isotype control.

## BACTERIAL SORT-PROTOCOL STANDARDIZATION (PS)

Bacterial sample outputs, flow cytometry voltage parameters, and fluorophore concentrations required optimization via protocol standardization experiments before reaching the final cell-sorting stages. These optima were tested in cell-sorting trials of SCD mice.

### PS1. Maximizing Bacteria Cell Numbers in Mice Ileal Samples via DTT and IEL Buffer Pre-treatment

Objective:

The first set of experiments to establish the Bacterial sort method determined the number of bacteria extracted from ileal samples, if a particular sample treatment improved these numbers, and if additionally washing treated samples would continue to yield viable bacteria numbers.

The objective of this first set of experiments was to determine a protocol to isolate the largest amount of intestinal bacteria from the small intestine. In the first set of experiments, we collected ileal and large intestine (LI) content from mice. These ileal and LI samples would undergo pre-treatment with Dithiothreitol (DTT) and Intraepithelial lymphocyte (IEL) extraction buffer to be compared to untreated contents. These buffers dissociate the epithelial layer, loosening the mucus to release bacteria and cells. Mouse ileum samples were split for one sample to be stained with IgA or IgG and undergo flow cytometry and the other to undergo quantitative PCR (qPCR) to quantify present bacterial genes. LI contents only underwent flow cytometry (**Figure 5**).

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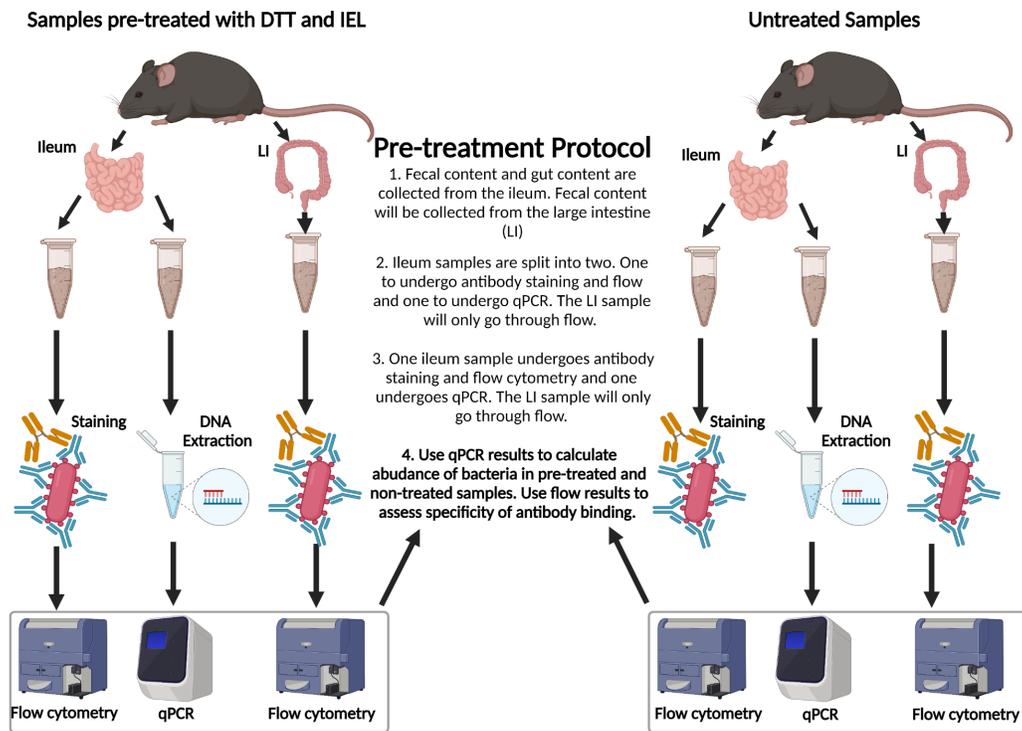
**Figure 5:** Pretreatment protocol. Ileum samples will go through qPCR and flow cytometry. Large intestine samples will only go through flow cytometry. Flow and qPCR results show us the specificity of antibody binding and copy number of bacterial genes, respectively.

**Experiment:**

We gathered two groups of four male Jax mice ($n$ = 4) fed and maintained on a SCD diet. One group's extracted ileal and LI contents received the pre-treatment, whereas the other did not. Pre-treated mice samples were split for one fraction to undergo additional washing and purification, known as the "wash treatment." Ileal and LI samples were stained with either anti-mouse IgA or anti-pig IgG antibodies tagged with the PE fluorophores. Samples were also stained with SYTO BC, a green fluorescent bacterial nucleic acid dye. These samples were measured in flow cytometry to detect IgA+ and IgA- consortia by seeing which sample contents had large enough PE and SYTO BC fluorescences.

DNA was extracted from each mouse sample that was destined for qPCR via the QIAmp DNA Stool mini (QIAGEN) kit. DNA samples underwent qPCR to quantify the number of 16s and SFB gene copies among pre-treated, untreated, and wash-treated mice.

The wash-treatment aimed to emulate how much bacteria would be available after cell-sorting as the cytometer sheath fluid dilutes cells. Furthermore, enumerating our samples' SFB gene copy numbers would indicate sufficient and representative amounts of bacteria extraction as SFB deeply embed into the ileal epithelium surface[13] and are thus difficult to extract.

**Results:**

The flow cytometry analyses return plots showing SYTO BC and antibody fluorescence of all sample entities. The flow cytometry software, FACSDiva, recognizes these two dyes as FIT-C and PE-A, respectively.

Each flow cytometry plot for a particular sample contains four quadrants that categorize sample contents into one of four groups: **Q1:** non-bacteria bound by the antibody of interest, **Q2:** bacteria bound by the antibody of interest, **Q3:** bacteria lacking the antibody of interest, and **Q4:** non-bacteria lacking the antibody of interest (**Figure 6**). Cells fall into one of these quadrants if their fluorophore shines above or below a certain brightness threshold. Gates define these quadrants and are determined by analyzing the compensation controls used to compensate the cytometer that lack one or both of the fluorophores to determine where the fluorophores fluoresce. Q2 and Q3 are the most important quadrants, containing the IgA+ and IgA- populations, respectively, whose cells we seek to obtain.

SFB and 16s gene copy numbers were obtained from all samples and compared to one another (**Figure 7**).
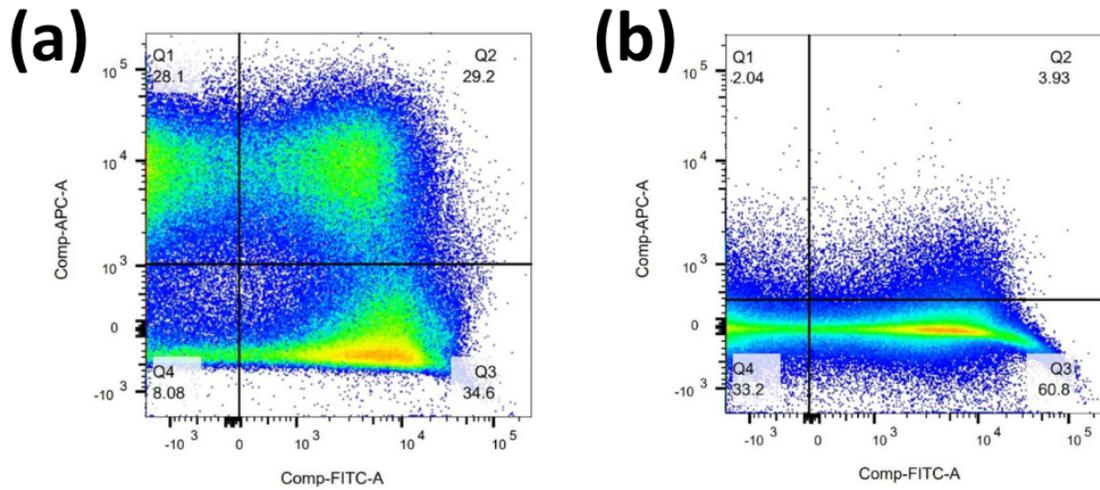
**Figure 6:** LI flow cytometry results for an untreated mouse's LI intestine contents stained with **(a)** IgA-PE or **(b)** IgG-PE gated into four quadrants. Compensation fluorophores are FITC-A and PE-A, which measure SYTO BC and secondary IgA fluorescence, respectively. The quadrant-defining gates are determined by comparison to the flow graphs of the compensation controls. **Q1:** non-bacteria bound by the antibody of interest, **Q2:** bacteria bound by the antibody of interest **Q3:** bacteria lacking the antibody of interest. **Q4:** non-bacteria lacking the antibody of interest. Each quadrant shows the percentage of the analyzed population belonging to it.
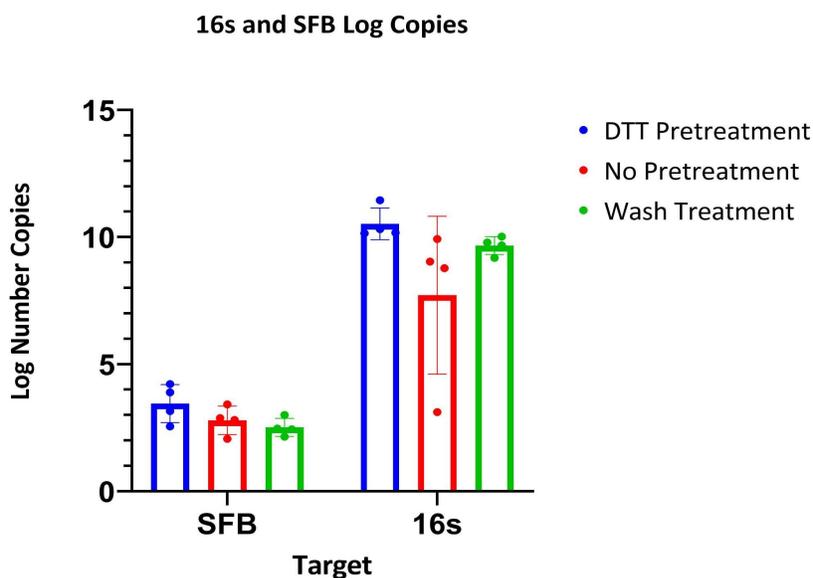
**Figure 7:** qPCR Log number of 16s and SFB copies from the ilea of pre-treated mice, untreated mice, and pre-treated mice whose samples underwent additional washing and purification. Error bars are STDEV. A Student's *t*-test comparing the SFB and 16s copy numbers of each treatment to each other revealed no significant differences at α = 0.05.


**Conclusions:**

Our qPCRs found that SFB and 16s copy numbers do not significantly differ among each treatment. Therefore, the pre-treatment does not significantly increase the copy number despite resulting in the largest copy number out of each treatment. Furthermore, the wash treatment does not significantly decrease the number of bacteria available, meaning we should have sufficient bacteria to sequence after cell sorting.

Despite not statistically significantly increasing the available gene copy number, we chose to pre-treat our samples due to the qualitatively larger number of bacteria it yields.


**PS2. Optimizing Flow Cytometry Fluorescein Isothiocyanate (FIT-C), Side Scatter (SSC), and Forward Scatter (FSC) Parameters to Maximally Reveal Locations of Bacteria in Ileal Samples**


**Objective:**

After developing a sample treatment that improves bacterial availability, our next objective was to ensure the flow cytometer can detect bacteria accurately in ileal samples that contain significant amounts of non-bacterial material. Intializing the flow cytometer requires setting and optimzing the voltages that will accurately identify IgA+ and IgA- fractions among the bacteria interacting with IgA.


**Experiment:**

To prepare the cytometer to accurately identify bacterial populations, we needed to measure pure bacteria samples void of debris and host cells. Therefore, we prepared *E. coli* and *L. reuteri* cultures, stained them with a 1:2000 SYTO BC concentration on ice for 5 minutes, and processed them loosely following the IgA staining protocol in the bacterial sort method to

ultimately measure at different voltages to find the optimal voltages at which the bacterias' locations are most visible.

Specifically, we prepared *E.coli* and *L. reuteri* samples stained with a 1:2000 SYTO BC concentration and a 50% *E.coli* and 50% *L.reuteri sample* stained with SYTO BC as well. Sample controls excluding SYTO BC were included for each SYTO BC-containing sample. Pure bacteria samples were not IgA stained because they were not mouse-native and thus lacked the IgA required by the anti-IgA antibody.

**Results:**

We had to manually adjust the FIT-C, SSC, and FSC voltages while measuring each sample to visually determine which set of parameters optimally identified the bacteria. FIT-C, fluorescein isothiocyanate, is a fluorescent probe that visualizes bacteria and is the name of the wavelength channel it corresponds to. In our experiments, SYTO BC serves as a FIT-C proxy. SSC and FSC refer to the directions of scattered light and canonically correspond to cell size and SS and cell granularity, respectively. Once we decided on an optimal set, we recorded and plotted the flow cytometry results in **Figure 9**.

**Conclusions:**

On measuring these samples, we visually appraised that the following parameters yielded optimally visible bacteria: FIT-C: 400, SSC: 600, and FSC: 230. We would use these voltages when measuring our ileal samples to locate the residing bacteria.
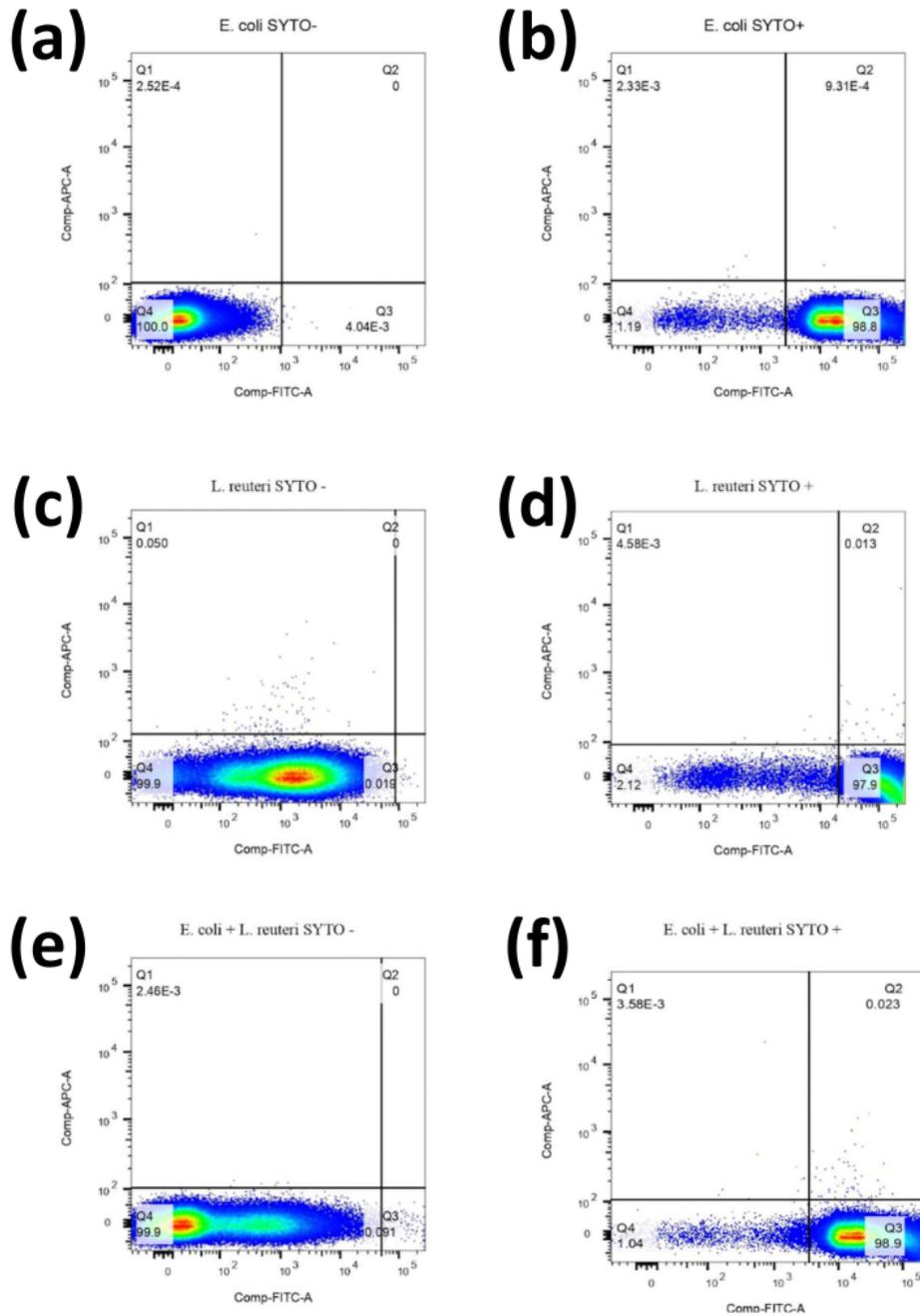
**Figure 8:** Flow cytometry results of **(a)** *E.coli* sans SYTO BC, **(b)** *E.Coli* stained with 1:2000 SYTO BC, **(c)** *L.reuteri* sans SYTO BC, **(d)** *L.reuteri* stained with 1:2000 SYTO BC, SYTO BC**, (e)** *E.coli + L. Reuteri* sans SYTO BC, and **(f)** *E.coli + L. Reuteri* stained with 1:2000 SYTO BC bacteria samples at optimal SSC and FSC voltages of 600 and 230, respectively.

**PS3. Titration of our Bacterial Nucleic Acid Dye, SYTO BC, Stained Onto Pure Bacteria Samples to Determine a Concentration that Optimally Distinguishes Bacteria and Non-Bacteria**

**Objective:**

After optimizing our flow voltages, our next objective was to determine the optimal SYTO BC concentration that would best distinguish bacteria and non-bacteria populations in bacterial cultures that we could now optimally visualize with the voltages derived from the last experiment.

Finding the SYTO BC concentration that most optimally identifies bacterial and non-bacterial fractions in a pure bacteria sample would help identify true bacterial cells in an ileal sample full with non-bacterial material. Therefore, we performed SYTO BC titrations on pure bacteria.

**Experiment:**

We stained seven replicates of a bacteria culture consisting of a 1 to 1 *E.coli* and *L.reuteri* mixture with serially diluted SYTO BC concentrations ranging from 1:2000 to 1:64000. We brought these samples to the cytometer and measured them at the voltages derived from PS2.

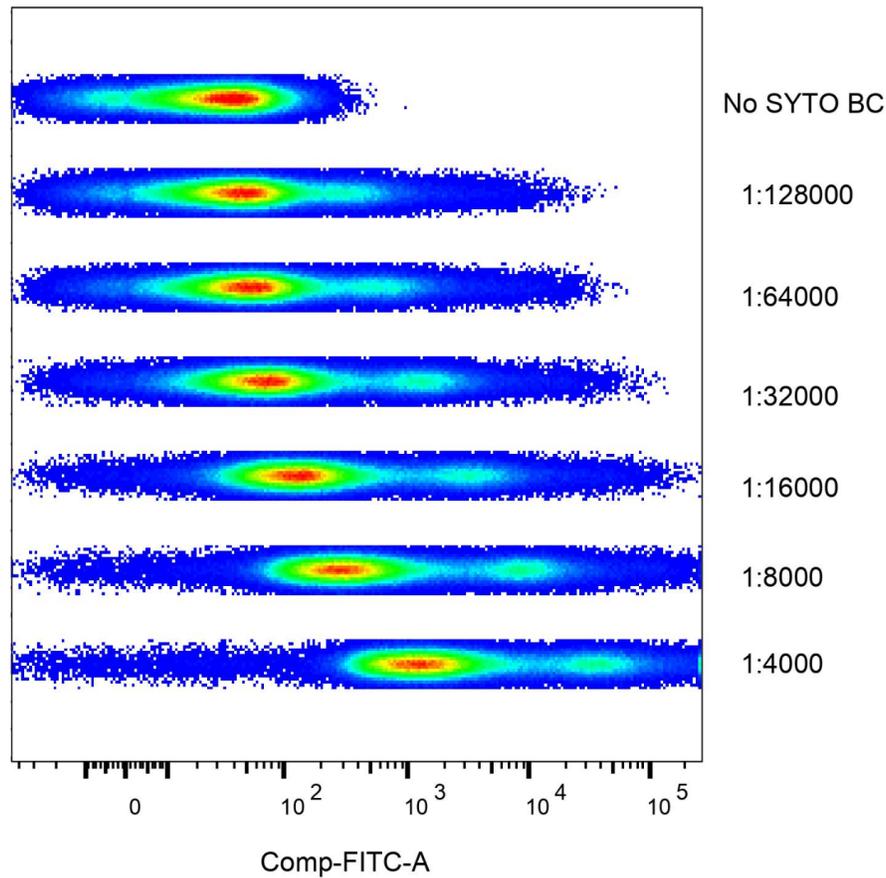**Results:**

Flow cytometry of these samples showed that a 1:8000 concentration yielded the best positive and negative population separation (**Figure 10**).

**Conclusion:**

A 1:8000 concentration yielded the best positive and negative separation.

**Figure 9:** Concatenation of flow cytometry results showing the performance of different SYTO BC concentrations stained onto bacterial samples consisting of a 1 to 1 *E.coli* and *L.reuteri* ratio.

**PS4:  Assessing our Optimal SYTO BC Concentration on Serially Diluted Ileal Samples**

**Objective:**

Next, we sought to stain undiluted, 1:10, and 1:100 ileum sample replicates with this SYTO BC concentration and two more serial dilutions, 1:16000 and 1:32000. Our objective was to see if this optimal SYTO BC concentration could sufficiently distinguish bacteria and non-bacteria in an ileum sample and yield a separation similar to PS3's results. Additionally, we included serially diluted ileal samples to see if a particular dilution would enhance the

separation of bacteria and non-bacteria fractions. Furthermore, we included 1:16000 and 1:32000 to see if these concentrations would outperform 1:8000.

**Experiment:**

We prepared 1:10 and 1:100 serial dilutions of extracted mice ileal contents. Differentially concentrated ileal samples were brought to a 96-well Corning plate, where they were stained with the appropriate SYTO BC concentration in a matrix fashion (**Figure 10**). All samples were then analyzed with flow cytometry.



| | | 1:8000 SYTO BC | 1:16000 SYTO BC | 1:32000 SYTO BC | Blank SYTO BC | Compensation |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| Undiluted Ileum | A | | | | | FITC |
| Ileum 1:10 | B | | | | | 1:10 Unstained Control |
| Ileum 1:100 | C | | | | | |

**Figure 10:** 96-well plate setup for experiment PS4. In each colored entry, a diluted mouse ileum sample is stained with the appropriate SYTO BC concentration. The blank column samples are not stained with SYTO BC. Column 5 contains the compensation controls used to initialize the cytometer.

**Results:**

We organized our samples by dilution and then looked at the performance of each SYTO BC concentration for ileal dilution (**Figure 11**).

