THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

THE EFFECTS OF VITAMIN A STATUS ON GUT MICROBIOTA POPULATIONS

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A thesis
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in Biology
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ABSTRACT

Vitamin A deficiency is a public health concern in many parts of the world and is especially prevalent in Africa and South-East Asia. This deficiency can cause blindness, a compromised immune system, and reduced immune responses. It has also been shown that vitamin A deficient (VAD) mice are unable to clear an enteric infection caused by the murine pathogen *Citrobacter rodentium*. Before studying the effects of vitamin A on gut microbiota, a new protocol was derived in order to isolate DNA from fecal samples obtained from gel-fed mice. Using the standard techniques, low amounts of DNA were extracted from the gel-fed fecal samples. The new protocol increased DNA yield by 1,957%. The study went on to examine the effects of vitamin A status on gut microbiota populations as a potential causal factor for the reduced ability of VAD mice to clear infection. The results indicated no significant change in bacteria populations from the Firmicutes, Bacteroides, Actinobacter, and Gammaproteobacter phyla. There were more total bacterial DNA in A- mice but the result did not reach significance. This thesis was successful in modifying the DNA isolation protocol to increase DNA yields, but did not find any significant effects of vitamin A status on gut microbiota.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

Introduction

The human gastrointestinal tract is comprised of a diverse ecosystem of microorganisms. Along with the skin, mouth, and vagina, the gut is one of the principal contact points with bacterial populations. Interactions between the intestinal tract and bacterial populations have evolved over time to produce a mutualistic relationship. The bacteria in the gut metabolize indigestible compounds, regulate immune function, and provide defense against the colonization of pathogenic species. Although it is not known exactly how these relationships evolved, the microbiome is a vital aspect of host health and immune function. Dysregulation of the microbiota has been associated with various diseases such as inflammatory bowel disease (IBD), metabolic syndrome, and irritable bowel syndrome.[1]

Gut microbiota have a variety of vital functions in the body. One of the most important functions of the commensal flora is as mediators of intestinal immune function. Various bacterial populations can exert pro-inflammatory or anti-inflammatory effects. Proper maintenance of a healthy and balanced microbiome can be vital to proper immune functioning within the host.[1] Additionally, germ free organisms have shown increased susceptibility to infection.[2] Germ free mice infected with *Listeria monocytogenes* have demonstrated reduced resistance to infection as well as impaired bacterial clearance compared to colonized mice.[2] The reduced ability to resist infection by *L. monocytogenes* is attributed to deficiencies in T-cell homing to the site of infection.[2] Gut microbiota are an essential component for the maintenance of a proper immune response in the intestinal tract.

The microbiota have also been shown to heavily influence the integrity of the intestinal epithelial barrier. Germ free mice demonstrate altered morphology compared to conventionally raised mice.[2] One of the most profound defects observed in germ free mice was the altered development of the intestinal mucosal cells (IEC). Germ free mice exhibited changes in the microvilli morphology and reduced turnover rates of IEC.[2] Compared to colonized mice, germ free mice also had poorly developed villus capillaries.[2] Bacterial colonization of germ free mice increased the rate of angiogenesis and the villus capillaries formed correctly.[3] Studies have also illustrated the role of the microbiota in augmenting intestinal motility and enzymatic activity.[4] The gut microbiota play a dynamic role in the intestinal tract and have developed a symbiotic relationship to aid in the proper development and functioning of the gastrointestinal system.

The composition of intestinal bacteria can differ widely from one individual to another. Recent studies have suggested that discrepancies between microbial compositions between members of the same species are likely due to environmental factors rather than host genetics. Twin studies have described a substantial differentiation between bacterial communities in identical twins, signifying the importance of environmental influences.[5] Previous research points to environmental factors having the greatest influence over the composition of microbiota in the gut.

One of the most importance environmental factors affecting bacterial composition in the gut is diet. A study by Hildebrandt et al.[6] demonstrated that changing the diet could alter the microbial community structure in a single day in a mouse model. Wild-type mice on standard chow diet were switched to a high-fat diet and 16s primers were utilized to investigate the effects on the microbiota. The diet change resulted in decreased Bacteroides populations and increased

Firmicutes and Proteobacter.[6] The rapid change in microbial composition suggests that diet has a substantial influence on regulation of commensal bacteria populations.

Before the gut microbiota could be studied in this experiment, the Qiagen DNA Isolation Kit[7] protocol needed to be modified in order to increase DNA yields. The DNA isolation kit was designed to isolate DNA from the feces of mice fed a chow diet; however, mice used in the Cantorna Laboratory are fed a purified gel diet that interferes with the kit's ability to extract high concentrations of DNA. Part of the honors thesis involved altering the DNA isolation protocol to improve DNA yield. By altering the existing protocol, this study aimed to make the protocol usable on samples from mice fed a gel diet.

After altering the DNA extraction protocol, this study aimed to determine how the gut microbiota was affected by host vitamin A status. Previous research had shown that vitamin A deficient mice have a reduced ability to clear *Citrobacter rodentium* infection, whereas vitamin A sufficient mice cleared the infection within two weeks.[8] The purpose of this study was to determine whether shifts in bacterial populations were occurring in vitamin A deficient mice, which could possibly affect their ability to clear the *Citrobacter rodentium* infection. Previous work by another group had demonstrated significantly lower numbers of intestinal bacteria for several phyla in vitamin A deficient mice.[9] The study also showed significantly lower number of total bacteria in vitamin A deficient mice.[9] The goal of this study was to expand on these findings to determine if changes in microbiota, due to vitamin A status, could be associated with protection from infection.