**Conclusions:**

Our flow cytometry results showed that a 1:8000 SYTO BC gave the best bacteria to non-bacteria population separation, which a 1:100 ileum dilution enhanced. Unfortunately, attempted repeats of this trial were full of experimenter errors and were discarded. Due to time constraints, we did not repeat this experiment; therefore, we continued with this concentration despite being uncertain about its validity.
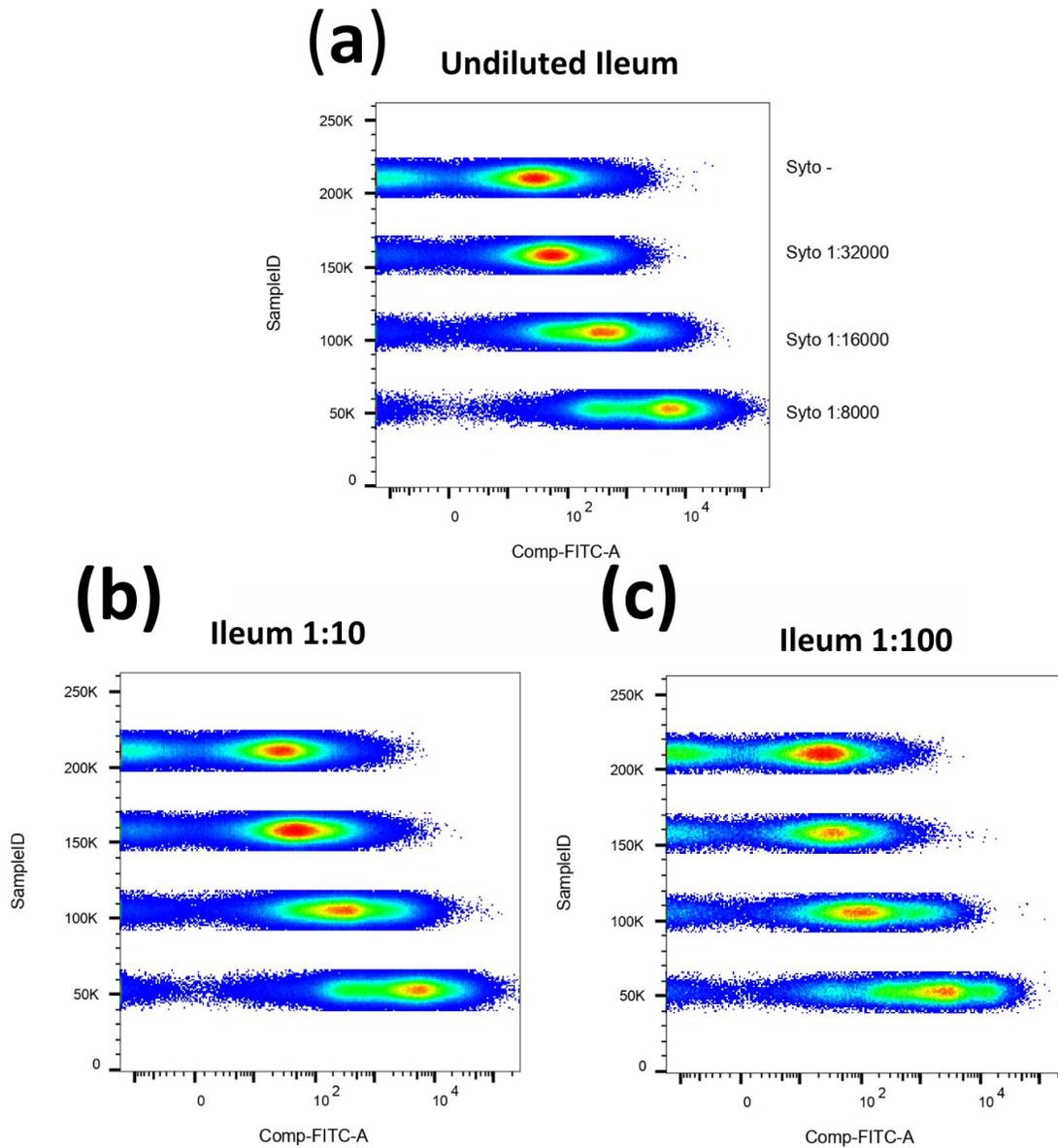
**Figure 11:** Concatenation of flow cytometry results showing the performance of SYTO BC concentrations ranging from 1:8000 to 1:3200 (top to bottom) stained onto **(a)** undiluted, **(b)** 1:10, **(c)** 1:100 ileal samples.

**PS5: Titration of IgA-PE and IgA-BV650 Immunostainers to Determine a Better Fluorophore and a Concentration that Optimally Distinguishes IgA+ and IgA- bacteria**

**Objective:**

Now that we found an optimal SYTO BC concentration that optimally distinguished bacteria and non-bacteria in ileal samples, our next objective was to find a concentration of our IgA-PE antibody that optimally separates IgA+ and IgA- fractions within bacteria that we can now accurately identify within ileal samples. We also included another antibody and fluorophore pair, BV650-IgA, to see if this fluorophore could better distinguish the IgA populations relative to PE. Determining which fluorophore performed better and at what concentration would improve IgA+ and IgA- cell-sorting accuracy such that we could have large numbers of obtainable bacteria in our samples we bring to cell-sorting.

**Experiment:**

We stained mouse ileum sample replicates diluted from 1:10 to 1:1000 with 1:8000 SYTO BC and serial dilutions of the isotype control and IgA-PE and IgA-BV650, ranging from 1:25 to 1:800. Like PS4, SYTO BC, and antibody staining were done in a 96-well Corning plate from which all samples were brought to flow cytometry (**Figure 12**).



**Figure 12:** 96-well plate setup for IgA-PE and IgA-BV650 titration experiment. In each colored entry, a mouse ileum sample is stained with 1:8000 SYTO BC and the appropriate antibody and fluorophore concentration, as determined by the identities of columns 1 through 7 and rows 1 through 3, respectively. Column 8 contains the compensation controls that calibrate the cytometer. A 1:100 ileum sample was stained with the appropriate fluorophore in each of

column 8's wells. The unstained control consisted of a 1:100 ileum sample "stained" with only FACS buffer.

**Results:**

Flow cytometry results showed PE outperforming BV650 in producing a higher SYTO BC+ percentage for IgA concentrations from 1:25 to 1:100 and at 1:400 for all three ileal sample dilutions. At IgA concentrations of 1:200 and 1:800, BV650 produced a higher SYTO BC+ percentage for all ileal dilutions. Interestingly, BV650's SYTO BC+ percentage spiked at a 1:800 IgA concentration across all ileal dilutions. Furthermore, for both fluorophores, the SYTO BC+ percentage increased across each ileal dilution when decreasing the IgA concentration from 1:400 to 1:800.

However, despite BV650's strong performance, we saw that all samples stained with BV650 resembled those lacking the fluorophore (See **Figure 13** comparing PE and BV650 for a 1:10 ileum dilution and see **Supplementary Figures S8-S9** comparing PE and BV650 for 1:100 and 1:1000 ileum dilutions, respectively).

Therefore, there was high IgA+ fluorescence in these blank samples despite lacking the antibody and fluorophore. Contamination could not explain this, as we analyzed the ileal samples from the SYTO BC titration experiment with the BV650 wavelength channel and saw the same high IgA+ fluorescence despite IgA not being used in this experiment.
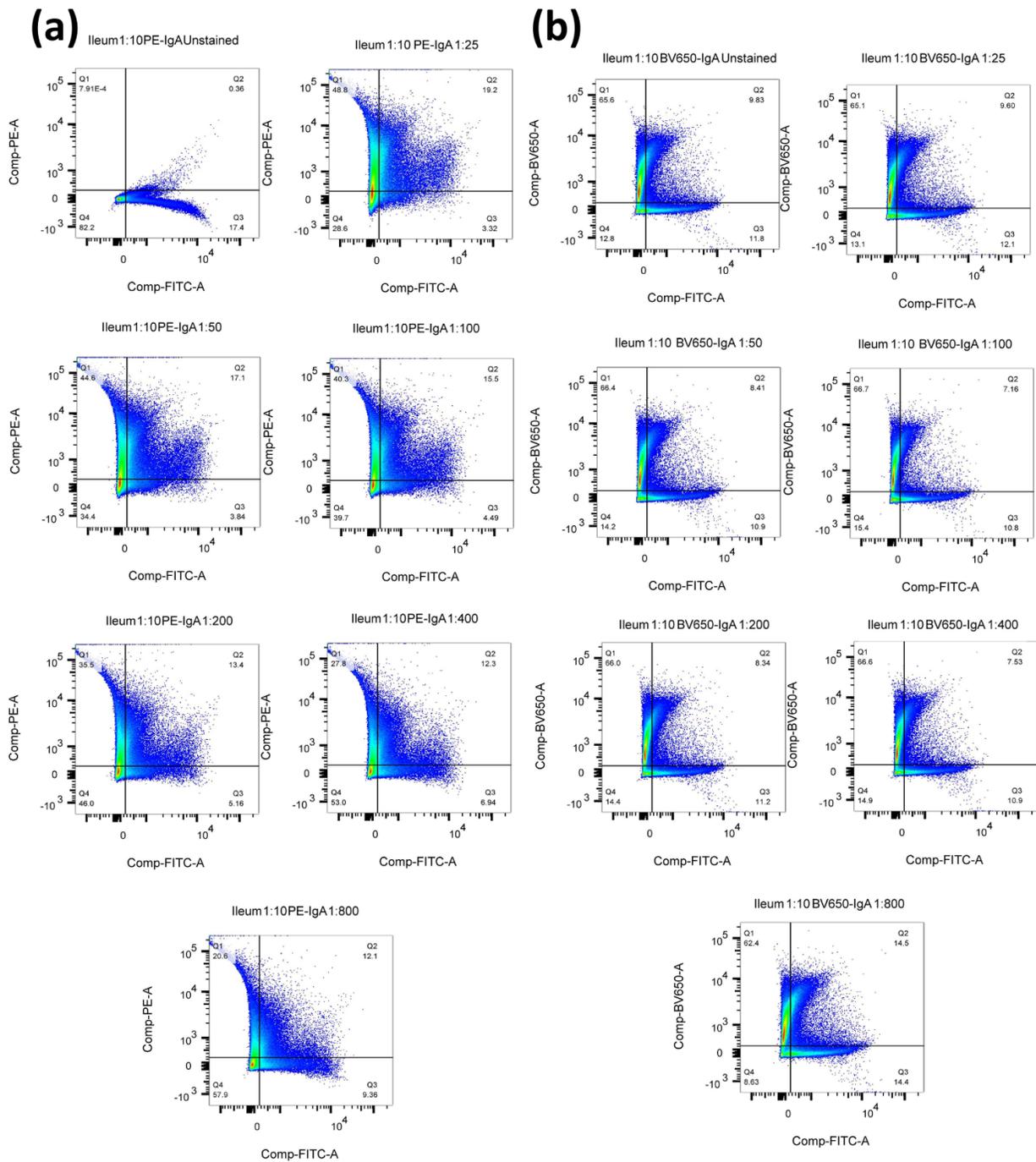
**Figure 13:** Flow cytometry results from the first IgA titration trial testing PE-IgA and BV650-IgA.

**(a)** Results for 1:10 diluted ileal sample replicates stained with PE-IgA ranging from a

concentration of 1:25 to 1:800. **(b)** Results for 1:10 diluted ileal sample replicates stained with PE-BV650 ranging from a concentration of 1:25 to 1:800.
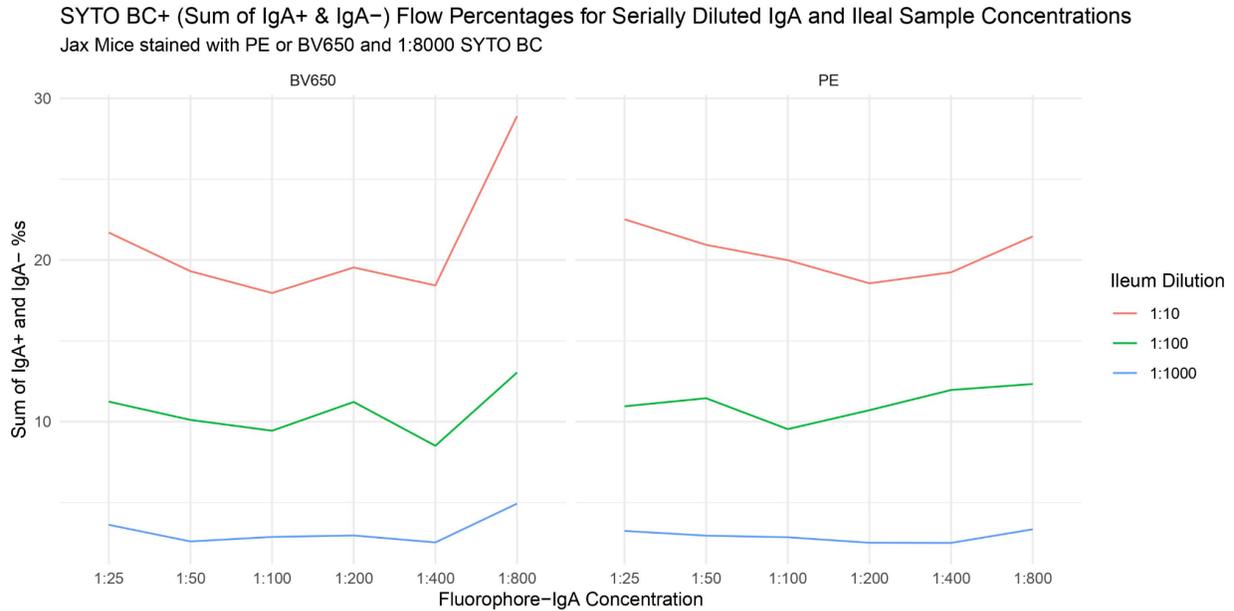


**Figure 14:** The SYTO BC+ (sum of IgA+ and IgA-) percentage from each flow cytometry graph for serially diluted IgA and ileal sample concentrations. The left panel contains the sum of the IgA+ and IgA- population percentages for the BV650 fluorophore tested one mouse. The right panel contains the sum of the IgA+ and IgA- population percentages for the PE fluorophore tested on the same mouse.

**Conclusions:**

BV650 proved to be an unreliable fluorophore because our samples' bacteria auto-fluoresce in the 650 wavelength channel. PE had promising performance and we sought to test it further on four more mice.

**PS6: Repeat of PS5 for PE-IgA Only**
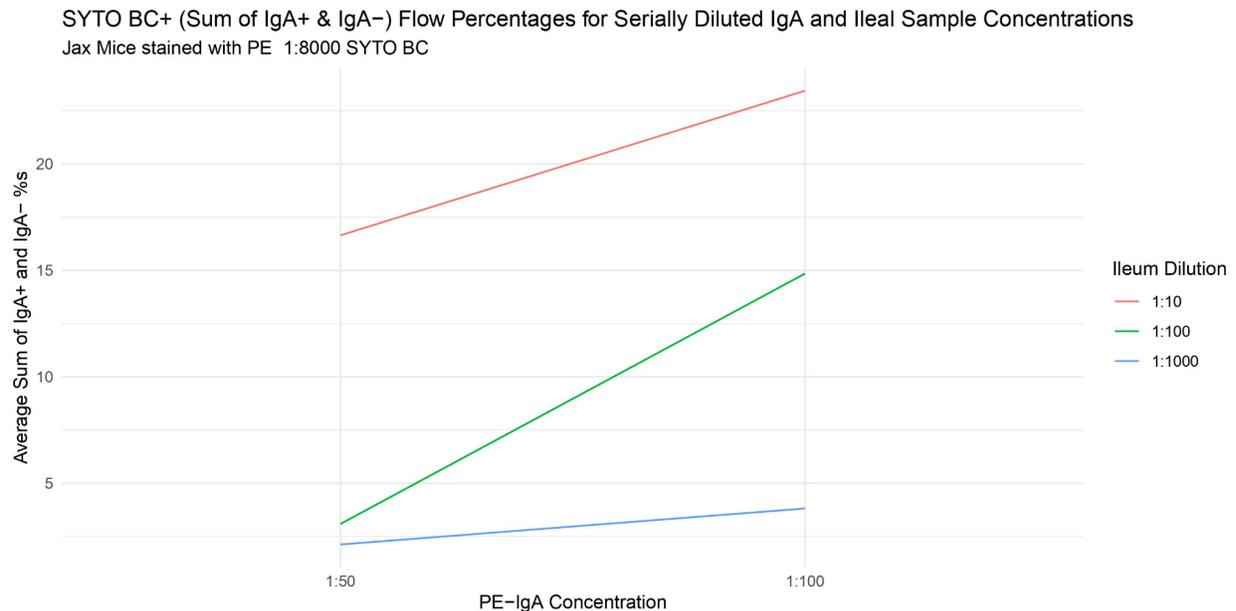
**Objective:**

After excluding BV650, we sought to conduct two more IgA titration trials with two mice each to see if PE produced the same strong IgA+ and IgA- populations and proportions again.

**Experiment:**

In these next two experiments, we tested only two antibody concentrations, 1:50 and 1:100, instead of the entire 1:25 to 1:800 range to first determine if this antibody binds specifically and has low background staining.

**Results:**

From the flow cytometry results of four mice whose ileal samples were stained with PE (**Figures 16-17**), we averaged the IgA+ and IgA- percentage sum for each IgA and ileum sample serial dilution and compared these averages (**Figure 15**).

SYTO BC+ (Sum of IgA+ & IgA−) Flow Percentages for Serially Diluted IgA and Ileal Sample Concentrations
Jax Mice stained with PE  1:8000 SYTO BC



**Figure 15:** The average SYTO BC+ (sum of IgA+ and IgA-) percentage from each flow cytometry graph for serially diluted IgA and ileal sample concentrations among four Jax mice. **I need to include error bars.**

Interestingly, the average SYTO BC+ percentage increased when the PE-IgA concentration was serially diluted from 1:50 to 1:100. Furthermore, the average SYTO BC+ percentage produced by PE decreased significantly across each IgA concentration as the ileal dilution increased from 1:10 to 1:100. At a 1:100 ileal dilution, there was nearly a non-existent bacterial population.
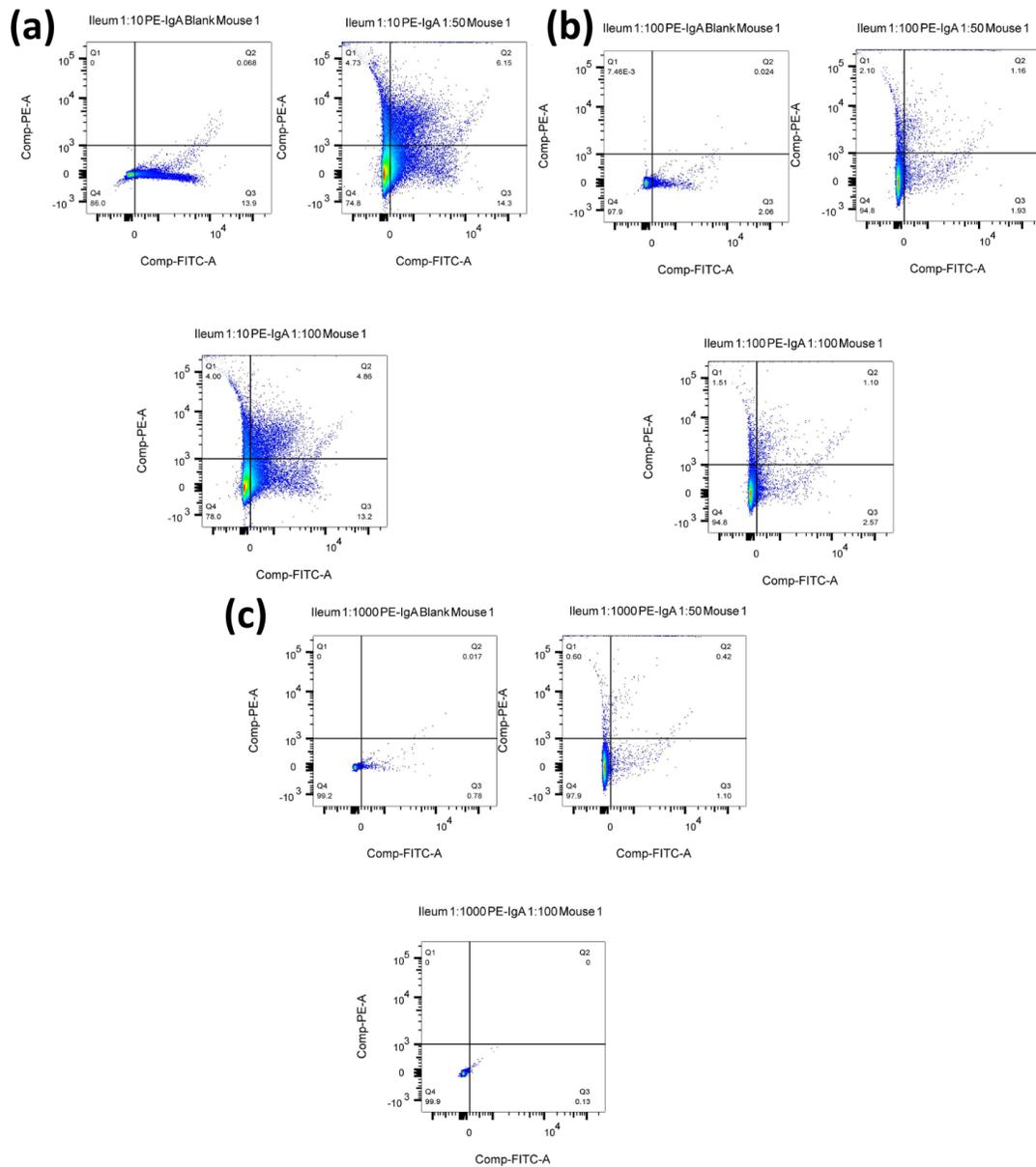
**Figure 16.1:**  Flow cytometry results from the first IgA titration trial testing PE-IgA only for
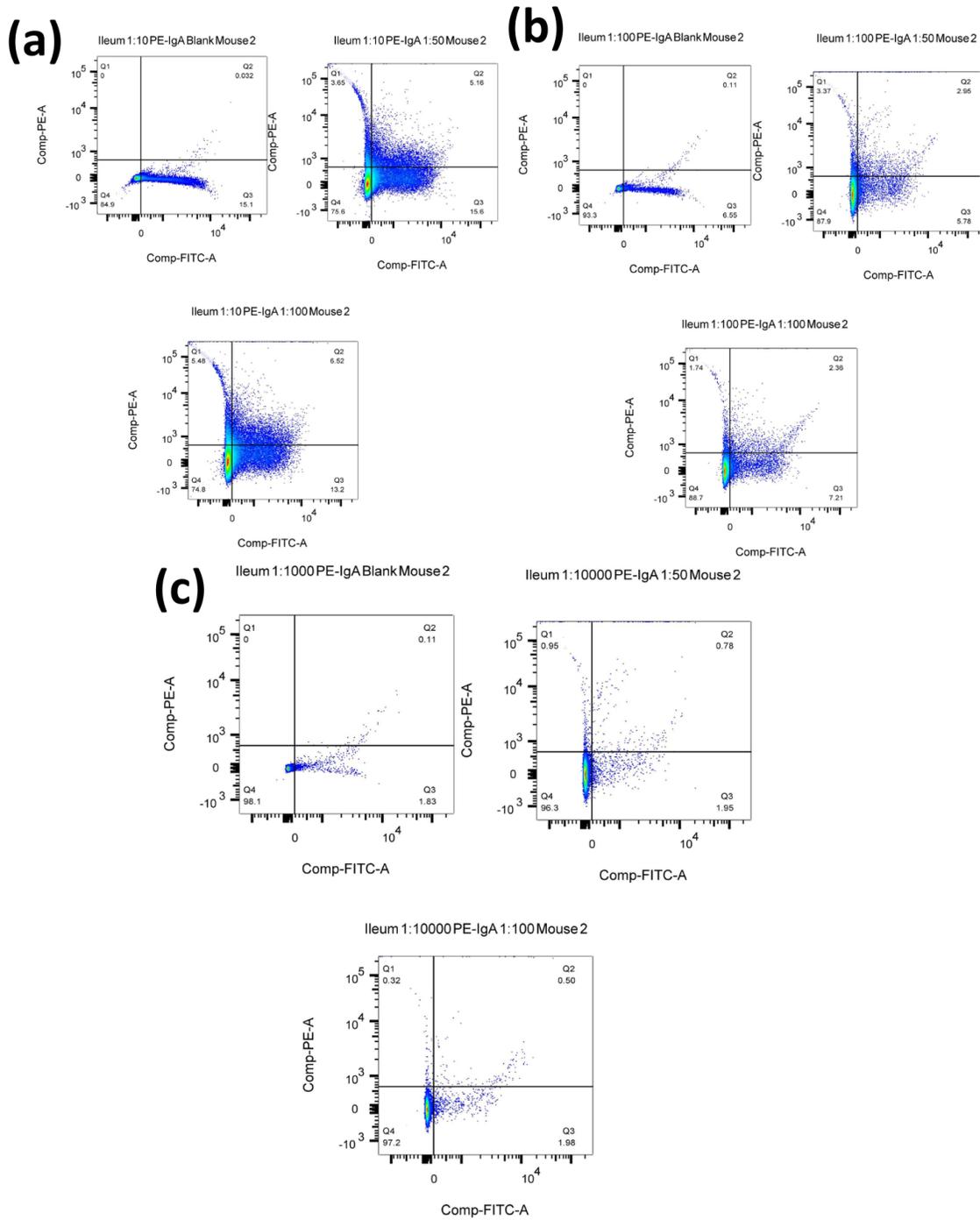
mouse 1.

**Figure 16.2:** Flow cytometry results from the first IgA titration trial testing PE-IgA only for mouse 2.
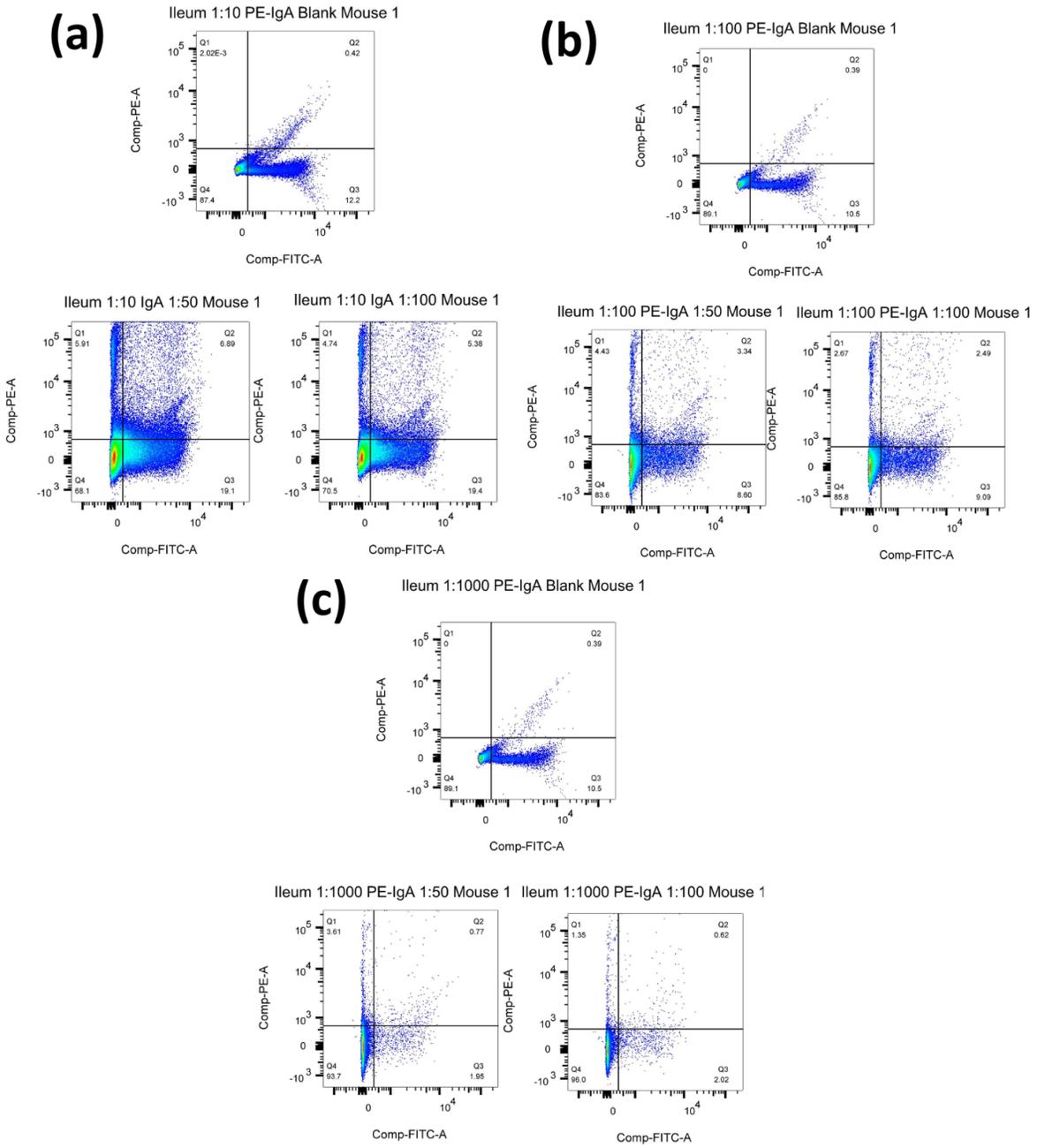
**Figure 17.1:** Flow cytometry results from the second IgA titration trial testing PE-IgA only for mouse 1.

**Figure 17.2:** Flow cytometry results from the second IgA titration trial testing PE-IgA only for mouse 2.

**Conclusions:**

Despite PE's strong performance in producing large average SYTO BC+ percentages we saw that it overlapped 105% with the FIT-C wavelength in each flow cytometry session. Therefore, we were concerned ab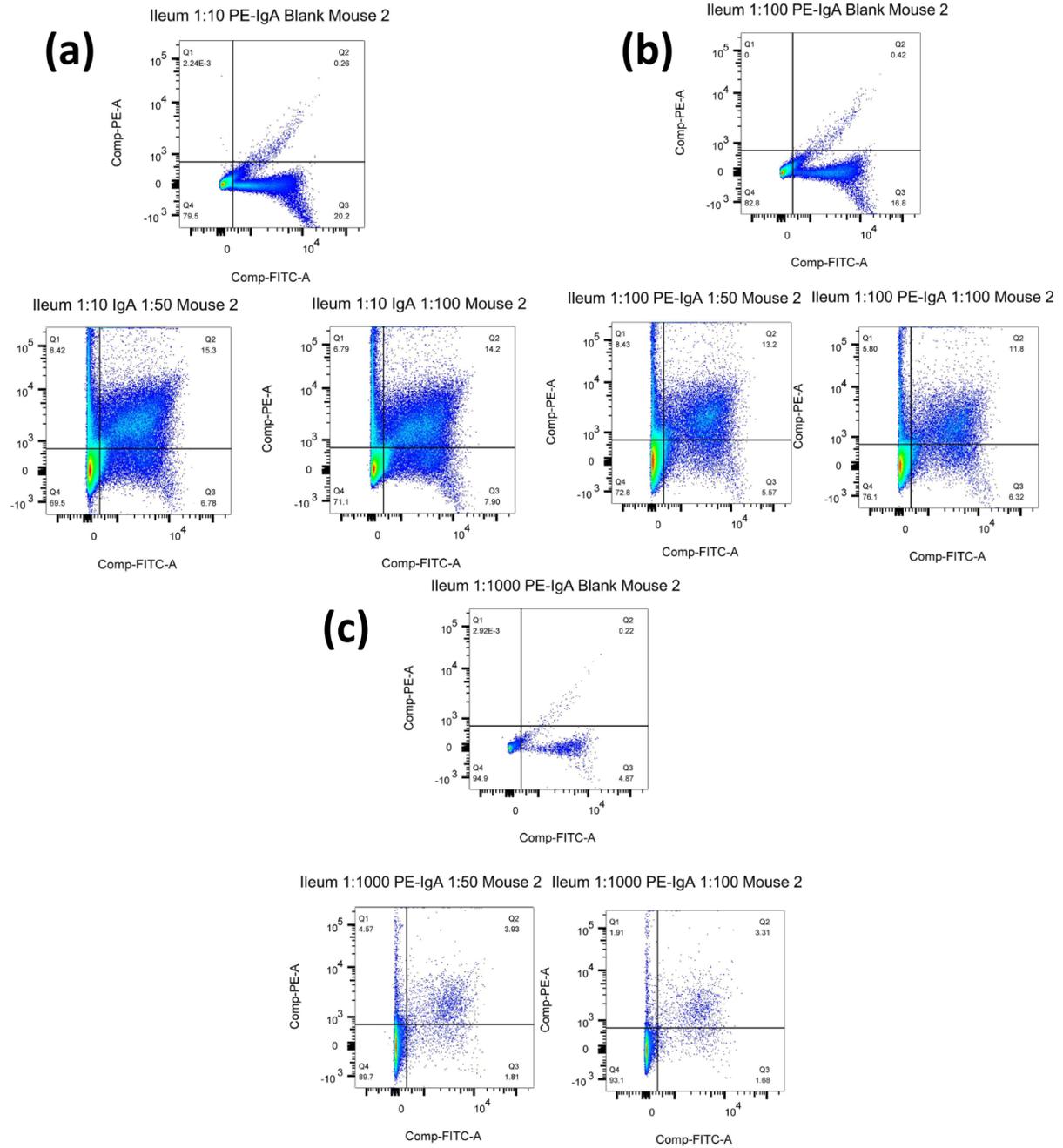out these fluorophores overlapping such that the IgA+ and IgA- events PE returned were simply artifacts. Therefore, our next step was to test a fluorophore that fluoresced in a channel far from FIT-C but yielded a similar performance.

**PS7: Titration of IgA-Biotin-Streptavidin-AF647 to Determine a Concentration that Optimally Distinguishes IgA+ and IgA- Bacteria**

**Objective:**

One such fluorophore whose wavelength does not overlap with FIT-C is Alexa Fluor® 647 (AF647). Therefore, we sought to test the anti-IgA-Biotin and Streptavidin-AF647 fluorophore complex. In this molecular multiplex, biotin is attached to an anti-IgA molecule and three streptavidin molecules. Biotin, Streptavidin, and the anti-IgA form a complex that is resistant to extreme pHs, temperatures, and other denaturing agents, making it a stable molecule. Another favorable feature of this complex is that it may have higher detectable fluorescence than PE because each Biotin has three Streptavidin molecules bound to an AF647 fluorophore.

Therefore, due to its preferable distance from the FIT-C wavelength channel, its high signal amplification, and resistance to non optimal environmental conditions, we aimed to see if this complex could be a robust immunostainer in our experiments.

**Experiment:**

Samples were stained with 1:8000 SYTO BC and 1:50 to 1:100 IgA-Biotin in the 96-well plate (see **Supplementary Figure S10** for illustration of this plate layout), incubated on ice, and stained with 1:400 Streptavidin presenting the AF647 fluorophore.

**Results:**

The AF647 fluorophore was analyzed using the APC-A channel in FACSDiva as AF647 is spectrally similar to APC.

The flow cytometry results from this experiments showed IgA-Biotin-Streptavidin-AF647 yielding a lower SYTO BC+ proportion relative to PE (**Figures 18-19**).
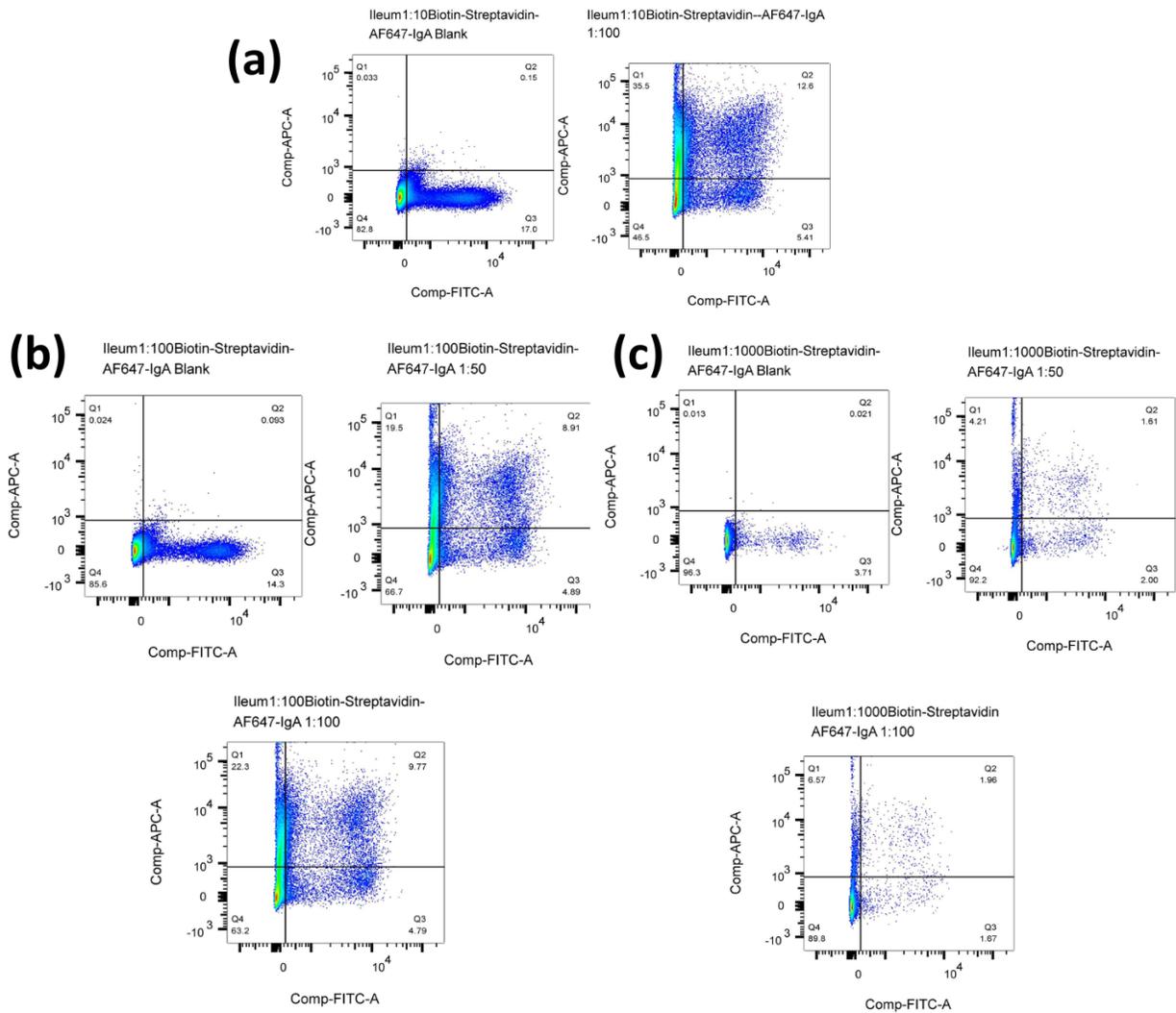


**Figure 18:** Flow cytometry results from the first IgA titration trial utilizing IgA-Biotin-Streptavidin AF647. **(a)** 1:10 Diluted ileal samples stained with no antibody or a 1:100 concentration. There was an error in flowing the 1:50 sample. **(b)** 1:100 Diluted ileal samples stained with antibody

concentrations ranging from 0 (blank) to 1:100. **(c)** 1:100 Diluted ileal samples stained with the same antibody concentrations as in (c).
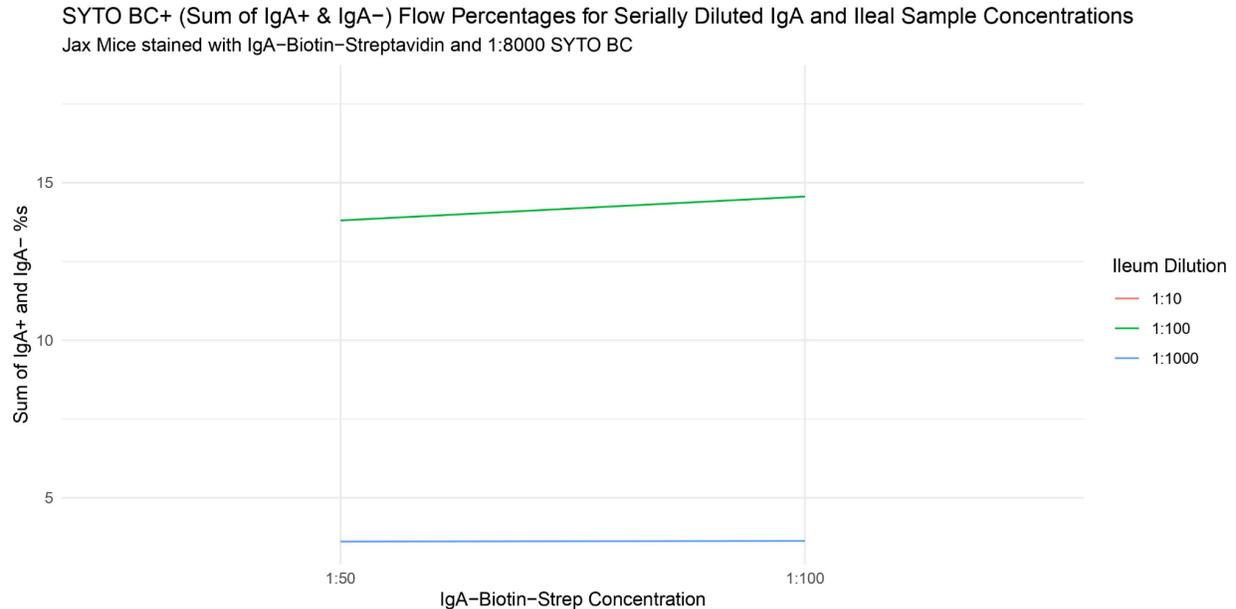


**Figure 19:** The SYTO BC+ (sum of IgA+ and IgA-) percentage from each flow cytometry graph for serially diluted IgA-Biotin-Streptavidin-AF647 and ileal sample concentrations from one mouse. At a 1:10 ileum and 1:100 IgA-Biotin-Streptavidin dilution, the SYTO BC+ percentage was 18.01 but is not shown in this figure.

**Conclusions:**

These results were concerning as we expected a similar or better performance from Biotin and Streptavidin. Therefore, we hypothesized that the low SYTO BC+ proportion was due to our SYTO BC becoming less effective at a 1:8000 concentration due to experiencing frequent freeze-thawing throughout our experiments. If this SYTO BC concentration could no longer efficiently stain bacteria, then the flow cytometer would not be able to detect significant IgA+ and IgA- fractions.

Overall, this trial yielded less desirable results than the PE runs due to SYTO BC degradation potentially. Therefore, our next direction was to test if a higher SYTO BC

concentration could yield an average IgA+ and IgA- percentage that matched or outperformed that of PE's.

**PS8: Testing if 1:4000 SYTO BC Outperforms 1:8000 SYTO BC in Aiding IgA-Biotin-Streptavidin-AF647 Yield a Favorable IgA+ and IgA- Average Percentage**

**Objective:**

We aimed to see if a higher concentration of SYTO BC would improve the SYTO BC+ signal. If it did, then we would reduce our working SYTO BC concentration for future experiments. If not, then that suggests our SYTO BC has degraded or that IgA-Biotin-Streptavidin inherently weakly identifies IgA+ and IgA- populations.

Experiment:

We repeated the titration experiment with IgA-Biotin-Streptavidin on a mouse ileal sample stained with either 1:4000 or 1:8000 SYTO BC.

**Results:**

1:4000 SYTO BC yielded a trivially larger SYTO BC+ signal for each ileum and antibody dilution; however, all flow panels from each trial resulted in low IgA+ and IgA- signals once more, which would lead to cell-sorted IgA+ and IgA- samples low in bacteria were these samples brought to flow (**Figures 20-21**).
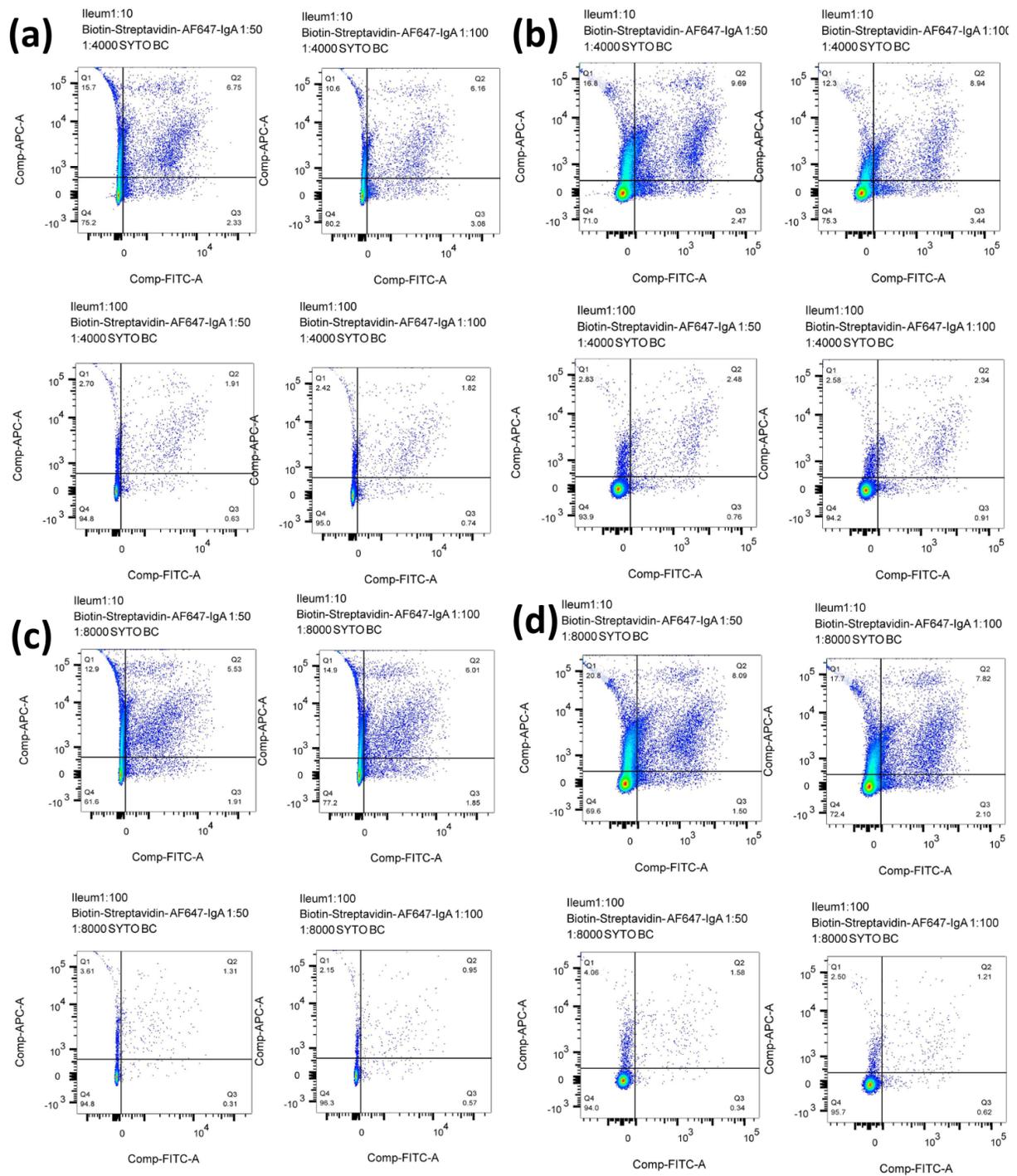
**Figure 20:** Flow cytometry results from two IgA-titration trials (trial 1 testing old SYTO BC stock on left column and trial 2 testing new SYTO BC stock on right column) assessing

IgA-Biotin-Streptavidin AF647 effectiveness at 1:4000 SYTO BC (top row) or 1:8000 SYTO BC (bottom row). **(a)** Trial 1: 1:10 to 1:00 diluted ileal samples stained with 1:4000 SYTO BC and 1:50 or 1:100 IgA-Biotin. **(b)** Trial 2: 1:10 to 1:00 diluted ileal samples
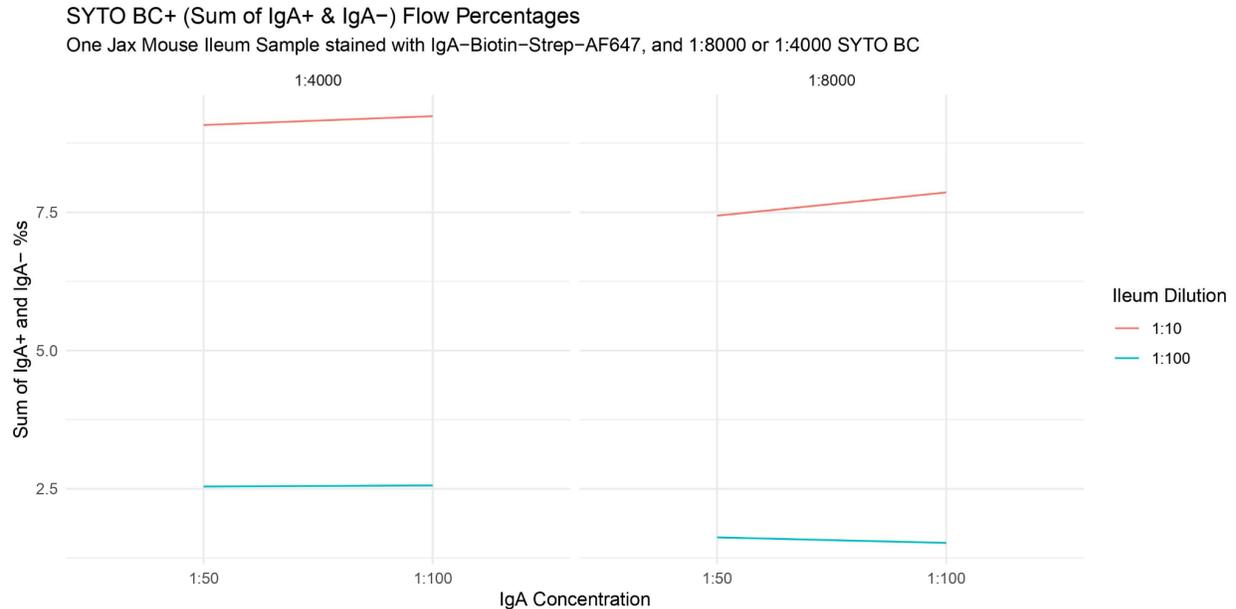


**Figure 21:** The SYTO BC+ (sum of IgA+ and IgA-) percentage from each flow cytometry graph for serially diluted IgA-Biotin-Streptavidin-AF647 and ileal sample concentrations from one mouse. The left panel contains the sum of the IgA+ and IgA- population percentages for sample stained with 1:4000 SYTO BC. The right panel contains the sum of the IgA+ and IgA- population percentages for sample stained with 1:8000 SYTO BC.

**Conclusions:**

A 1:4000 SYTO BC concentration did not outperform a 1:8000 SYTO BC concentration significantly. Therefore, we hypothesized that our SYTO BC had degraded due to frequent freeze-thawing, and could potentially jeopardize the amount of cells we would get in IgA+ and IgA- samples if we continued to use it in our cell-sorting experiments. Therefore, we purchased a new 200 uL stock and transferred 5 uL volume into 40 sterile PCR tubes. We aimed to see if this new SYTO BC stock could aid IgA-Biotin-Streptavidin-AF647 in detecting the IgA+ and IgA- populations.

**PS9: Repeating PS8 with a new SYTO BC Stock**


**Objective:**

We aimed to see if our old SYTO BC stock had degraded by repeating the previous experiment with a new stock. If this new SYTO BC yielded significantly higher IgA+ and IgA- signals at either concentration relative to the older one that emulated the results we saw in the PE-IgA-titration trials, then we would continue using this new SYTO BC at the optimal 1:8000 concentration previously determined by our SYTO BC titration experiments. However, if this new stock yielded the same or worse results relative to the previous trial, then our old SYTO BC stock did not degrade and then we would reduce our working SYTO BC concentration for future experiments. If not, then perhaps there was some other flaw in our experimental design.


Experiment:

We repeated PS9 but using our new SYTO BC.


**Results:**

Once more, 1:4000 SYTO BC yielded a trivially larger SYTO BC+ signal for each ileum and antibody dilution. Furthermore, all flow panels from each trial resulted in low IgA+ and IgA- signals again (**Figure 20 - Right Panel** and **Figure 22**).
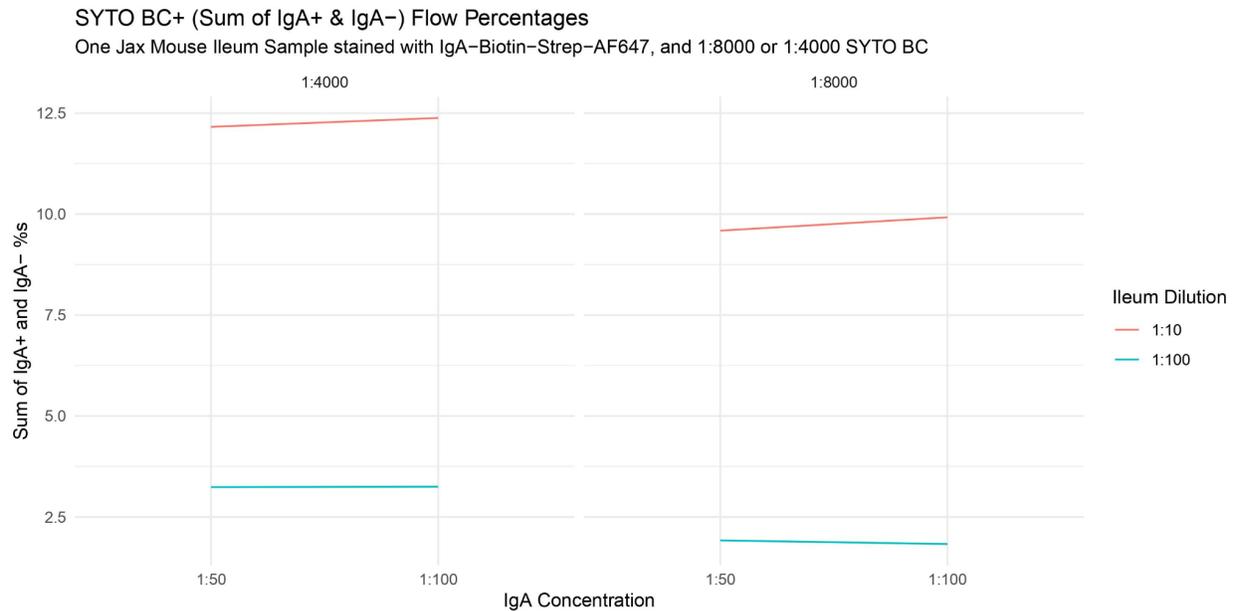
**Figure 22:** The SYTO BC+ (sum of IgA+ and IgA-) percentage from each flow cytometry graph for serially diluted IgA-Biotin-Streptavidin-AF647 and ileal sample concentrations from one mouse. The left panel contains the sum of the IgA+ and IgA- population percentages for sample stained with 1:4000 new SYTO BC stock .The right panel contains the sum of the IgA+ and IgA- population percentages for sample stained with 1:8000 new SYTO BC stock.


Conclusions:

Using a new SYTO BC stock increased the IgA+ and IgA- percentage for both concentrations relative to the previous experiment, by not by a significant margin. Therefore, we should expect a low average SYTO BC+ percentage when using 1:8000 SYTO BC and IgA-Biotin-Streptavidin.

The common pattern in these titration trials was an overall low SYTO BC + signal that reduced the overall IgA+ and IgA- signals and population sizes. We also considered any experimental errors on our part. In particular, we used multi-channel pipettes to stain multiple samples in the 96-well plate at once. However, these pipettes vacuumed inconsistent volumes in each tip or would permanently retain volume and have to be discarded, thus potentially jeopardizing sample availability and impacting our SYTO BC+ and, therefore, IgA+ and IgA-

population sizes. Therefore, we abandoned the usage of these multi-channel pipettes altogether for further experimentation after PS10.

In addition to producing undesirable IgA+ and IgA- populations, all flow cytometry results from the IgA-Biotin-Streptavidin titration trials showed that both 1:50 and 1:100 IgA concentrations yielded similar SYTO BC+ percentages for each ileum dilution. **Therefore, we continued with 1:50 IgA because it gave the best signal to noise ratio.**

Overall, despite getting low SYTO BC+ signals in each IgA-Biotin-Streptavidin-AF647 titration trial, we opted to use this immunostainer because we hypothesized it would yield more accurate results than PE due to the lack of wavelength overlap and potentially confounding interactions with FIT-C. Therefore, we continued with using 1:50 IgA-Biotin-Streptavidin in our subsequent cell-sorting trials and accepted the trade-off in the number of sorted cells in our IgA+ and IgA- and accuracy in identifying these populations.

**PS10: Cell-Sorting Trial 1 with One Jax SCD Mouse utilizing 1:8000 SYTO BC and 1:50 IgA-Biotin-Streptavidin**

Objective:

We aimed to see if our optimized SYTO BC and IgA-Biotin concentrations would yield large enough IgA+ and IgA- populations in the BD FACSAria™ II cell sorter.

Experiment:

In our first cell-sorting trial, we stained ileal contents from one SCD Jax mouse with 1:8000 SYTO BC and 1:50 IgA-Biotin-Streptavidin. Furthermore, we had to identify optimal flow cytometry voltages as the laser setup from this instrument differs from the flow cytometer we used in all previous experiments. Therefore, after ileum sample isolation and fluorophore staining, we initialized the cell-sorter's flow cytometry voltages with a cultured *L.reuteri* sample stained with 1:2000 SYTO BC. This procedure had the same purpose as PS2, as we had to find the optimal flow cytometry voltages on the cell-sorter that would best reveal the bacteria in our

ileal samples. We only used *L.reuteri* this time as our *E.coli* cultures were contaminated by the time we scheduled to perform cell-sorting.

Results:

  The optimal voltages resulting from measuring bacterial cultures were FIT-C: 425, SSC: 615, and FSC: 230

  The flow cytometry plots generated using these voltages showed significantly low SYTO BC+ signals in the ileal samples resulting in the same small IgA+ and IgA- populations. These IgA+ and IgA- populations were no larger than 200,000 cells. However, the *L.reuteri* sample showed a strong SYTO BC+ signal and a large Q3 population of 99.1%. (**Figure 23**).
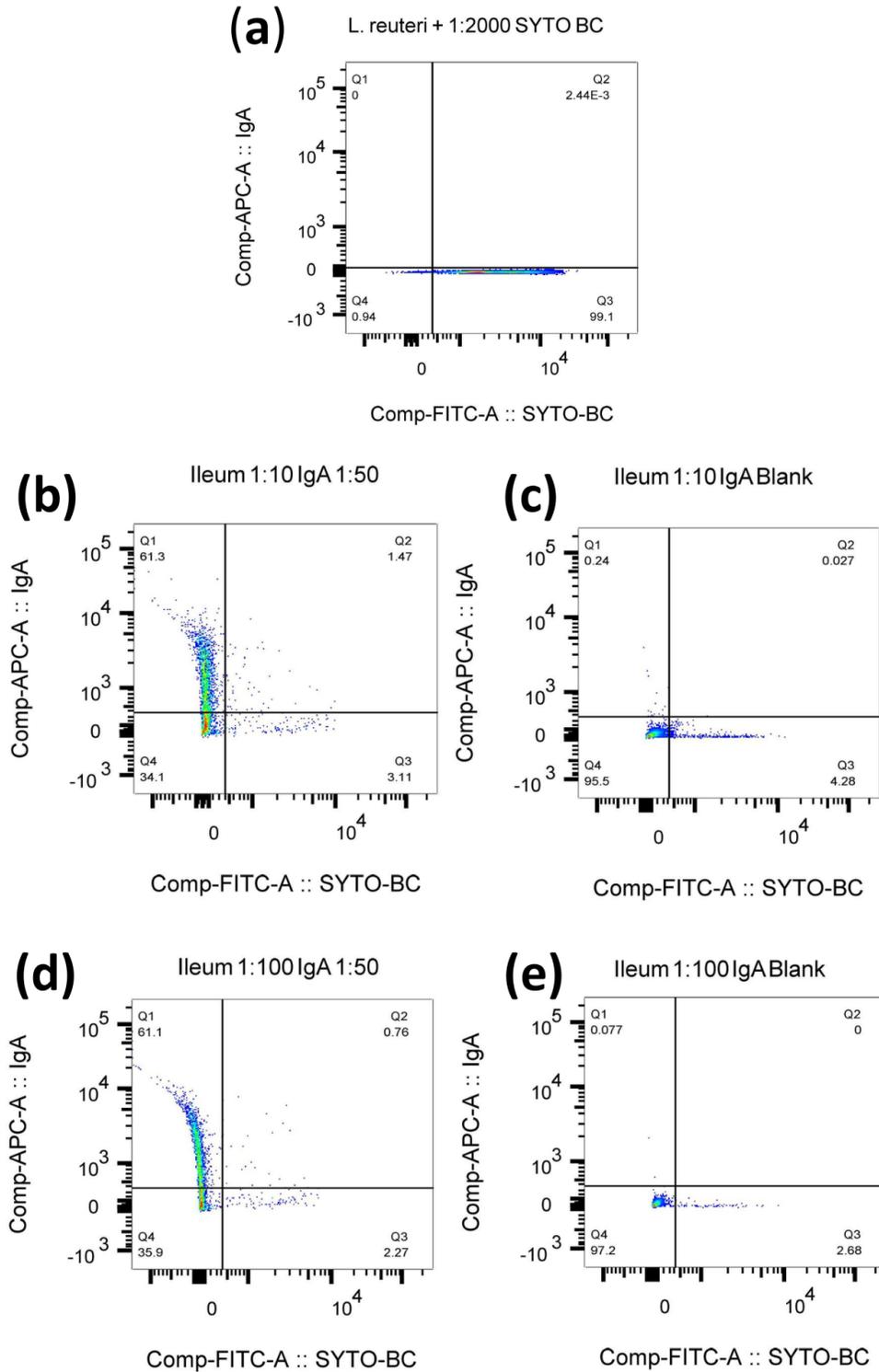
**Figure 23:** Cell-sorting trial 1 flow cytometry results showing an **(a)** *L.reuteri* culture with a strong SYTO BC+ signal, **(b-c)** 1:10 ileum samples stained with 1:50 IgA or no IgA (blank), and **(d-e)** 1:100 ileum samples stained with the same IgA concentrations.

Conclusions:

        Our SYTO BC+ signal in this cell-sorting trial was significantly weaker than in the IgA-Biotin-Streptavidin trials and resulted in IgA+ and IgA- cell counts that would yield low amounts of genetic material to analyze in 16s sequencing. Therefore, we sought to make changes in our ileum isolation and SYTO BC and IgA staining protocol.

        Specifically, we sought to increase the SYTO BC staining period on ice from 5 to 20 minutes to ensure that the flurophore fully saturates the samples. Additionally, our next step was to stain ileal samples with 1:2000 SYTO BC in the next trial to see if that could improve the SYTO BC+ signal. Seeing how strongly the pure bacteria fluoresced with a 1:2000 SYTO BC concentration, and 1:8000 SYTO BC underperforming, even without using the multi-pipetter, prompted us to test this 1:2000 SYTO BC concentration.

**PS11: Cell-Sorting Trial 2 with One Jax SCD Mouse utilizing 1:2000 SYTO BC and 1:50 IgA-Biotin-Streptavidin**

Objective:

        We aimed to see if our increased SYTO BC staining time and concentration would yield large enough IgA+ and IgA- populations in the BD FACSAria™ II cell sorter.

Experiment:

        We stained one SCD Jax mouse's ileal contents, diluted to 1:10 and 1:100, with 1:2000 SYTO BC and 1:50 IgA-Biotin. Additionally, we included an undiluted LI sample to increase the material we worked with. All samples were incubated on ice for 20 minutes after being stained with SYTO BC. Cell-sorted samples were collected into 1.5 mL collection tubes containing 500 uL Inhbitex buffer.

Results:

This trial yielded significantly larger SYTO BC+ percentages and IgA+ and IgA- populations (**Figure 24**) that resembled the results we saw when using IgA-PE. Specifically, the 1:10 ileum sample stained with 1:50 IgA-Biotin yielded a SYTO BC+ percentage of 29.19%, composed of IgA- events mostly. The 1:100 ileum sample stained with 1:100 IgA-Biotin yielded a 49.33% SYTO BC+ percentage, composed of IgA- events once more. However, the IgA+ and IgA- populations appeared to be very small themselves. The undiluted LI sample stained with 1:50 IgA-Biotin yielded a 26.75% SYTO BC+ percentage, composed of IgA- events mostly once more. However, this sample had visibly larger IgA+ and IgA- populations than the other two.

After analyzing the flow cytometry plots of each sample, we flowed the ileum 1:10 IgA 1:50 sample again and cell-sorted it. The cell-sorted IgA+ and IgA- populations from this sample had 1.2 million and 450,000 cells, respectively.

We isolated the DNA from the IgA+ and IgA- cell-sorts for the ileum 1:10 IgA 1:50 sample samples as they had sufficient numbers of cells. We quantified the number of 16s and SFB gene copies in them via qPCR, which showed that all samples had fewer than $10^3$ SFB gene copies and $10^7$ 16s gene copies at least. We consider $10^3$ to be a basal level, meaning that these SFB gene copies are nearly undetectable due to their low abundances. (**Figure 25.**)
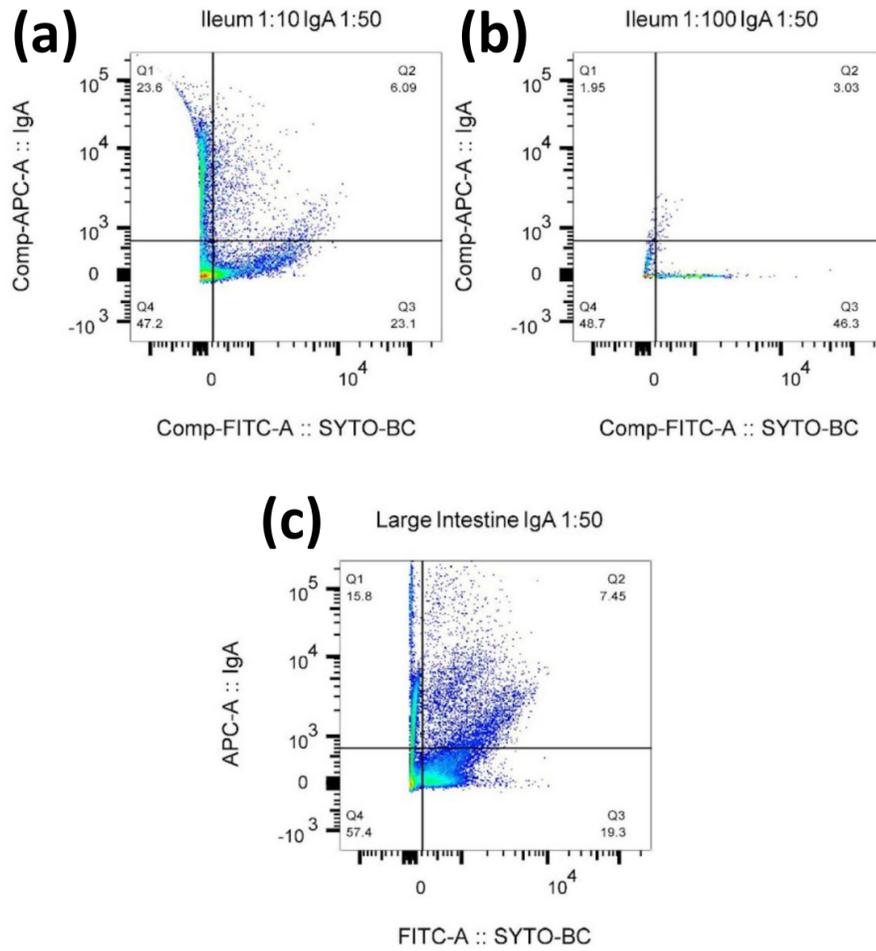
**Figure 24:** Cell-sorting trial 2 flow cytometry showing **(a-b)** 1:10 ileum samples stained with 1:50 or no IgA (blank)and **(c)** an undiluted large intestine sample stained with 1:50 IgA.
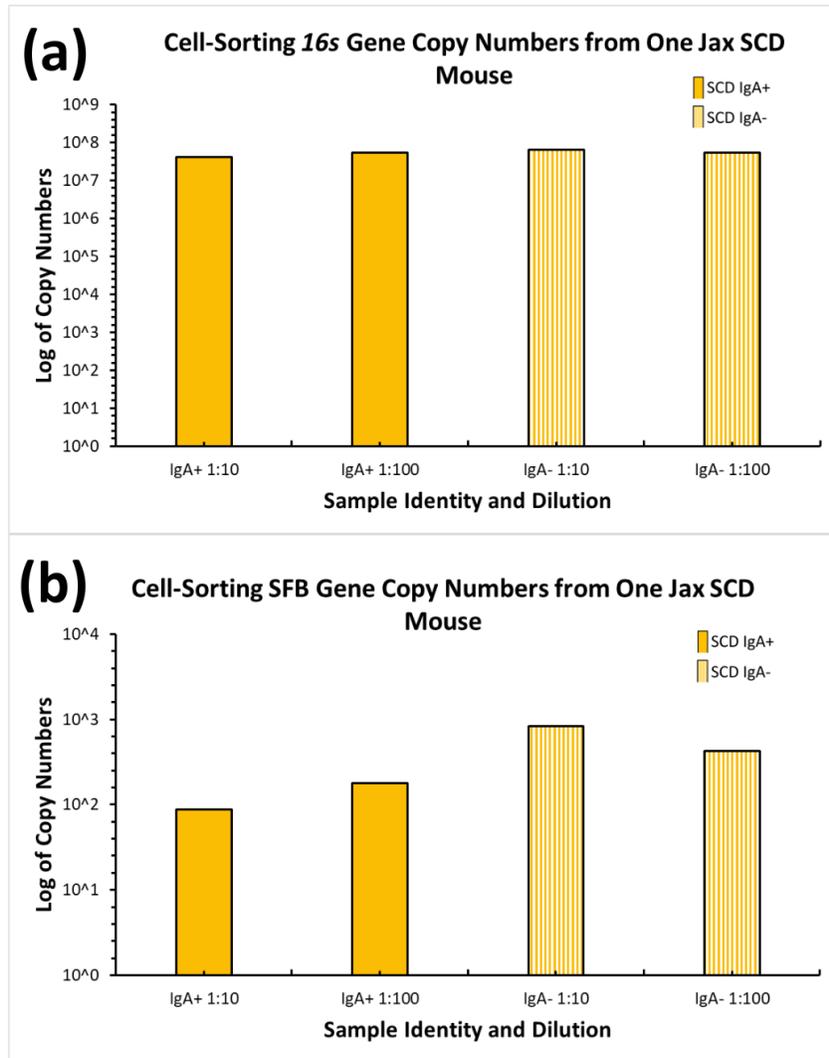
**Figure 25:** Number of **(a)** 16s and **(b)** SFB gene copy numbers calculated from qPCR of the last cell-sorting trial using a Jax mouse. The ileum 1:10 IgA 1:50 IgA+ and IgA- samples were diluted diluted to 1:10 and 1:100. SFB gene copy numbers are no higher than the basal level of $10^3$.

Conclusions:

The 1:100 ileum sample yielded the largest SYTO BC+ percentage but the smallest IgA+ and IgA- populations visibly whereas the undiluted LI sample yielded the smallest SYTO BC+ percentage but the largest IgA+ and IgA- populations visibly. The 1:10 ileum sample lied in the middle, producing the second largest SYTO BC+ percentages and IgA+ and IgA- populations whose sizes were in between those of the other two samples.

The large IgA+ and IgA- populations in the undiluted LI sample suggested that we could increase these population sizes if we ceased diluting the ileum samples. Therefore, we sought to only stained undiluted ileal contents in the next cell-sorting trial to see if we could increase the number of available bacteria and 16s and SFB gene copies in our IgA+ and IgA- samples.

Furthermore, we saw that SFB occurs at undetectable abundances in the Jax mice we have analyzed. Since we are interested in exploring the relationship between SFB and IgA production and availability further, we must ensure we have sufficient SFB numbers in our samples. Therefore, we sought to use a CR mouse in the next cell-sorting trial, as they characteristically carry large numbers of SFB bacteria.

**PS12: Cell-Sorting Trial 3. Staining a CR SCD Mouse's Undiluted Ileum Sample with 1:2000 SYTO BC and 1:50 IgA-Biotin**

**Objective:**

Our objective was to see if we could increase the number of cells and bacterial genes in our IgA+ and IgA- sorted samples by only staining an undiluted ileal sample and if we would have more SFB gene copies available to analyze in a CR mouse. Therefore, we worked with a CR mouse.

**Experiment:**

We repeated the same experiment as in the previous cell-sorting trials but used a CR mouse and stained only undiluted ileal contents.

Results:

This trial yielded the largest IgA+ and IgA- populations yet, leading to over 1.5 million IgA+ and 500k IgA- cells being sorted into our sample collection tubes. (**Figure 26**) qPCR of these IgA+ and IgA- samples showed there were at least $10^8$ 16s and $10^5$ SFB gene copies present in each sample (**Figure 27** - "SCD2 Mouse"). From this trial, the diluted IgA+ samples

contain slightly more 16s genes than the IgA- samples. However, the IgA- diluted samples contain slightly more SFB genes than the IgA+ ones.
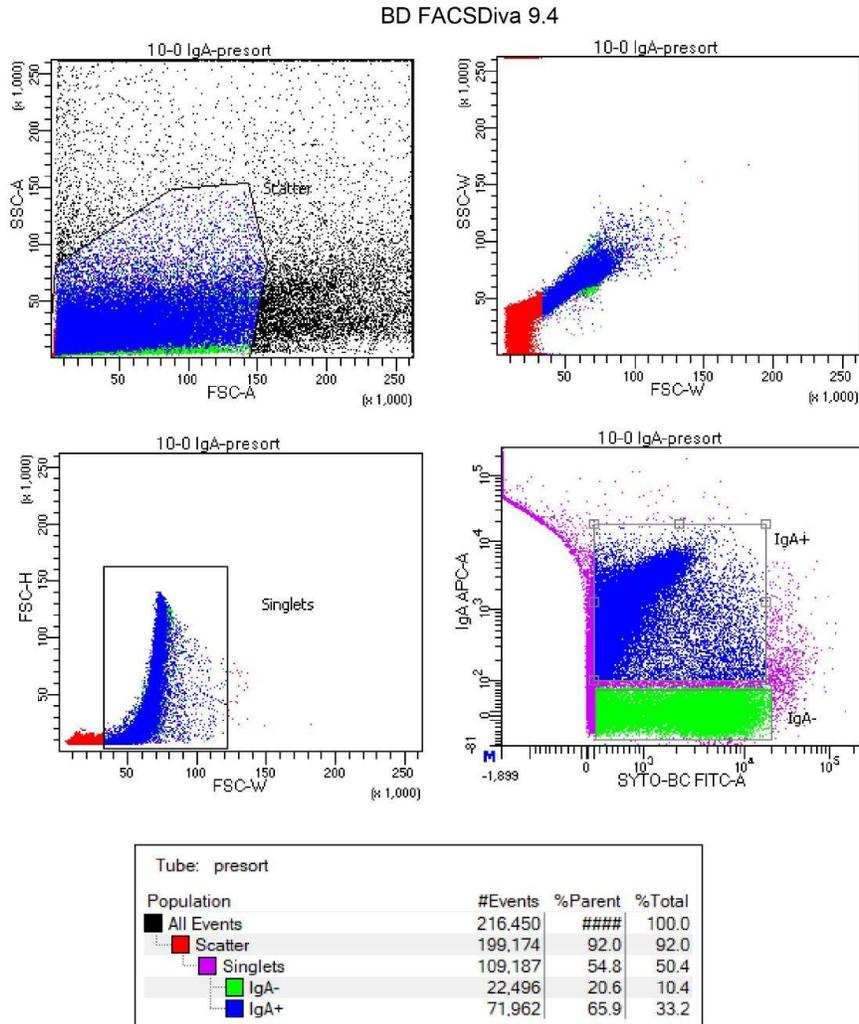


**Figure 26:** Cell-sorting results from a CR SCD mouse undiluted ileum sample stained with 1:2000 SYTO BC and 1:50 IgA-Biotin. The bottom-right graph is the sample's flow cytometry panel with IgA+ and IgA- gates which were determined by comparison to compensation controls. Cells falling within these gates were sorted and released to sample collection tubes in the cell-sorter. The bottom data table shows the percentage of flow events composed by those two gates and the number of cells sorted by those two gates out of 100,000

Conclusions:

We obtained more bacteria cells and genes in undiluted ileal samples stained with 1:2000 SYTO BC and IgA-Biotin 1:50 compared to when we diluted the ileal contents in the previous experiment. Furthermore, our CR mouse contained sufficient SFB gene copies, and showed that its IgA- population contained more SFB than the IgA+ fraction.

Utilizing these parameters increases the output of bacteria cells and genes in the IgA+ and IgA- sorted samples. Therefore, we chose to use these parameters in our standardized and final cell-sorting experiments with SCD and LFF CR mice.


**STANDARDIZED FINAL BACTERIAL SORT EXPERIMENTS**

Objective:

After standardizing our Bacterial sort protocol, we aimed to identify and collect IgA+ and IgA- bacteria in the ileal samples of littermate mice reared on the SCD diet and weaned onto the SCD or LFF diet via cell-sorting using our optimized flow cytometry parameters.


Experiments:

We gathered *n = 5* SCD/LFF and *n = 5* SCD/SCD littermate mice. On five separate days, we took a random LFF and SCD mouse pair, isolated their ileal contents, stained them with SYTO BC and IgA-Biotin-Streptavidin, and cell-sorted their stained ileal contents into IgA+ and IgA- samples.

We aliquoted replicates of each mouse's ileal sample into multiple wells in a 96-well plate. Each replicate was stained with the same SYTO BC and IgA-Biotin-Streptavidin-AF647 concentrations and volumes and resuspended in 200 uL FACS buffer. Blank replicates were stained with SYTO BC but not antibody whereas isotype control blank replicates were stained with SYTO BC and the same antibody-fluorophore concentration of TFNγ-AF647. The same compensation controls were prepared. All replicates pertaining to the same mouse were transferred and combined into one cell-sorting collection tube. We prepared different numbers of mice sample replicates across all Bacterial Sort experiments.

In experiments one and two, we prepared four wells for each mouse's ileal contents, resulting in a final sample volume of 800 uL for all mice analyzed in these experiments. In experiment three, we increased the number of replicates to eight, resulting in a final sample volume of 1.6 mL for both mice. These replicate numbers were increased to ten and 11 in experiments four and five, respectively. Therefore, the final sample volumes for the mice in experiments four and five were 2 mL and 2.2 mL , respectively.

The number of sample replicates was increased from four to eight as we began extracting low ileal content amounts from the LFF mice. Therefore, we wanted to use as much sample as possible from the LFF mice due to the low samples amounts as well as keep the number of sample replicates consistent for both mice. We extracted the lowest amount of LFF ileal contents in experiments four and five, and therefore increased the replicate numbers significantly in those runs (see **Supplementary Figure S11** for an illustration of the final bacterial sort experiment's 96-well plate layout).

During cell-sorting, all samples were collected into sterile 1.5 mL Eppendorf tubes containing 200 uL PBS buffer. After each experiment, we spun the collection tubes at max speed for ten minutes to pellet the cells. Then, we aspirated the supernatant from each sample, aliquoted 500 InhibitEx buffer into one tube, and then transferred that to each tube with the sample identity. All collection tubes were stored in -80°C to await DNA extraction and 16s sequencing. DNA from these IgA+ and IgA- samples was isolated using the QIAGEN kit.

We extracted the DNA from experiment one's samples a day after cell-sorting and measured the DNA concentrations using a NanoDrop® ND-1000 UV-Vis Spectrophotometer. Afterwards, we stored these samples in -80°C. DNA extraction and concentration measurement of experiments two to five were performed simultaneously the day after the final cell-sorting experiment was performed. 50 uL of the DNA extracted from each sample was aliquoted in 20 1.5 mL Eppendorf tubes and placed on dry ice until they were shipped for 16s sequencing.

## CELL-SORTING RESULTS FROM BACTERIAL-SORT EXPERIMENTS

Bacterial Sort Experiment 1:

Our first experiment showed a larger IgA+ population and percentage in the LFF mouse. The LFF mouse had 44046 IgA+ cells out of 100000 and 36.1% of its sample consisted of IgA+ bacteria cells. Conversely, the SCD mouse had only 29245 IgA+ cells out of 100000 and a total IgA+ percentage of 23.5%. This experiment also showed a larger IgA- population and percentage in the SCD mouse. The SCD mouse had had 14680 IgA- cells out of 10,000 and 11.8% of its sample consisted IgA+ bacteria cells whereas the LFF mouse had 4286 IgA- cells out of 100000 and a total IgA- percentage of 3.5% (**Figure 28.a**). These observations contrasted previous experiments where LFF mice housed considerably fewer IgA+ cells.

qPCR of 1:10 and 1:100 dilutions of the DNA extracted from these samples showed that both SCD IgA+ diluted DNA samples contained slightly more 16s and SFB genes than their LFF counterparts. However, the SCD IgA- 1:10 sample had a trivially larger number of 16s genes than its LFF equivalent, but had fewer SFB genes. Furthermore, the SCD IgA- 1:100 sample had fewer 16s genes than its LFF complement. Unfortunately, a comparison between the SFB gene numbers for the SCD IgA- 1:100 and LFF IgA- 1:100 samples could not be made because the qPCR melt curve used to generate the latter's copy number estimate was invalid. (**Figure 27**)

Overall, the first experiment revealed a large number of IgA+ and IgA- cells that yielded abundant 16s and SFB gene copies.
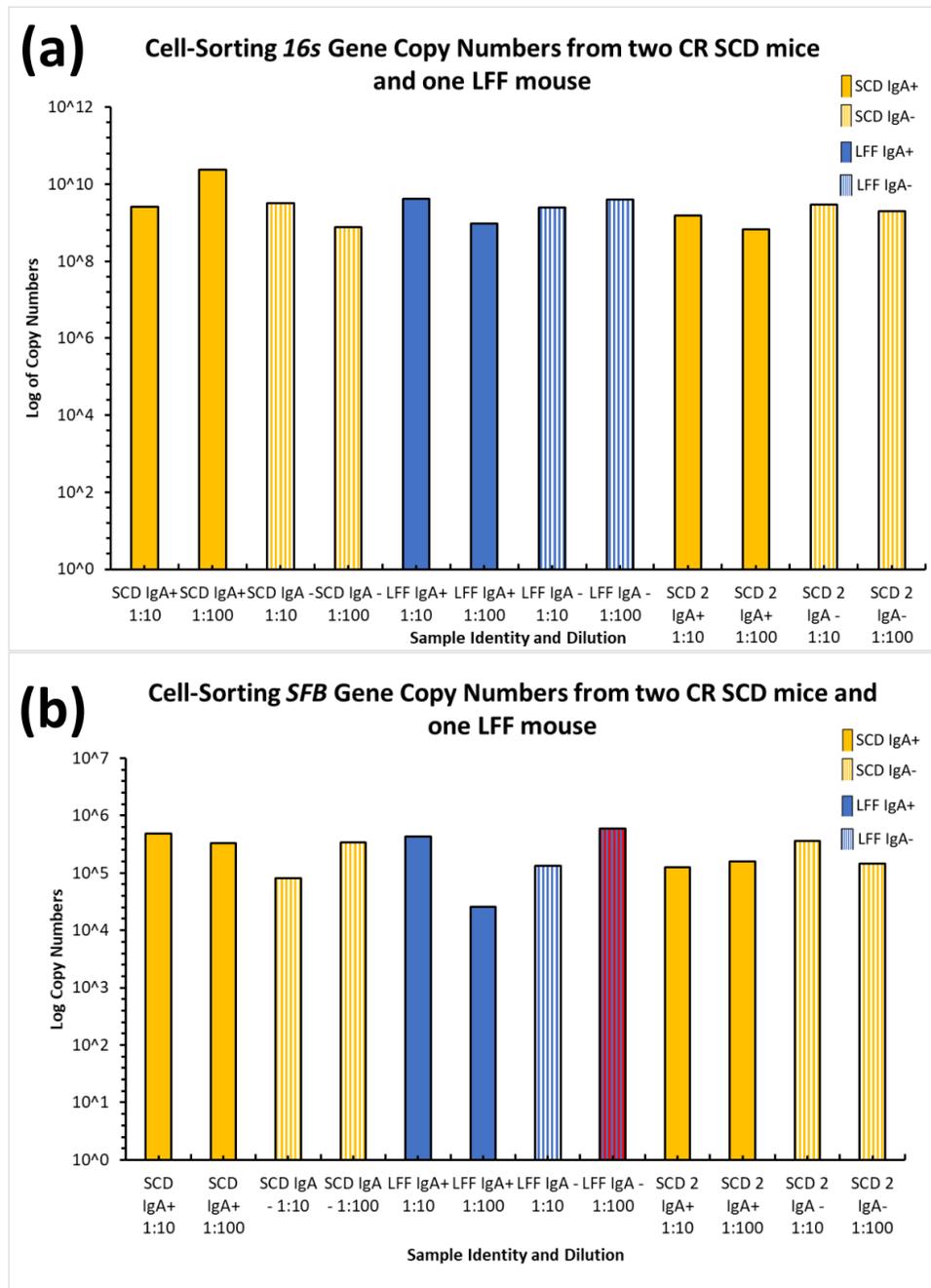
**Figure 27: (a)** 16s and **(b)** SFB Gene Copy Numbers from two CR SCD Mice and one LFF mouse. SCD2 refers to the mouse from the trial before the first official Bacterial Sort experiment. The red bar signifies an unreliable copy number as the melt curve for that sample varied significantly from the melt curves for the SFB plasmid samples used in the qPCR.

Bacterial Sort Experiments 2 to 5:

In the second, third, and fifth Bacterial Sort experiments, the SCD mice showed larger IgA+ and IgA- population sizes and percentages than the LFF mice. However, in experiment 4, the IgA- population size and percentage was larger in the LFF mouse.
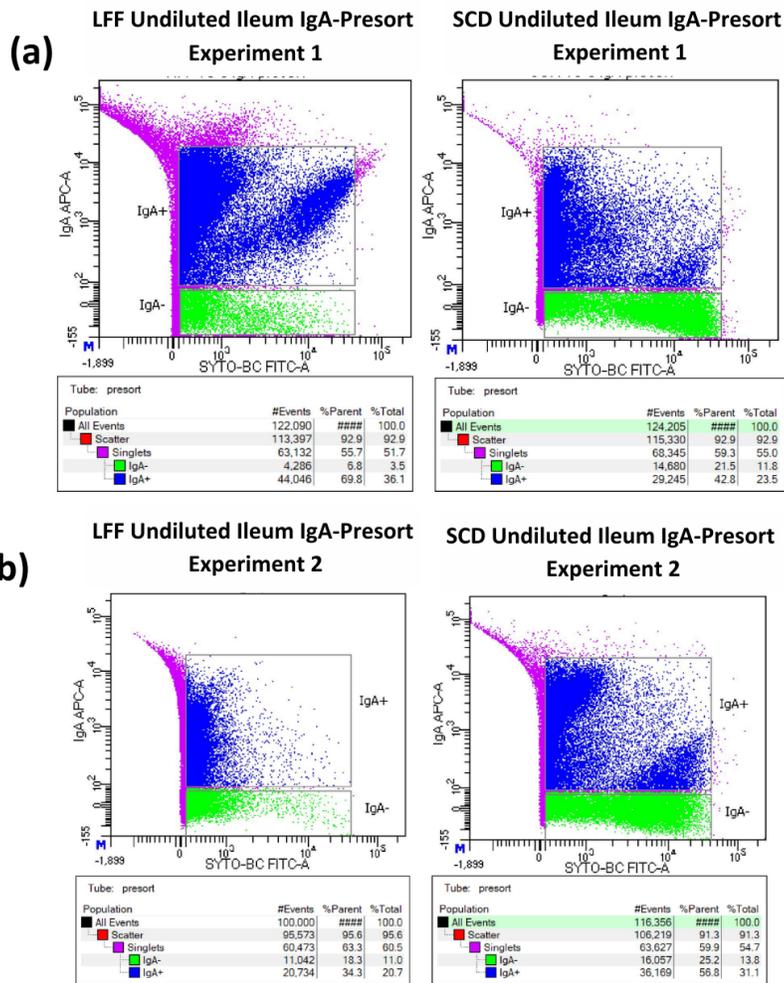
Bacterial Sort Experiments 1 to 5:

Flow cytometry and cell-sorting results for all experiments are shown in **Figures 28 to 30**. We did not run qPCRs on the rest of these experiments due to time-constrains and concerns that we did not have sufficient qPCR reagent volumes to analyze all samples from each experiment. However, the 16s sequencing will return qPCR results for these experiments.

A metadata summary table in **Figure 31** contains the DNA concentrations measured from each sample, sample weights, the final number of bacteria cells extracted from each sample, and total IgA+ and IgA- percentages.

The SCD mice had larger IgA+ and IgA- populations than the LFF mice, on average. IgA-average population sizes were lower than IgA+ for mice from both diets. However, a two-tailed two-sample student's *t*-test ($\alpha = 0.05$) revealed insignificant differences among these comparisons (**Figure 32**).

The LFF IgA+ and IgA- had the same average DNA concentration (ng/μL). However, the SCD IgA+ samples had a higher average DNA concentration than the SCD IgA- samples. Both SCD IgA+ and IgA- samples had higher DNA concentration averages than their LFF counterparts. A two-tailed two-sample student's *t*-test revealed insignificant differences for any of these comparisons (**Figure 33**).

**Figure 28:** Flow cytometry panels and cell-sorted populations from the first **(a)** and second **(b)** Bacterial Sort experiments.

**(a)**

**LFF Undiluted Ileum IgA-Presort Experiment 3**

IgA APC-A

IgA+

IgA-

SYTO-BC FITC-A

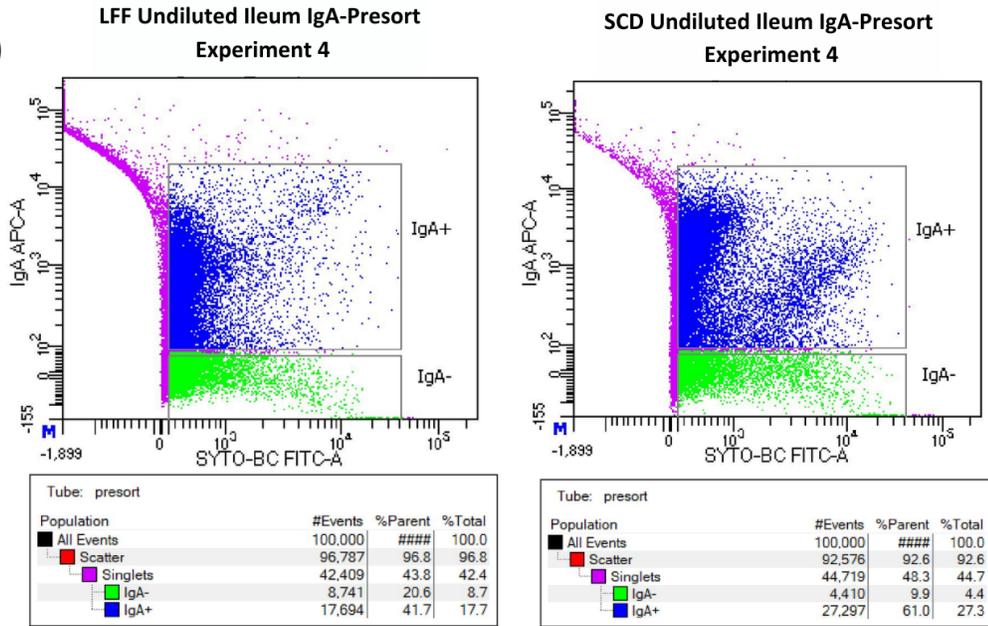| Tube: presort | | | |
|---|---|---|---|
| Population | #Events | %Parent | %Total |
| All Events | 111,747 | #### | 100.0 |
| Scatter | 106,087 | 94.9 | 94.9 |
| Singlets | 51,377 | 48.4 | 46.0 |
| IgA- | 9,831 | 19.1 | 8.8 |
| IgA+ | 24,859 | 48.4 | 22.2 |

**SCD Undiluted Ileum IgA-Presort Experiment 3**

IgA APC-A

IgA+

IgA-

SYTO-BC FITC-A

| Tube: presort | | | |
|---|---|---|---|
| Population | #Events | %Parent | %Total |
| All Events | 133,135 | #### | 100.0 |
| Scatter | 120,640 | 90.6 | 90.6 |
| Singlets | 74,785 | 62.0 | 56.2 |
| IgA- | 19,567 | 26.2 | 14.7 |
| IgA+ | 43,137 | 57.7 | 32.4 |

**(b)**

**LFF Undiluted Ileum IgA-Presort Experiment 4**

IgA APC-A

IgA+

IgA-

SYTO-BC FITC-A

| Tube: presort | | | |
|---|---|---|---|
| Population | #Events | %Parent | %Total |
| All Events | 100,000 | #### | 100.0 |
| Scatter | 96,787 | 96.8 | 96.8 |
| Singlets | 42,409 | 43.8 | 42.4 |
| IgA- | 8,741 | 20.6 | 8.7 |
| IgA+ | 17,694 | 41.7 | 17.7 |

**SCD Undiluted Ileum IgA-Presort Experiment 4**

IgA APC-A

IgA+

IgA-

SYTO-BC FITC-A

| Tube: presort | | | |
|---|---|---|---|
| Population | #Events | %Parent | %Total |
| All Events | 100,000 | #### | 100.0 |
| Scatter | 92,576 | 92.6 | 92.6 |
| Singlets | 44,719 | 48.3 | 44.7 |
| IgA- | 4,410 | 9.9 | 4.4 |
| IgA+ | 27,297 | 61.0 | 27.3 |

**Figure 29:** Flow cytometry panels and cell-sorted populations from the third **(a)** and fourth **(b)** Bacterial Sort experiments.
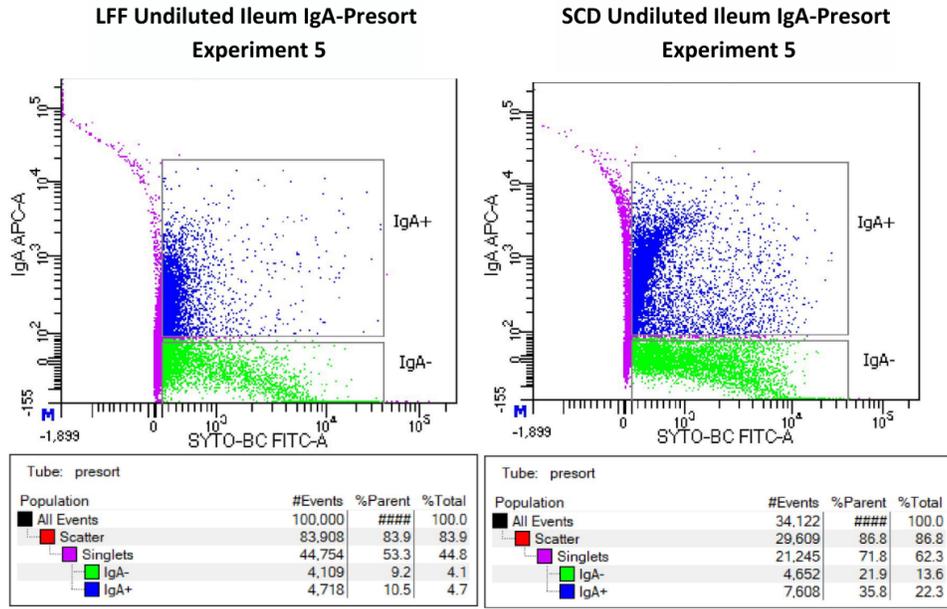
**Figure 30:** Flow cytometry panels and cell-sorted populations from the fifth and final Bacterial Sort experiment.
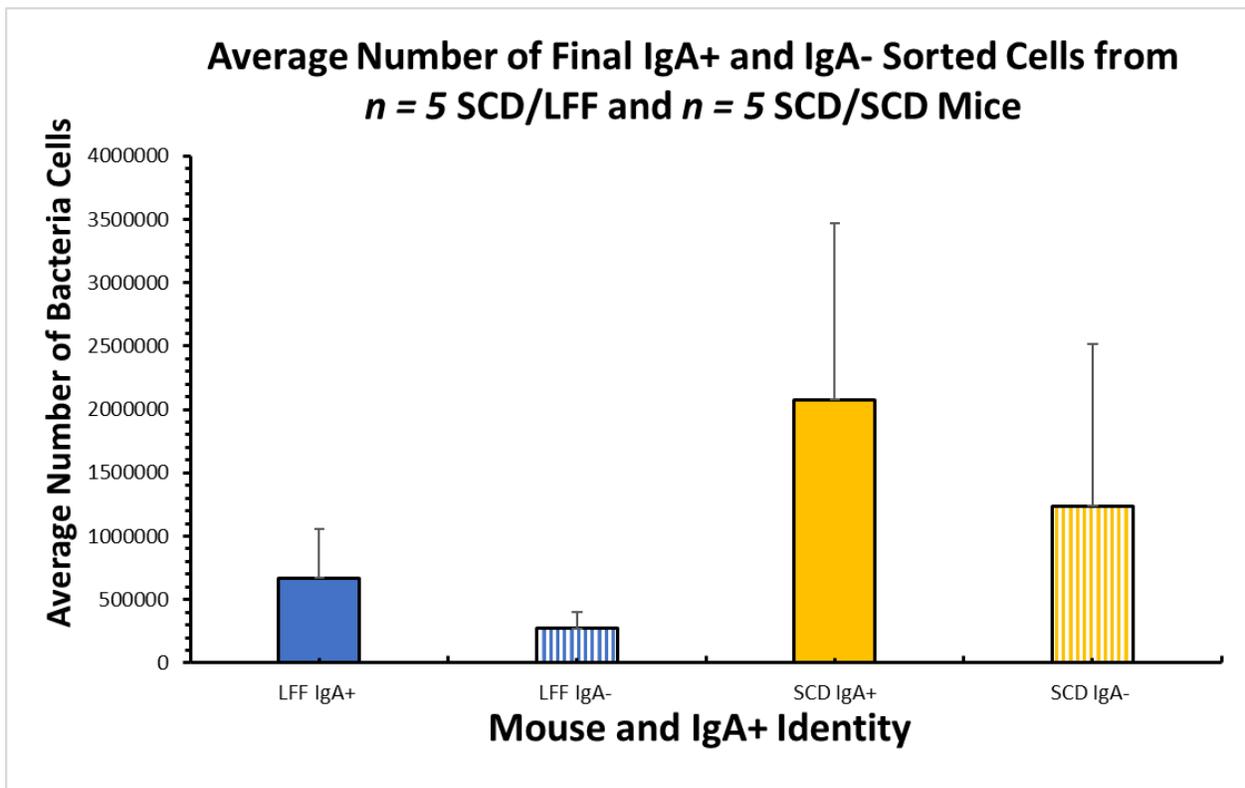


**Figure 31:** Final cell-sorting experiment metadata summary table. **(a)** Metadata for SCD mice. **(b).** Metadata for LFF mice.

**Figure 32:** The average number of bacteria cells obtained from the final IgA+ and IgA- samples from LFF and SCD mice across all five FACS experiments. Error bars are STDEV. A two-tailed two-sample unequal variance student's *t*-test revealed no significant differences in average population numbers for any comparison between IgA and mice diet groups.
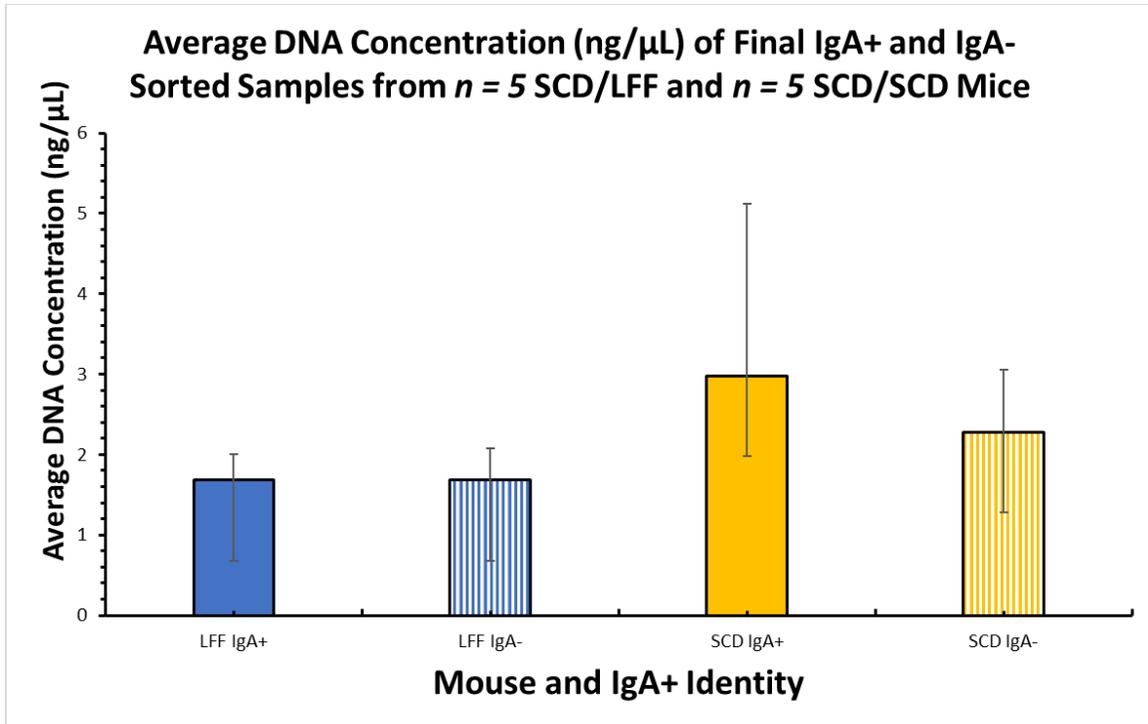
**Figure 33:** The average DNA concentration (ng/µL) obtained from the DNA isolated from the final IgA+ and IgA- samples from LFF and SCD mice across all five FACS experiments. Error bars are STDEV. A two-tailed two-sample unequal variance student's *t*-test revealed no significant differences in average DNA concentrations for any comparison between IgA and mice diet groups.

**Conclusions:**

We obtained an average number of 668848 and 2078705 LFF IgA+ and SCD IgA+ bacteria cells, respectively, and an average number of 274827 and 1237508 LFF IgA- and SCD IgA- bacteria cells, respectively, across all mice tested in these five experiments. Despite SCD mice having larger IgA+ and IgA- populations than LFF mice, these differences were not significant.

We obtained an average DNA concentration of 1.68 ng/µLin both LFF IgA+ and IgA- samples and an average concentration of 2.98 ng/µL and 2.28 ng/µL in the SCD IgA+ and IgA- samples, respectively.

Overall, the SCD mice housed larger IgA+ and IgA- bacteria populations than the LFF mice in our five Bacterial Sort experiments.

**A PREDICTIVE MODEL FOR IGA PLASMA CELL AND B CELL FREQUENCIES GIVEN BACTERIA ORDER RELATIVE PROPORTIONS**

In Figures 32 and 34, we pulled smaller IgA+ and IgA- populations in LFF mice relative to SCD mice. Therefore, It is possible that these reduced numbers in LFF mice may reflect lower bacterial diversity, which in turn may lead to the fecal and ileal IgA concentration differences we observed in **Figure 1**. Unfortunately, we do not have the 16s taxonomic profiles of IgA+ and IgA- consortia from our bacterial sorted samples to see if there is such a relationship between bacterial diversity and immune functioning yet. However, we do have data from a previous set of experiments.

In our previous experiments assessing the impact of dietary fiber on intestinal Th17 and intraepithelial T cell development, we saw that a reduced intake of dietary fiber changes small intestinal microbiota composition and impairs T cell development in the small intestine (**Figure 34**). These results further corroborate a relationship between immune functioning and bacterial diversity impacted by changes in dietary fiber, and suggests that a similar relationship could exist between bacterial diversity and the abundance of plasma and B cells that produce IgA.

To explore this relationship quantitatively, we developed a model from a previous independent experiment in which we measured the frequencies of Lamina propria (LP) plasma and B cells, out of total lymphocytes, that produce IgA in *n = 5* SCD/SCD and *n = 5* SCD/LFF mice. Specifically, we combined the immune cell data from this experiment with the 16s bacteria order reads from five randomly chosen SCD/SCD and five SCD/LFF mice from the data in **Figure 34 (d)**, and generated a Least Absolute Shrinkage and Selection Operator (LASSO) regression model to predict IgA plasma and B cell abundances based off the the proportion of reads for a particular order from each mouse's profile of bacteria orders.

LASSO regression is a regularization method, meaning that it shrinks the size of the Ordinary Least Square (OLS) coefficients by applying a penalty term, $\lambda$, to the OLS mean squared error (MSE) loss function, and increasing it. An optimal $\lambda$ is determined by conducting cross-fold validation on the training data, and seeing which $\lambda$ generates the minimum MSE. When applied in the LASSO model, this $\lambda$ will shrink the sizes of some coefficients and reduce others to zero. The explanatory variables whose coefficients are greater in magnitude than zero best explain

the output when coefficient sizes are penalized. Overall, the LASSO method encourages sparse models and is well-suited for models containing multiple variables showing high multicollinearity.[15]

Developing such a model will provide insight into which bacterial orders best predict immune cell frequencies in mice fed the SCD or LFF diet. Identifying these bacteria may guide us on which bacteria to more thoroughly investigate in the IgA experiment 16s results.
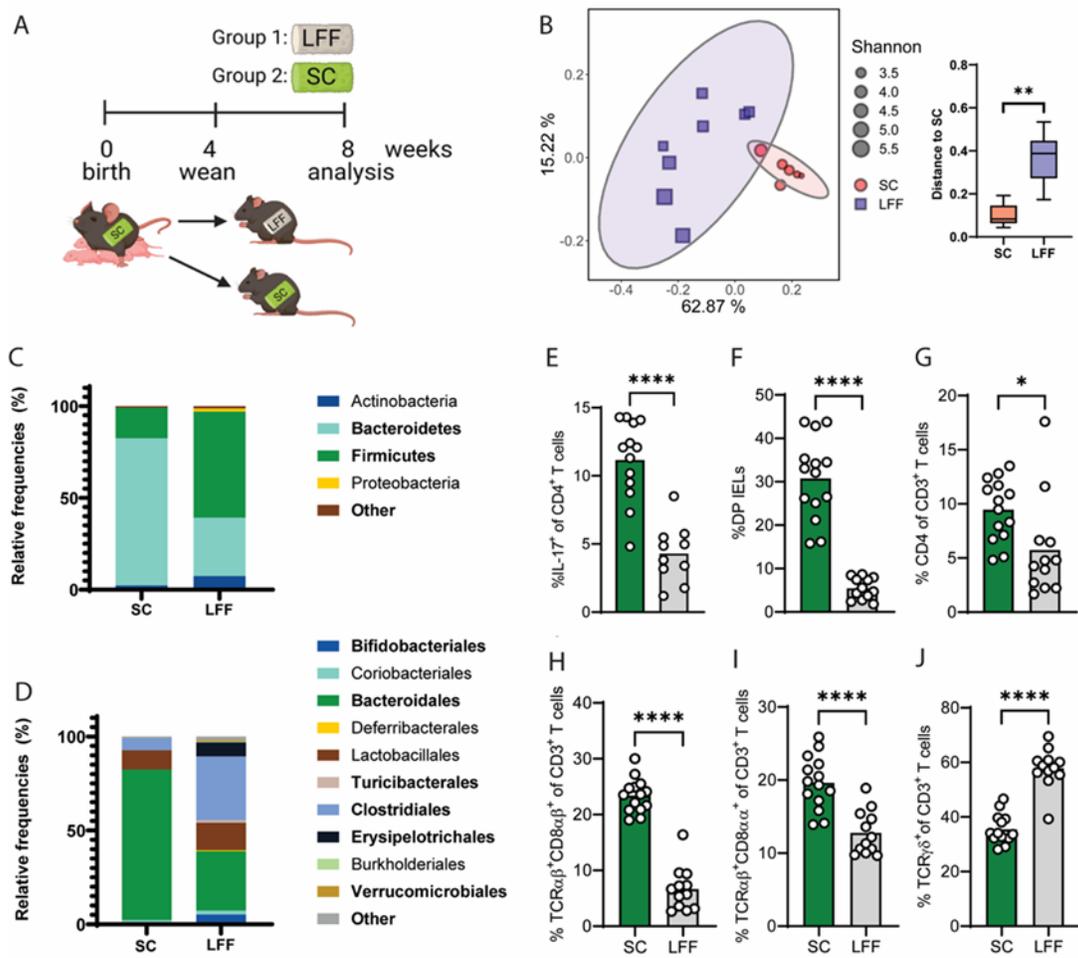


**Figure 34:** Sex-matched, littermate B6 mice were weaned at 4 weeks of age and received standard chow (SC) or low fermentable fiber diets (LFF) for four weeks. At the end of the four weeks, ileal content and small intestines were isolated for analysis A) Experiment design.  B) PCoA of weighted UniFrac distances, box-and-whisker graphs of significant distances to SC

calculated with PERMANOVA. C-D) Taxonomy plots at phylum and order levels of 16s RNA sequencing of ileal contents. Phyla and orders in bold were significantly different. E-J) Small intestinal leukocytes were isolated and analyzed by flow cytometry. Graphs show the frequencies of the indicated populations of CD3+ T cells in the lamina propria (E) and intraepithelial lymphocyte fraction (F-J). Bars represent means, symbols represent single mice. Data are representative of 2-3 independent experiments (n=6-8). Statistical analysis was performed using Student's t test or Mann-Whitney test (*, *p<0.05*; ****, *p<0.0001*).

## PREDICTIVE MODEL CONSTRUCTION

We used the glmnet[16,17] and dplyr[18] packages in R[19] to construct LASSO Predictive Models for the four following groups:

1. **SCD/SCD Mice IgA Plasma Cell Frequencies**
2. **SCD/SCD Mice IgA B Cell Frequencies**
3. **SCD/LFF Mice IgA Plasma Frequencies**
4. **SCD/LFF Mice IgA B Cell Frequencies**

The explanatory variables were the proportion of a mouse's 16s reads that belonged to a particular bacterial order, as proxies for bacterial order relative abundances within a mouse's intestinal environment. To begin this analysis, we imported the bacteria order data used to construct **Figure 34 (d)**, which contained taxonomic data for eight SCD/LFF and six SCD/SCD mice bacteria taxonomic data, and the IgA plasma and B cell data from five SCD/LFF and five SCD/SCD mice. Mice from both experiments were different mice entirely but belonged to the same dietary regimes.

After importing the datasets, we selected five random SCD/LFF and five random SCD/SCD mice and their taxonomic data observations from the former dataset to combine with the latter. This final dataset was named "IgA_Plasma_B_Cell_and_G1_Taxonomic_Data" (**Figure 35**).

| | group | diet born | weaned to | swap | LP IgA Plasma Cells | LP IgA B Cells | k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales | k_Bacteria;p_Firmicutes;c_Bacilli;o_Lactobacillales |
|---|---|---|---|---|---|---|---|---|
| 1 | RNN | CON | NFF | CON-NFF | 0.0420 | 0.0400 | 0.1755415 | 0.15949829 |
| 2 | RNN | CON | NFF | CON-NFF | 0.0615 | 0.4475 | 0.2235541 | 0.01162935 |
| 3 | RNN | CON | NFF | CON-NFF | 0.0640 | 0.6300 | 0.3617836 | 0.09287739 |
| 4 | RNN | CON | NFF | CON-NFF | 0.0690 | 0.7300 | 0.1369425 | 0.06543301 |
| 5 | RNN | CON | NFF | CON-NFF | 0.0710 | 0.3900 | 0.1403210 | 0.66695786 |
| 6 | CR_RRR | CON | CON | none CON | 0.2500 | 3.4300 | 0.7838708 | 0.15470657 |

**Figure 35:** A glance at the dataset containing the taxonomic data from five randomly chosen SCD/SCD and five LFF/LFF mice combined with the immune cell frequency data from an independent experiment analyzing five SCD/SCD and five SCD/LFF mice. "CON" refers to the SCD/SCD diet and "NFF" refers to the SCD/LFF diet. The first column contains the colloquial names we used to describe the dietary regimes. The last two columns are bacteria orders where each entry is the proportion of 16s total reads in that particular mouse that belonged to a specific order.

Then, we split the dataframe into two datasets based on the dietary regime of the mice. These two datasets were called "RNN_IgA_Taxonomic_Data" and "CR_RRR_IgA_Taxonomic_Data," where RNN and CR_RRR refer to the SCD/LFF diet and SCD/SCD diet, respectively.

Summary statistics of the LP IgA plasma and LP IgA B cells data for SCD/SCD and SCD/LFF mice are shown below (**Figure 36**).

**(a)**     **SCD/SCD Mice**

```
LP IgA Plasma Cells  LP IgA B Cells
Min.    :0.0780      Min.    :0.500
1st Qu.:0.1200       1st Qu.:0.590
Median :0.1900       Median :1.040
Mean    :0.1796      Mean    :1.406
3rd Qu.:0.2500       3rd Qu.:1.470
Max.    :0.2600      Max.    :3.430
```

**(b)**     **SCD/LFF Mice**

```
LP IgA Plasma Cells  LP IgA B Cells
Min.    :0.0420      Min.    :0.0400
1st Qu.:0.0615       1st Qu.:0.3900
Median :0.0640       Median :0.4475
Mean    :0.0615      Mean    :0.4475
3rd Qu.:0.0690       3rd Qu.:0.6300
Max.    :0.0710      Max.    :0.7300
```

**Figure 36:** Summary statistics of LP IgA plasma and B cells for **(a)** SCD/SCD and **(b)** SCD/LFF mice.

We split each dataset into a training and test set, where the training set consisted of four observations and the test set consisted of only one. Then, for each training set, at least 3-fold

cross-validation (CV) was performed to find the optimal λ that produced the minimum CV MSE when predicting the four output variables listed above (**Figure 36**). The optimal λ values from each CV were used to construct the four final LASSO regression models for the four training sets.
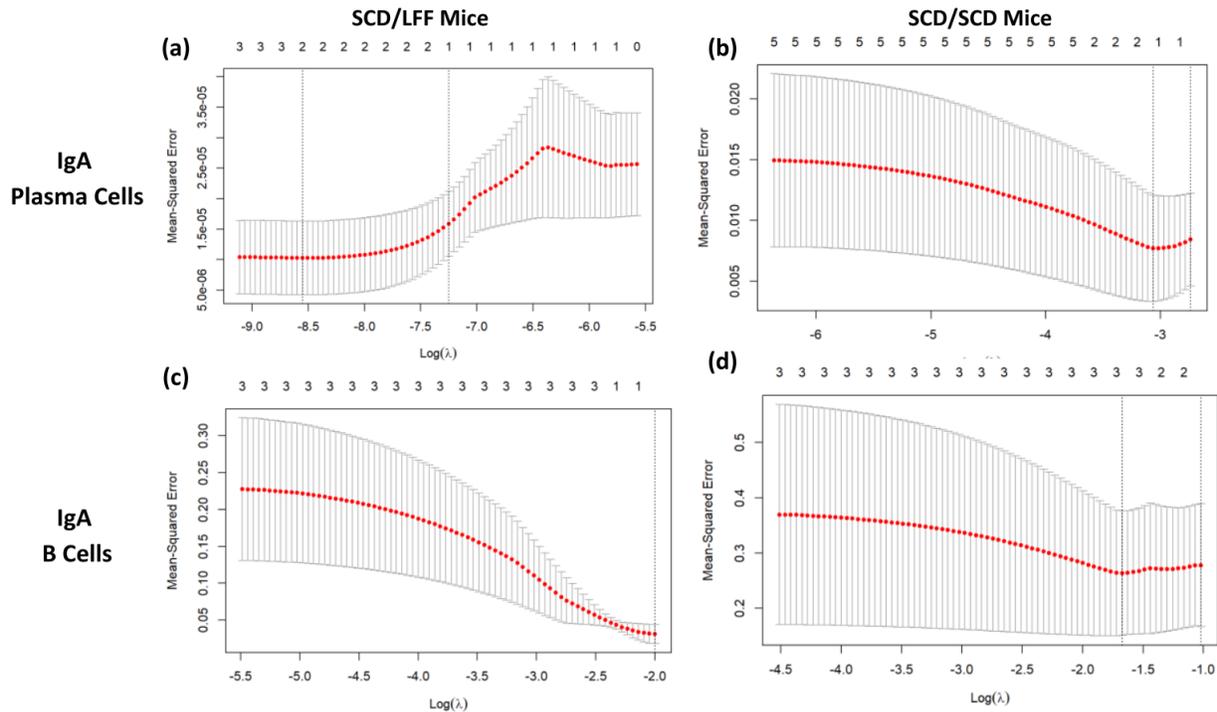


**Figure 37:** Cross-Validation of LASSO regression models predicting IgA plasma cells or IgA B cell frequencies for SCD/LFF and SCD/SCD mice based on the relative read proportions of their bacterial orders. One dashed line indicates the λ value that minimized the CV MSE. The other dashed line indicates the λ value that produces a MSE that falls within 1 standard deviation of the minimum MSE. We use the former λ to construct our LASSO models.

## PREDICTIVE MODEL RESULTS

**Predicting LP IgA Plasma Cells for SCD/LFF Mice:**

The LASSO model for predicting LP IgA Plasma Cells for SCD/LFF Mice based off bacteria taxonomic order relative read proportions using the optimal λ = 0.00019 that minimized the CV MSE was:

*SCD/LFF LP IgA Plasma Cells = 0.0709 - 1.2658\*CyanobacteriaMLE12 - 46.87\*EW055*

At an optimal penalty term of **λ = 0.00019** , a one percentage point increase in the relative proportion of reads for CyanobacteriaMLE1-12 decreases SCD/LFF LP IgA Plasma Cells by 1.2658, on average, EW055 read proportions are held fixed. Conversely, a one percentage point increase in the relative proportion of reads for EW055 , decreases SCD/LFF LP IgA Plasma Cells by 46.87, on average, when CyanobacteriaMLE1-12 read proportions are held fixed.

The $R^2$ for this model was 0.9970603. The out of sample error for one test Point was 0.01917212, meaning the predicted LP IgA Plasma Cell was 0.019 off from the actual value for the test SCD/LFF mouse observation.


**Predicting LP IgA Plasma Cells for SCD/SCD Mice:**

The LASSO model for predicting LP IgA Plasma Cells for SCD/SCDMice based off bacteria taxonomic order relative read proportions using the optimal λ = 0.0467 that minimized CV MSE was:

*SCD/SCD LP IgA Plasma Cells = 0.135 + 0.32\* Clostridiales*


At an optimal penalty term of λ = 0.0467, a one percentage point increase in the relative proportion of reads for Clostridiales increases SCD/SCD LP IgA Plasma Cells by 0.32, on average.

The $R^2$ for this model was 0.4172103. The out of sample error for one test Point was −0.09924327, meaning the predicted LP IgA Plasma Cell was 0.099 off from the actual value for the test SCD/SCD mouse observation.


**Predicting LP IgA B Cells for SCD/LFF Mice:**

The LASSO model for predicting LP IgA B Cells for SCD/LFF Mice based off bacteria taxonomic order relative read proportions using the optimal λ returned no bacterial order coefficients. The $R^2$ for this model was 0.

**Predicting LP IgA B Cells for SCD/SCD Mice:**

The LASSO model for predicting LP IgA B Cells for SCD/SCD Mice based off bacteria taxonomic order relative read proportions using the optimal λ = 0.18768 that minimized CV MSE was:

*SCD/SCD LP IgA B Cells = 0.604 - 0.43\* Clostridiales + 223.878\*Enterobacteriales + 892.801\*Xanthomonadales*

At an optimal penalty term of λ=0.18768, a one percentage point increase in the relative proportion of reads for Clostridiales decreases CR RRR LP IgA B Cells by 0.43, on average, when the relative read proportions of the other two orders are held fixed.

A one percentage point increase in the relative proportion of reads for Enterobacteriales increases CR RRR LP IgA B Cells by 223.878, on average, when the relative read proportions of the other two orders are held fixed. A one percentage point increase in the relative proportion of reads for Xanthomonadales increases CR RRR LP IgA B Cells by 892.80, on average, when the relative read proportions of the other two orders are held fixed.

The $R^2$ for this model was 0.7184672.The out of sample error for one test Point was 0.8439052, meaning the predicted LP IgA B Cell was 0.843 off from the actual value for the test SCD/SCD mouse observation.

## PREDICTIVE MODEL SUMMARY

Assuming that the relationship between LP IgA Plasma and B Cells and Order Relative Read Proportions Follows can be modeled by a linear functional form, the bacterial orders with the highest explanatory powers are:

1. *CyanobacteriaMLE1-12* and *EW055* for Predicting LP IgA Plasma Cells for SCD/LFF Mice
2. *Clostridiales* for Predicting LP IgA Plasma Cells for SCD/SCD Mice
3. None for Predicting LP IgA B Cells for SCD/LFF Mice
4. *Clostridiales*, *Enterobacteriales*, and *Xanthomonadales* for Predicting LP IgA B Cells for SCD/SCD Mice

The out-of-sample test errors for each model were:

1. 0.01917212

2. **−0.09924327**

3. **NA**

4. **0.8439052**

**DISCUSSION AND FUTURE DIRECTIONS**

At the time of writing this manuscript, the DNA from all Bacterial Sort SCD and LFF IgA+ and IgA- samples are currently undergoing 16s rRNA gene sequencing. The V3 and V4 region of the 16S rRNA gene will be PCR-amplified using barcoded primers and sequenced using the Illumina NovaSeq Platform (2 × 250–bp paired-end reads, LC Sciences).

Once we obtain our data, we will conduct bioinformatics analyses using the QIIME 2 2023.2 pipeline consisting of denoising with DADA236, construction of phylogenetic trees with mafft37 and fasttree238, calculations of Shannon diversity index, weighted UniFrac analyses with q2-diversity, and production of phylogenetic assignments with q2-feature-classifier classify-sklearn36, to assign taxonomy using the Greengenes 13_8 sequence database37.

From our LASSO predictive models, *CyanobacteriaMLE12*, *EW055*, *Clostridiales*, *Enterobacteriales*, and *Xanthomonadales* were the only bacterial orders with non-zero coefficients that best explained IgA-producing immune cell frequencies. The model with the best performance was the one predicting IgA plasma cell frequencies for SCD/LFF mice; its $R^2$ was 0.99 and prediction was 0.02 off from the test mouse's observed plasma cell frequency. The model predicting IgA B cell frequencies for SCD/SCD mice had the second best $R^2$ (0.718) but the worst out-of-sample predictive performance, being 0.84 off from the test mouse's observed B cell frequency. The model predicting IgA plasma cell frequencies for SCD/SCD mice had the worst $R^2$ (0.417) but the second best out-of-sample predictive performance, being 0.1 off the test mouse's observed plasma cell frequency.

Overall, a linear relationship between bacterial orders with non-zero coefficients and IgA-producing immune cell frequencies best explained plasma cell and B cell frequencies for SCD/LFF and SCD/SCD mice, respectively. Conversely, the same linear relationship weakly explained B cell and plasma cell frequencies for SCD/LFF and SCD/SCD mice, respectively.

A downfall in these modeling efforts was that we could not robustly gauge the out-of-sample predictive performance of each model as we tested each model on one test observation only. Therefore, our estimates of out-of-sample predictive errors are not representative of each model's generalizability. Furthermore, our models were only trained on four observations, which may not be a sufficient sample size to determine how appropriate these linear fits are.

From these bacterial orders with non-zero coefficients, we will see if they appear once more when we use LASSO predictive models to predict the same outputs with the 16s taxonomic data from the IgA experiment. If they do, they significantly explain IgA producing cell frequencies within different mice diets in a linear context. Identifying bacteria that best explain immune cell frequencies may provide insights into bacteria that interact significantly with IgA plasma and B cells and, therefore, impact IgA concentrations. Therefore, we can experimentally explore the relationships between these bacteria and IgA immune cells and concentrations to determine if there are any causal relationships. Doing so will help us uncover the specific mechanisms underlying decreased IgA in mice fed the LFF diets. The findings from continued research will further our understanding of how diet alters the immune system and may inform future studies delineating the possible causes of human IgA deficiency and developing appropriate treatments and therapies.

**MATERIALS AND METHODS SUMMARY**

All mice ileal material extraction, sample washing and staining, and sample transferring to flow cytometry tubes, was performed using Eppendorf Research® plus mechanical pipettes, filtered VWR® universal pipette tips, and in sterile conditions in the Purifier Logic + Class II, TYPE A2 Biosafety Cabinet (BSC).

**PS1: Maximizing Bacteria Cell Numbers in Mice Ileal Samples via DTT and IEL Buffer Pre-treatment**

*n = 8* male Black-6 SCD CR mice were used in these tests, where half received a DTT and IEL buffer pretreatment. We extracted each mouse's ileal and large intestine contents, collected the feces, scraped the intestinal walls with a sterile razor blade, and placed all contents in 1.5 mL Eppendorf collection tubes.

While the sample tubes sat on ice, we prepared the DTT and IEL buffer pre-treatment by making a DTT stock of 6mg DTT and 400 uL of IEL buffer. 5 uL of this stock was combined with 495 uL IEL buffer to create a DTT master mix. The DTT master mix volume was increased depending on how many samples we worked on in a given run of these experiments.

500 uL of the master mix was aliquoted onto each ileum and LI sample. Samples were vortexed and incubated for 20 minutes on ice. Then, 250 uL of ileal contents were removed from each ileum sample into a separate tube post-incubation. Tubes were vortexed, and bacteria were pelleted via centrifugation at 15060 RPM in a Eppendorf microcentrifuge 5425 for three minutes. Supernatant from tubes was removed, and pellets were resuspended in 1 mL Inhbit EX buffer if they were to undergo qPCR or 500 uL of 1X PBS if they were to undergo flow cytometry.

200 uL of supernatant from each flow sample was pipetted off, filtered over sterile, 5 mL round bottom polystyrene FACS tubes with 35 um strainer caps and washed with no more than 1.3 ml of 1X PBS. Each sample's resulting filtrate was transferred to a separate 1.5 ml tube for max speed centrifugation for 3 minutes, washing with PBS, and supernatant removal.

The samples were split into two 500 uL aliquots. Each half received 100 ulLof a 1:50 concentration of IgA-APC or IgG-APC. Each sample underwent incubation, further washing with

PBS, and was stained with 200 uL of 1:400 ThermoFisher Invitrogen™ SYTO™ 9 Green Fluorescent Bacteria Nucleic Acid Stain. Stained samples were ultimately resuspended in 200 uL FACS to be measure be in the BD LSRFortessa™ X-20 Cell Analyzer.

DNA was extracted from each qPCR sample via the QIAGEN kit. Extracted and eluted DNA samples underwent qPCR to quantify the number of 16s and SFB gene copies in the samples.

We assessed the impact of additional washing on pre-treated samples' bacteria gene copy numbers in our samples. *n = 4* CR mice had their samples pre-treated and then washed with materials used to prepare samples for antibody staining and flow cytometry. This wash treatment would further reduce mucus in the samples and is the same procedure used in cell sorting. Therefore, the wash treatment would replicate the results we get back from cell sorting.

**PS2: Optimizing Flow Cytometry FIT-C, Side Scatter (SSC), and Forward Scatter (FSC) Parameters to Maximally Reveal Locations of Bacteria in Ileal Samples**

We prepared LB and MRS liquid media in which to grow our *E.coli* and *L.reuteri*, respectively. MRS broth was made using 150  ml of Milli-Q® (MQ) ultrapurified water, 12.75 grams of MRS powder, and 250 microliters of Tween 80. LB broth was made with 150 ml of MQ water, 2.5 grams of Tryptone powder, 1.25 grams of yeast extract, and 2.5 grams of salt. All contents were placed inside bottles and autoclaved for about 47 minutes at 121 °C.

After autoclaving, two 15 mL tubes were gathered for each bacteria, and 10 mL broth were aliquoted in each. One tube in each pair served as control samples lacking bacteria. For the experimental tubes, we pricked -80 °C frozen cryo-samples of *E.coli* and *L. reuteri* using a sterile filtered pipette tip and submerged and agitated them in the tubes. Control tubes were pricked with blank sterile tips. All samples were inoculated overnight; *E.coli* were shaker incubated while *L.reuteri* were idly incubated.

Blank controls showed no growth. Two new 15 mL empty tubes were gathered for each bacteria sample. We transferred 10 mL of broth into each new tube and 500 uL of bacteria. Samples were incubated for four hours for the bacteria to grow to the log phase.

We transferred 200 uL aliquots of our cultures and blank samples into a sterile 96-well plate to analyze via optical density (OD600) to estimate the number of cells per mL in each strain. $1\times10^7$ cells of each bacteria were added to 5 mL PBS each in new 15 mL tubes. A combination of both bacteria containing $2.5\times10^6$ cells for each strain was added in 5 mL PBS. Samples were centrifuged in the Eppendorf Centrifuge 5810R Benchtop Centrifuge at 2200 RPM for 10 minutes at 32 °C. The supernatant was aspirated from the top of each sample and transferred to sterile 1.5 mL tubes containing 500 uL PBS.

We prepared a SYTO buffer stock consisting of 45 mL water, 0.45 g NaCl, and 5 mL 1M Hepes to prepare our SYTO BC 1:2000 dilution. 1 mL SYTO buffer was aliquoted in each tube to wash the samples. Samples were spun at 10000 RPM for 3 minutes.

The supernatant from each sample was vacuumed off once more so that each sample that would be stained with SYTO BC could be washed with 200 uL of SYTO buffer and 200 uL of 1:2000 SYTO BC. Samples were stained on ice in darkness for 5 minutes. All samples were spun one last time at 10000 RPM for 3 minutes, resuspended in 200 uL FACS buffer, and then transferred to FACS tubes for flow cytometry.

**PS3: Titration of our Bacterial Nucleic Acid Dye, SYTO BC, Stained Onto Pure Bacteria Samples to Determine a Concentration that Optimally Distinguishes Bacteria and Non-Bacteria**

We followed the same procedure in PS2 for this experiment. We prepared a 96-well plate for the titrations, where we added 25 uL of bacteria to seven wells in one row. We added 25 uL of 1:2000 SYTO BC to the first well and serially diluted it. One well did not receive SYTO BC to serve as a blank control.

The plate was incubated in an ice bucket and was spun at 2200 RPM for 10 minutes afterward. Each well was resuspended in 200 uL FACS buffer and transferred to flow cytometry tubes to be brought to flow cytometry.

**PS4: Assessing our Optimal SYTO BC Concentration on Serially Diluted Ileal Samples**

Ileal samples were extracted using the same bacterial sort protocol in PS1 sans antibody staining. Ileal samples were diluted from 1:10 to 1:1000 using SYTO buffer. 25 uL of each dilution was plated in a 96-plate well with different SYTO BC concentrations from 1:8000 to 1:3200. The plate was incubated in an ice bucket and was spun at 2200 RPM for 10 minutes. Each well was resuspended in 200 uL FACS buffer and transferred to flow cytometry tubes. See **Figure 10** from the Bacterial Sort Protocol Standardization section for an illustration of the 96-well plate layout.

**PS5: Titration of IgA-PE and IgA-BV650 Immunostainers to Determine a Better Fluorophore and a Concentration that Optimally Distinguishes IgA+ and IgA- bacteria**

1:25 to 1:800 PE-IgA and BV650-IgA dilutions were made using FACS buffer. Ileal samples were extracted and processed using the bacterial sort protocol described in PS1 and diluted from 1:10 to 1:1000 using SYTO buffer.

They were brought to the 96-well plate, where they were stained with 1:8000 SYTO BC, incubated on ice for 5 minutes, and then stained with the appropriate antibody and concentration. Blanks lacking the antibody were prepared for each antibody and ileum concentration. The PE, BV650, FIT-C, and unstained flow cytometry compensation controls were plated in one column where each well had 25 uL of 1:100 ileal sample. Both IgA-fluorophore wells were given 25 uL of a 1:25 concentration of the appropriate fluorophore. The FIT-C compensation well received 25 uL of 1:4000 SYTO BC. The unstained control received 25 uL FACS buffer. After preparing all samples, the plate sat on ice in darkness for 30 minutes to give sufficient time for antibody staining and saturation.

After incubating, the plate was spun at 2200 RPM for 10 minutes. Then, the plate's wells were resuspended in 200 uL FACS buffer for it to undergo another spin and resuspension cycle. After this second cycle, all well samples were transferred to flow cytometry tubes. See **Figure 12** for the 96-well plate layout for this protocol standardization experiment.

**PS6: Repeat of PS5 for PE-IgA Only**

We followed the same procedure in PS5 but with PE-IgA only. Furthermore, we only stained the ileal samples with 1:50 and 1:100 PE-IgA instead of using the 1:25 to 1:800 range.

**PS7: Titration of IgA-Biotin-Streptavidin-AF647 to Determine a Concentration that Optimally Distinguishes IgA+ and IgA- Bacteria**

We followed the same procedure in PS6 but with IgA-Biotin and TFNγ-AF647, the isotype control for this experiment. 1:50 to 1:100 IgA-Biotin and TFNγ-AF647 dilutions and a 1:400 Streptavidin-AF647 stock were made using FACS buffer. Ileal samples were extracted and processed using the antibody staining protocol described in PS1 and diluted from 1:10 to 1:1000 using SYTO buffer.

They were brought to the 96-well plate, where they were stained with 1:8000 SYTO BC, incubated on ice for 5 minutes, and then stained with the appropriate antibody and concentration. The APC (AF647), FIT-C, and unstained flow cytometry compensation controls were plated in one column. The AF647 compensation well received 20 uL of 1:100 ileal sample whereas the other two wells received the usual 25 uL. The AF647 compensation well received 20 uL of IgA-Biotin. The plate sat on ice for 30 minutes to give sufficient time for antibody staining and saturation.

Afterward, the plate was spun at 2200 RPM for 10 minutes. Supernatant from each well was aliquoted off. The wells that received IgA-Biotin were resuspended in 40 uL FACS buffer and 40 uL 1:400 Streptavidin-AF647. The wells lacking IgA-Biotin were resuspended in 40 uL FACS buffer. The plate was incubated on ice for 30 minutes for Streptavidin to bind to and saturate the Biotin molecules.

The wells were resupended in 200 uL FACS buffer, spun at 2200 RPM for 10 minutes, and went through one more resuspension and spin cycle to be ultimately transferred to flow cytometry tubes.

**PS8: Testing if 1:4000 SYTO BC Outperforms 1:8000 SYTO BC in Aiding IgA-Biotin-Streptavidin-AF647 Yield a Favorable IgA+ and IgA- Average Percentage**

We followed the same procedure in PS7 but split all samples to be stained with 1:4000 or 1:8000 SYTO BC .

**PS9: Repeating PS8 with a new SYTO BC Stock**

We followed the same procedure in PS8 but using a new SYTO BC stock that we transferred in 40 PCR tubes with 5 uL SYTO BC each that were kept in -20 °C.

**PS10: Cell-Sorting Trial 1 with One Jax SCD Mouse utilizing 1:8000 SYTO BC and 1:50 IgA-Biotin-Streptavidin**

We conducted the bacterial-sort protocol and stained ileal contents from one SCD Jax mouse with 1:8000 SYTO BC and 1:50 IgA-Biotin-Streptavidin-AF647. The compensation controls were the same from PS7.

We intialized the BD FACSAria™ II Sorter's flow cytometry voltages with a cultured *L.reuteri* sample stained with 1:2000 SYTO BC and prepared using the same procedure from PS2. We only used *L.reuteri* this time as our *E.coli* cultures were contaminated by the time we scheduled to perform cell-sorting**.**

**PS11: Cell-Sorting Trial 2 with One Jax SCD Mouse utilizing 1:2000 SYTO BC and 1:50 IgA-Biotin-Streptavidin**

We followed the same procedure in PS10 but did not need to prepare bacteria cultures and changed the SYTO BC concentration from 1:8000 to 1:2000.

**PS12: Cell-Sorting Trial 3. Staining a CR SCD Mouse's Undiluted Ileum Sample with 1:2000 SYTO BC and 1:50 IgA-Biotin**

We followed the same procedure in PS11 but stained an undiluted ileum sample with the fluorophores instead of the 1:10 and 1:100 dilutions used in the previous experiments.

**Bacterial Sort Experiments**

The same procedure in PS7 was repeated. 1:100 ileal sample from the SCD mouse was still used to make the compensation controls. These experiments were explained in sufficient detail in the Standardized Final Bacterial Sort experiments section earlier in this manuscript.

DNA Extractions for Bacterial Sort Experiments:

We isolated DNA from cell-sorting experiments 1 to 5 using the QIAGEN kit.

qPCRs:

Quantitative PCR (qPCR) was performed using extracted DNA from intestinal contents, SFB 736F and SFB 844R oligonucleotides38 and iTaq Universal SYBR green supermix (Bio-Rad) in a CFX96 C100 real-time thermocycler (Bio-Rad). Genome copies were calculated using serial dilutions of a pUC57 plasmid expressing SFB target sequence as standard.

Mice and Diets:

C57BL/6 mice were purchased from Charles River Laboratories and Jackson Laboratories. Mice were bred in a pathogen-free facility at Emory University's Health Sciences Research Building. Sex-matched littermate animals at the indicated ages were used throughout the Bacterial sort experiments. All animal experiments were conducted according to the U.S.A. Public Health Service Policy of Humane Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee (School of Medicine, Emory University) based on the Guide for the Care and Use of Laboratory Animals. IACUC Protocol Numbers: PROTO201900010. Mice on a standard chow diet (SCD) received PicoLab Rodent Diet 5053 (LabDiet), and mice on a low fermentable fiber diet (LFF) received AIN-93G (Research Diets Inc.) ad libitum.

**DECLARATION OF INTERESTS**

The authors declare no conflict of interest.

**DATA AVAILABILITY**

All data can be found at https://github.com/waltermoisesavila/cervantes-barragan-lab.

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## SUPPLEMENTARY INFORMATION

**Specificity of Goat Derived Anti-Mouse IgA:**

The IgA antibodies we used in PS1 and the rest of the experiments are produced by inoculating goats with mice IgA. Goats then produce IgA antibodies in response to these foreign substances. Therefore, under optimal conditions, goat anti-mouse IgA antibodies will recognize and bind to IgA on bacterial cells in the mice fecal extracts.

From the flow cytometry plots, we saw that the IgA panels showed significantly higher antibody binding than the IgG panels; the percentage of flow cytometry events that were bacteria and bound by the antibody was significantly higher in the IgA panel, suggesting our IgA binding was specific (**Figure 6 and Supplementary Figures S1-S4**). To statistically evaluate binding specificity, we collected all IgA+ flow event percentages and compared average event percentages via student *t*-tests, which showed significant differences in this percentage among samples stained with IgA and IgG (*\*, p<0.05; \*\*, p<0.01*), demonstrating our IgA staining is indeed specific. **Supplementary Figure S7** shows a bar graph comparing these percentages among IgA and IgG stained samples and **Supplementary Figures S5-S6** contain the raw percentage data.

**Seeing High Bacteria Autofluorescence in PS5:**

Contamination did not explain the high autofluorescence trends in the mouse ileal samples lacking the BV650-IgA fluorophore and antibody because we analyzed the pure bacteria cultures from PS2 using the FIT-C and BV650 fluorophores on the X and Y axis, respectively. When viewed with these parameters, these samples showed the same high false IgA+ positive behavior even though no antibody-fluorophore pair was used in this experiment (**Supplementary Figure S**).
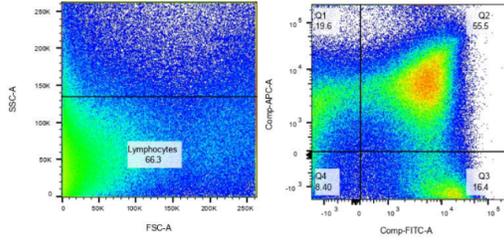
**Weighing Mice Ileal and Fecal Samples:**

In PS5, we began weighing the extracted ileal samples (in grams (g)) to delineate the relationship between sample weight and flow cytometry event rate. Modeling this relationship

could help standardize the ileal sample amount we use in potential future repeats of the
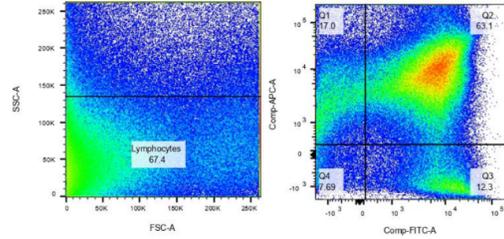
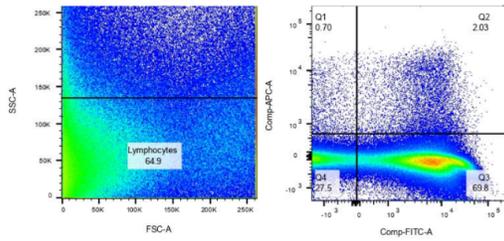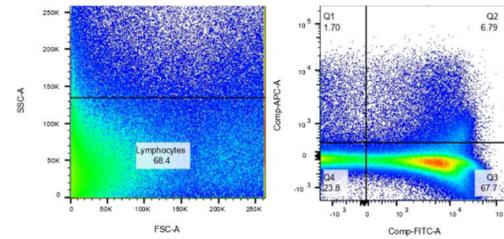Bacterial sort experiments in the future.

**SUPPLEMENTARY FIGURES**



**Supplementary Figure S1:** Flow cytometry graphs of ileal contents extracted from pre-treated

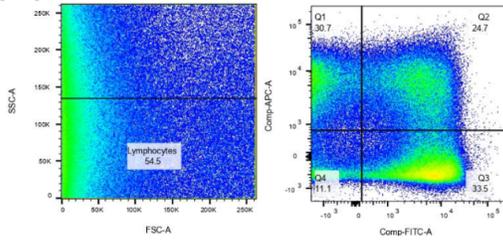mice. **(a)** Top: pretreated mouse 1 ileum contents stained with IgA. Bottom: pretreated mouse 1
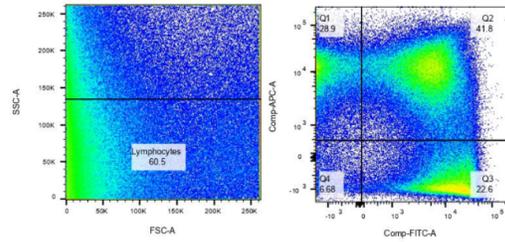
ileum contents stained with IgG. **(b)** Top: pretreated mouse 2 ileum contents stained with IgA. Bottom: pretreated mouse 2 ileum contents stained with IgG. **(c)** Top: pretreated mouse 3 ileum contents stained with IgA. Bottom: pretreated mouse 3 ileum contents stained with IgG.**(d)** Top: pretreated mouse 4 ileum contents stained with IgA. Bottom: pretreated mouse 4 ileum contents stained with IgG.



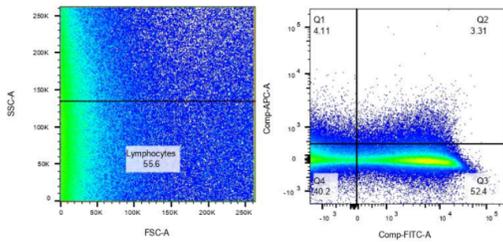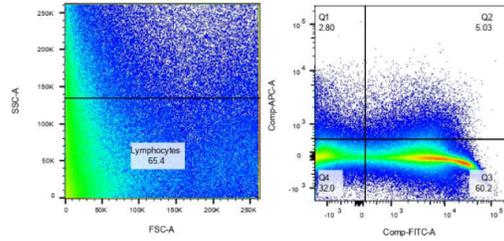**(a)** Pretreated Mouse 1 LI IgA
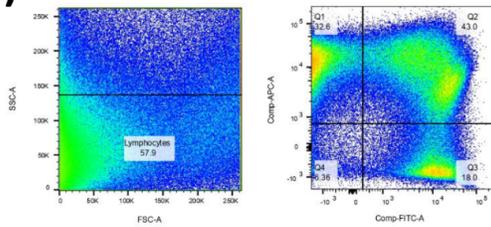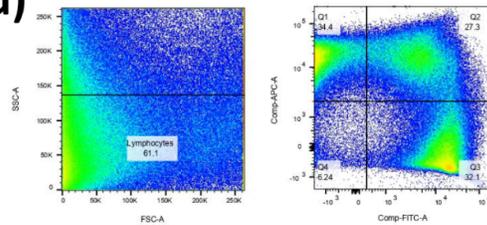Pretreated Mouse 1 LI IgG

**(b)** Pretreated Mouse 2 LI IgA
Pretreated Mouse 2 LI IgG
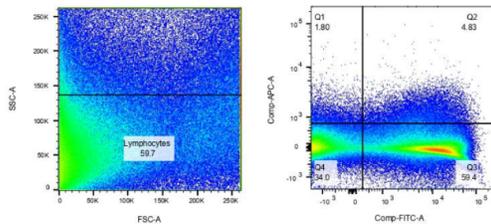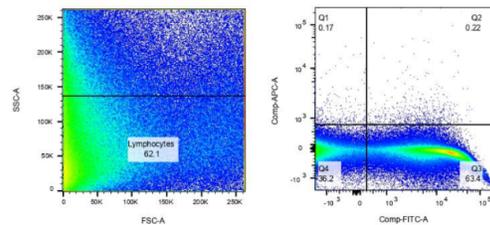
**(c)** Pretreated Mouse 3 LI IgA
Pretreated Mouse 3 LI IgG

**(d)** Pretreated Mouse 4 LI IgA
Pretreated Mouse 4 LI IgG

**Supplementary Figure S2:** Flow cytometry graphs of the large intestine (LI) contents extracted from pre-treated mice. **(a)** Top: pretreated mouse 1 LI contents stained with IgA. Bottom: pretreated mouse 1 LI contents stained with IgG. **(b)** Top: pretreated mouse 2 LI contents stained with IgA. Bottom: pretreated mouse 2 LI contents stained with IgG. **(c)** Top: pretreated mouse 3 LI contents stained with IgA. Bottom: pretreated mouse 3 LI contents stained with IgG. **(d)** Top: pretreated mouse 4 LI contents stained with IgA. Bottom: pretreated mouse 4 LI contents stained with IgG.



**(a)**

Untreated Mouse 1 Ileum IgA

Untreated Mouse 1 Ileum IgG

**(b)**

Untreated Mouse 2 Ileum IgA

Untreated Mouse 2 Ileum IgG

**(c)**

Untreated Mouse 3 Ileum IgA

Untreated Mouse 3 Ileum IgG

**(d)**

Untreated Mouse 4 Ileum IgA

Untreated Mouse 4 Ileum IgG
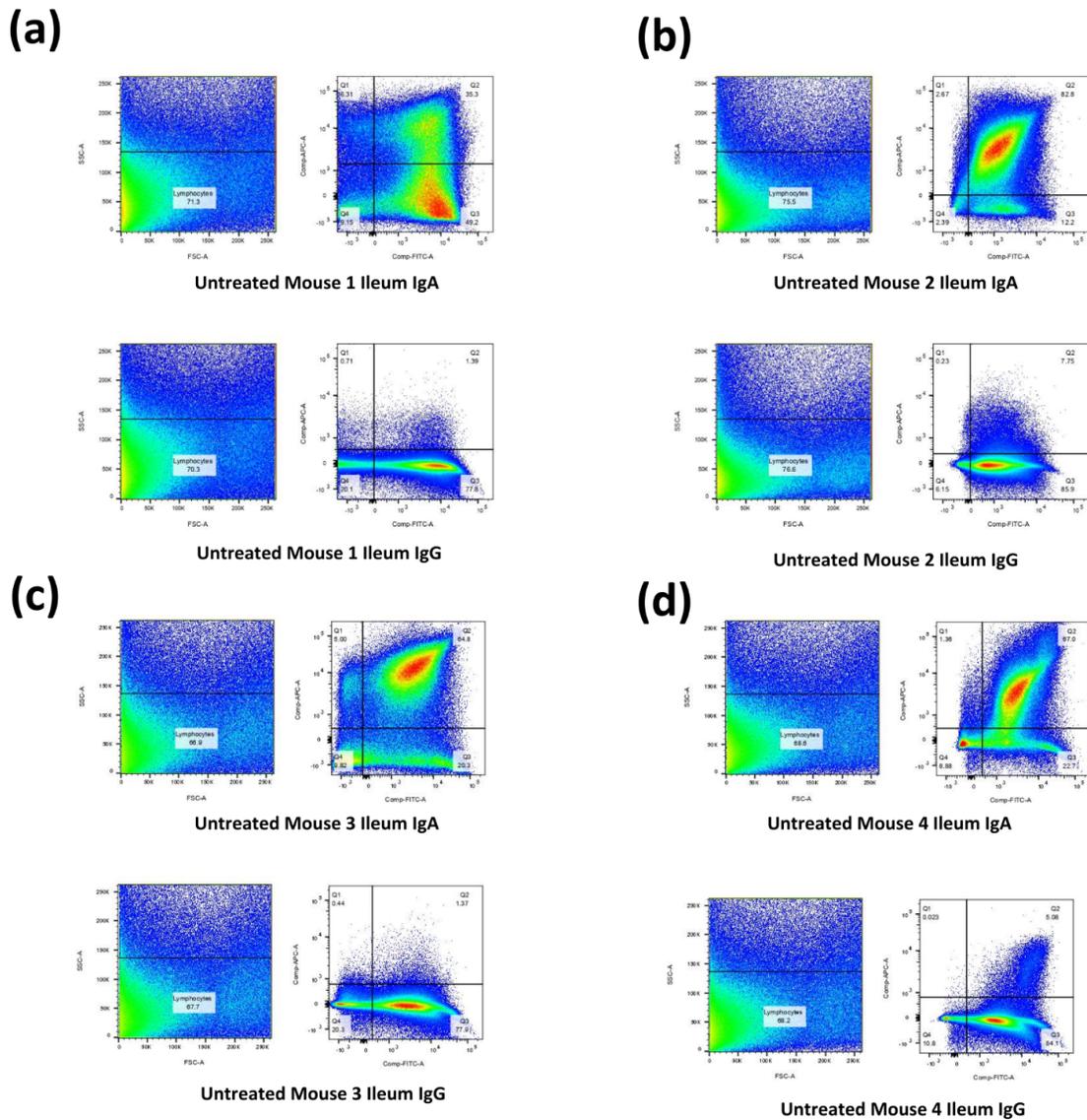
**Supplementary Figure S3:** Flow cytometry graphs of the ileal contents extracted from untreated mice. **(a)** Top: untreated mouse 1 ileum contents stained with IgA. Bottom: untreated mouse 1

ileum contents stained with IgG. **(b)** Top: untreated mouse 2 ileum contents stained with IgA. Bottom: untreated mouse 2 ileum contents stained with IgG. **(c)** Top: untreated mouse 3 ileum contents stained with IgA. Bottom: untreated mouse 3 ileum contents stained with IgG. **(d)** Top: untreated mouse 4 ileum contents stained with IgA. Bottom: untreated mouse 4 ileum contents stained with IgG.



**(a)** Untreated Mouse 1 LI IgA

Untreated Mouse 1 LI IgG

**(b)** Untreated Mouse 2 LI IgA

Untreated Mouse 2 LI IgG

**(c)** Untreated Mouse 3 LI IgA

Untreated Mouse 3 LI IgG

**(d)** Untreated Mouse 4 LI IgA

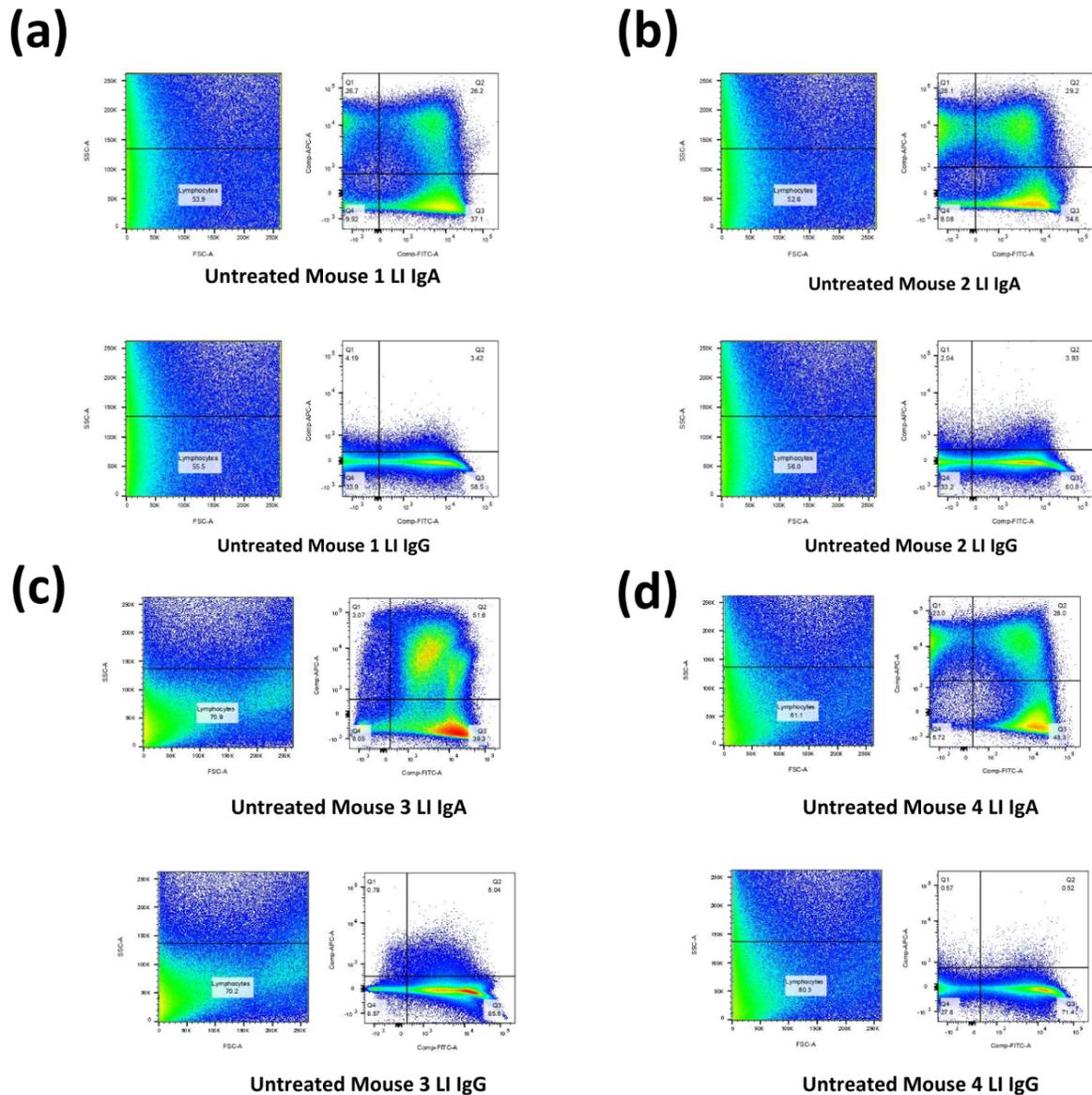Untreated Mouse 4 LI IgG

**Supplementary Figure S4:** Flow cytometry graphs of the LI contents extracted from untreated mice. **(a)** Top: untreated mouse 1 LI contents stained with IgA. Bottom: untreated mouse 1 LI

contents stained with IgG. **(b)** Top: untreated mouse 2 LI contents stained with IgA. Bottom: untreated mouse 2 LI contents stained with IgG. **(c)** Top: untreated mouse 3 LI contents stained with IgA. Bottom: untreated mouse 3 LI contents stained with IgG. **(d)** Top: untreated mouse 4 LI contents stained with IgA. Bottom: untreated mouse 4 LI contents stained with IgG.

| Mouse Sample | APC + SYTO BC - (Q1) | APC + SYTO BC + (Q2) | APC - SYTO BC - (Q3) | APC - SYTO BC + (Q4) |
|---|---|---|---|---|
| Untreated Mouse 1 Ileum IgA | 6.13 | 35.2 | 49.6 | 9.01 |
| Untreated Mouse 1 Ileum IgG | 0.71 | 1.39 | 77.8 | 20.1 |
| Untreated Mouse 1 Large Intestine IgA | 26.7 | 26.2 | 37.1 | 9.92 |
| Untreated Mouse 1 Large Intestine IgG | 4.19 | 3.42 | 58.5 | 33.9 |
| Untreated Mouse 2 Ileum IgA | 2.67 | 82.8 | 12.2 | 2.39 |
| Untreated Mouse 2 Ileum IgG | 0.23 | 7.75 | 85.9 | 6.15 |
| Untreated Mouse 2 Large Intestine IgA | 28.1 | 29.2 | 34.6 | 8.08 |
| Untreated Mouse 2 Large Intestine IgG | 2.04 | 3.93 | 60.8 | 33.2 |
| Untreated Mouse 3 Ileum IgA | 5 | 64.8 | 20.3 | 9.82 |
| Untreated Mouse 3 Ileum IgG | 0.44 | 1.37 | 77.9 | 20.3 |
| Untreated Mouse 3 Large Intestine IgA | 3.07 | 51.6 | 39.3 | 6.05 |
| Untreated Mouse 3 Large Intestine IgG | 0.78 | 5.04 | 85.6 | 8.57 |
| Untreated Mouse 4 Ileum IgA | 1.36 | 67 | 22.7 | 8.88 |
| Untreated Mouse 4 Ileum IgG | 0.023 | 5.08 | 84.1 | 10.8 |
| Untreated Mouse 4 Large Intestine IgA | 23 | 28 | 43.3 | 5.72 |
| Untreated Mouse 4 Large Intestine IgG | 0.57 | 0.52 | 71.4 | 27.6 |

**Supplementary Figure S5.1:** Flow cytometry quadrant percentages of untreated mice ileal and large intestine contents stained with IgA or IgG from the pre-treatment experiment.

| Ileum IgA Average Q2 % | STDEV |
|---|---|
| 62.45 | 19.8570726 |

| Large Intestine IgA Average Q2 % | STDEV |
|---|---|
| 33.75 | 11.9636951 |

| Ileum IgG Average Q2 % | STDEV |
|---|---|
| 3.8975 | 3.1046135 |

| Large Intestine IgG Average Q2 % | STDEV |
|---|---|
| 3.2275 | 1.92754377 |

**Supplementary Figure S5.2:** Average and standard deviation of the percentage of flow cytometry events for untreated mice ileal and large intestine contents that fall within Q2 (Antibody and SYTO BC+).

| Mouse Sample | APC + SYTO BC - (Q1) | APC + SYTO BC + (Q2) | APC - SYTO BC - (Q3) | APC - SYTO BC + (Q4) |
|---|---|---|---|---|
| Pretreated Mouse 1 Ileum IgA | 19.6 | 55.5 | 16.4 | 8.4 |
| Pretreated Mouse 1 Ileum IgG | 0.7 | 2.03 | 69.8 | 27.5 |
| Pretreated Mouse 1 Large Intestine IgA | 30.7 | 24.7 | 33.5 | 11.1 |
| Pretreated Mouse 1 Large Intestine IgG | 4.11 | 3.31 | 52.4 | 40.2 |
| Pretreated Mouse 2 Ileum IgA | 17 | 63.1 | 12.3 | 7.69 |
| Pretreated Mouse 2 Ileum IgG | 1.7 | 6.79 | 67.7 | 23.8 |
| Pretreated Mouse 2 Large Intestine IgA | 28.9 | 41.8 | 22.6 | 6.68 |
| Pretreateed Mouse 2 Large Intestine IgG | 2.8 | 5.03 | 60.2 | 32 |
| Pretreated Mouse 3 Ileum IgA | 13.4 | 44.2 | 8.33 | 34.2 |
| Pretreated Mouse 3 Ileum IgG | 0.12 | 5.94 | 8.29 | 85.7 |
| Pretreated Mouse 3 Large Intestine IgA | 32.6 | 43 | 6.36 | 18 |
| Pretreated Mouse 3 Large Intestine IgG | 1.8 | 4.83 | 34 | 59.4 |
| Pretreated Mouse 4 Ileum IgA | 25.1 | 41.5 | 5.07 | 28.4 |
| Pretreated Mouse 4 Ileum IgG | 0.75 | 8.61 | 26.5 | 64.2 |
| Pretreated Mouse 4 Large Intestine IgA | 34.4 | 27.3 | 6.24 | 32.1 |
| Pretreated Mouse 4 Large Intestine IgG | 0.17 | 0.22 | 36.2 | 63.4 |

**Supplementary Figure S6.1:** Flow cytometry quadrant percentages of pre-treated mice ileal and large intestine contents stained with IgA or IgG from the pre-treatment experiment.

| Ileum IgA Average Q2 % | STDEV |
|---|---|
| 51.075 | 10.0519899 |

| Large Intestine IgA Average Q2 % | STDEV |
|---|---|
| 34.2 | 9.54044024 |

| Ileum IgG Average Q2 % | STDEV |
|---|---|
| 5.8425 | 2.77497598 |

| Large Intestine IgG Average Q2 % | STDEV |
|---|---|
| 3.3475 | 2.22195672 |

**Supplementary Figure S6.2:** Average and standard deviation of the percentage of flow cytometry events for pre-treated mice ileal and large intestine contents that fall within Q2 (Antibody and SYTO BC+).
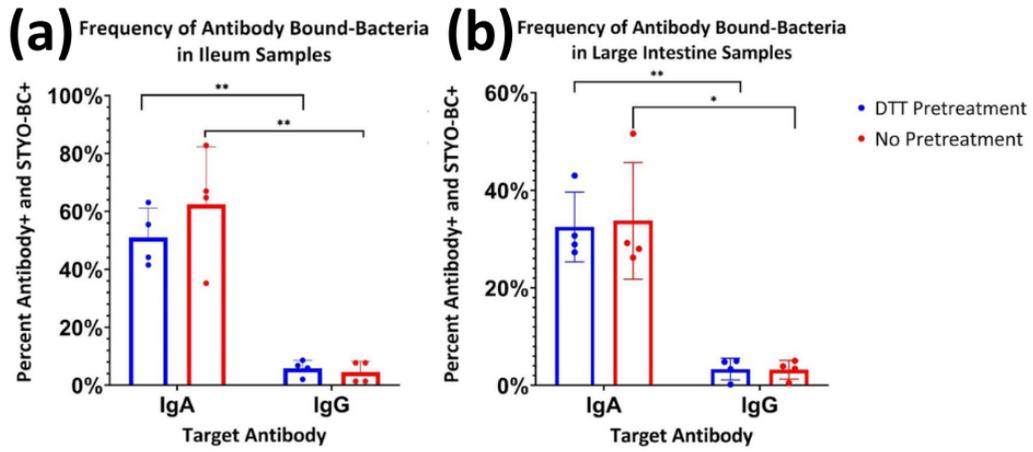
**Supplementary Figure S7:** Percentage of cells from the **(a)** ileum and **(b)** large intestine of pre-treated and untreated mice that are positive for IgA and bacteria (IgA+ consortium). Bars are STDEV
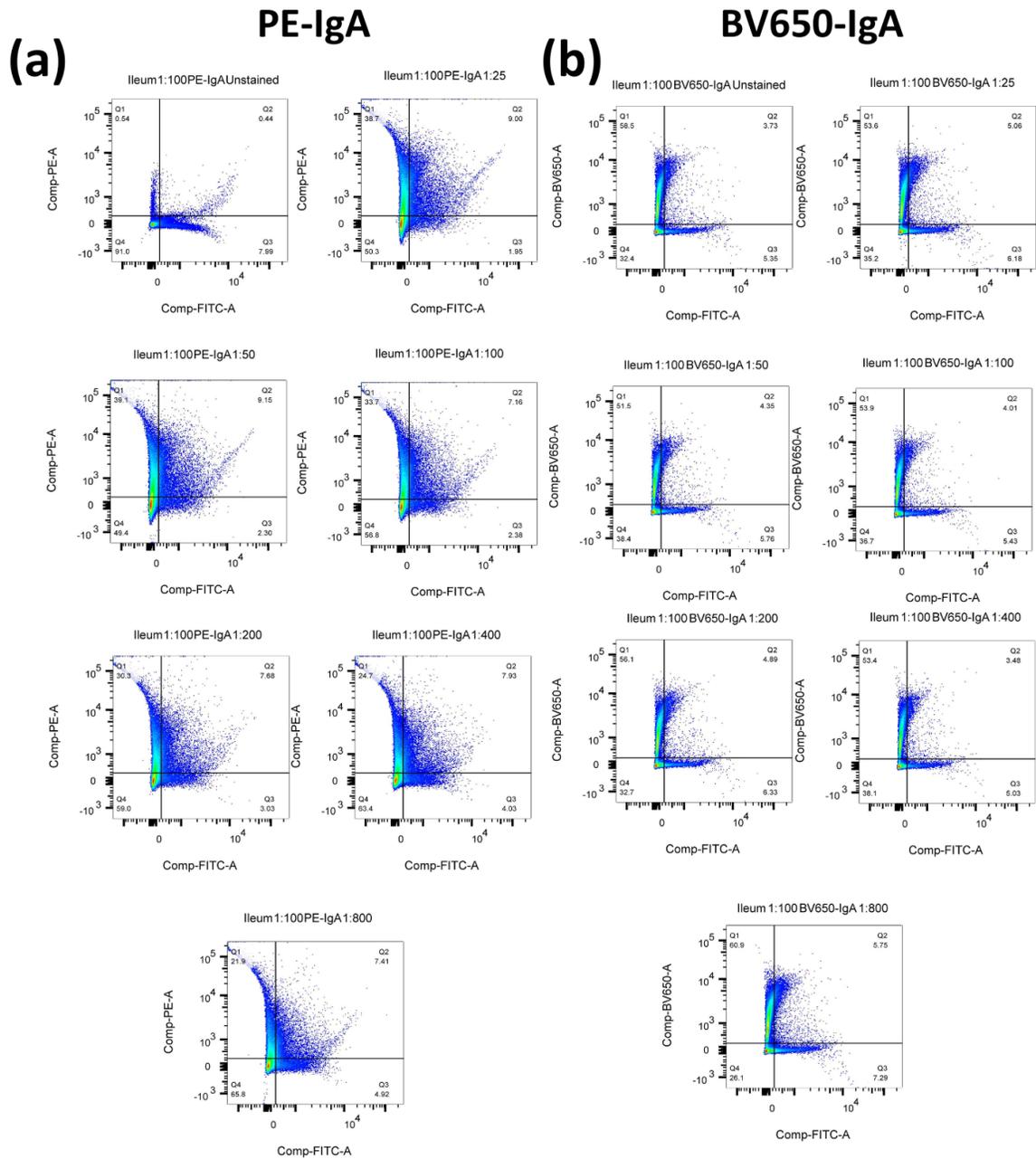
**Supplementary Figure S8:** Flow cytometry results from the first IgA titration trial testing PE-IgA and BV650-IgA. **(a)** Results for 1:100 diluted ileal sample replicates stained with PE-IgA ranging from a concentration of 1:25 to 1:800. **(b)** Results for 1:100 diluted ileal sample replicates stained with PE-BV650 ranging from a concentration of 1:25 to 1:800.
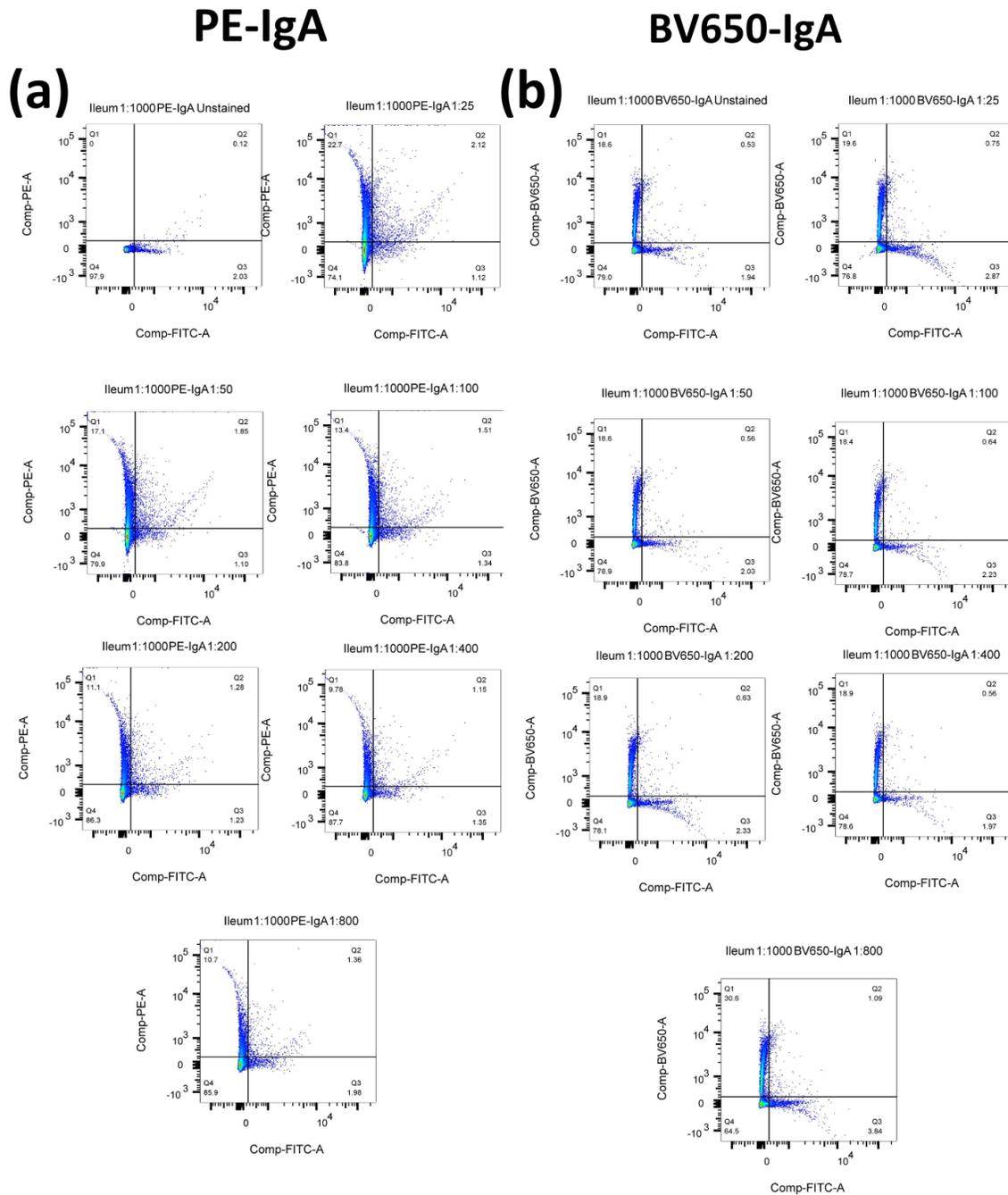
**Supplementary Figure S9:** Flow cytometry results from the first IgA titration trial testing PE-IgA and BV650-IgA. **(a)** Results for 1:1000 diluted ileal sample replicates stained with PE-IgA ranging from a concentration of 1:25 to 1:800. **(b)** Results for 1:1000 diluted ileal sample replicates stained with PE-BV650 ranging from a concentration of 1:25 to 1:800.

| | | IgA 1:50 | IgA 1:100 | IgA BLANK | Compensation | IFNY 1:50 | IFNY 1:100 | IFNY BLANK |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Ileum 1:10 | A | | | | APC (AF647) | | | |
| Ileum 1:100 | B | | | | FITC | | | |
| Ileum 1:1000 | C | | | | Unstained Control | | | |

**Supplementary Figure S10:** 96-well plate layout for the IgA-Biotin-Streptavidin-AF647 titration trials. 25 uL of SCD ileal samples diluted in the 1:10 to 1:100 range in the orange entries are stained with 1:8000 SYTO BC, 1:50 or 1:100 IgA-Biotin, and 1:400 Streptavidin. The same ileal replicates are stained with only 1:8000 SYTO BC and 1:50 TFNγ-AF647 in the blue entries. The third and seventh columns refer to samples lacking the antibody (blanks). The fourth column consists of the compensation controls. For the titration trials testing 1:8000 and 1:4000 SYTO BC, the same layout above was duplicated one row below sans the compensation column.

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SCD | A | | | | | | | | | | | |
| LFF | B | | | | | | | | | | | |
| Compensations | C | APC | FITC | Unstained | Isotype Control SCD | Isotype Control LFF | | | | | | |

**Supplementary Figure S11:** 96-well plate layout for the final cell-sorting experiment. 25 uL of undiluted SCD ileal sample in the orange entries are stained with 1:2000 SYTO BC, 1:50 IgA-Biotin, and 1:400 Streptavidin. LFF replicates are stained with the same contents in the blue entries. The 11th column refers to samples lacking the antibody (blanks). 25 uL of SCD and LFF ileum contents are stained with 1:2000 SYTO BC and 1:50 TFNγ-AF647. All compensation controls were made using 1:100 diluted SCD mouse ileal contents. The final volume of each blue, orange, and isotype control well is 200 uL.