Materials and Methods

Mice

C57BL/6 mice, ranging in age from 8-10 weeks old, were utilized in this experiment. Both groups were fed a purified gel diet prepared in the lab. The A+ diet was supplemented with corn oil containing retinyl acetate and the A- diet was supplemented with corn oil that did not contain retinyl acetate.[10] The A+ group contained eleven mice, including five females and six males. The A- group contained seven mice, in which four were female and three were male.

DNA Isolation

The DNA was isolated from fecal samples using an altered version of the Qiagen DNA Isolation Kit.[7] The new protocol included three major changes. The first alteration was to use 80-100mg of feces per sample instead of 180-200mg that the original protocol called for. The second alteration to the protocol involved adding an additional heating step before adding the InhibitEX tablet. The samples were to be heated for 5 min. at 95°C in a water bath before adding the InhibitEX tablet. The final change to the protocol involved adding an additional centrifugation after the InhibitEX tablet was added. The samples were centrifuged at 13,000G for 10 min. immediately after adding the InhibitEX tablet.

rtPCR

The DNA recovered from the fecal samples was analyzed by real-time PCR (rtPCR). Phyla-specific primers were used to analyze differences in Firmicutes, Bacteroides, Actinobacter, and Gammaproteobacter. Additionally, 16s Unibac primers were used as internal controls. The expression of each sample was compared to the Unibac control to give relative expression and fold change. The rtPCR was run using a SYBR Green PCR master mix and samples were diluted to $2ng/\mu L$. The master mix included $10\mu L$ of SYBR Green, $2.5\mu L$ of water, and $2.5\mu L$ of primer. It should be noted that the usual 4:1 ratio of water to primer in the master mix was changed to a 1:1 ratio, which gave more consistent results. Each well of the PCR plate included $15\mu L$ of master mix as well as $5\mu L$ of diluted sample. A control well was included for each phyla that consisted of $15\mu L$ of master mix and $5\mu L$ of DEPC water. The control is to test for contamination and should show no amplification.

Results

DNA Isolation Protocol Alteration

The Qiagen DNA Isolation Kit produced significantly lower concentrations of DNA when isolated from gel-fed mice compared to chow-fed mice. The protocol provided with the kit produced a concentration of 243ng/µL when used on chow diet fecal samples. However, when isolating DNA from gel-fed mouse fecal samples, the results were consistently in the 5ng/µL range, with the exception of A-2 which was 36.46ng/µL (Table 1). Three A+ and three A- mice were tested, and the average for the six gel-fed samples was 11 ± 5.2ng/µL.

The ratio of absorbance at 260nm and 280nm (260/280 ratio) was used to determine the quality of the extracted DNA. The 260/280 ratio measures quality because it accounts for RNA contamination. Accepted levels of pure DNA are around 2. Lower values indicate possible contamination by proteins, but slightly higher values are not believed to be indicative of a problem. None of the 260/280 ratios obtained using the original protocol were below 2, indicating quality DNA was extracted.

Sample	Concentration (ng/ul)	260/280
A+ 4	6.20	2.21
A+ 5	4.55	2.19
A+ 6	4.37	2.13
Avg. A+ Samples	5.04 ± 0.58	2.18 ± 0.024
A- 1	7.09	2.12
A- 2	36.46	2.09
A- 3	5.73	2.12
Avg. A- Samples	16.43 ± 10	2.11 ± 0.010
Chow 1	243.54	2.12
Avg. Gel-fed Samples	10.73 ± 5.2	2.14 ± 0.019

Table 1 Qiagen Isolation Kit Original Protocol Yields

The first alteration to the protocol included an additional heating step of 5 min. at 95°

before adding the InhibitEX tablet. This alteration gave a slightly higher concentration for chow

samples at 255ng/µL and 281ng/µL compared to 244ng/µL without the heating step. However,

an even larger increase was observed for gel-fed samples. The concentrations of the samples

ranged from 38 – 108ng/µL with an average of 73ng/µL. The averages for A+ samples and A-

samples were 62ng/µL and 83ng/µL respectively (Table 2). The addition of the heating step

resulted in a 700% increase in DNA concentration from gel-fed samples. Although yields were

much higher using the altered protocol, these concentrations were still less than half of those of

chow samples. Additionally, the average 260/280 ratios were around 2.1, which indicated that

the quality of DNA did not suffer with this added step.

Sample	Concentration (ng/ul)	260/280
A+1	70.52	2.11
A+2	76.41	2.12
A+3	38.1	2.19
Avg. A+ Samples	61.68 ± 11.91	2.14 ± 0.013
A-1	108.01	2.21
A-2	96.9	2.14
A-3	45.12	2.08
Avg. A- Samples	83.34 ± 19.38	2.14 ± 0.015
Chow 2	254.96	2.11
Chow 3	280.97	2.15
Avg. Chow Samples	267.97 ± 13.00	2.14 ± 0.020
Avg. Gel-fed Samples	72.51 ± 11.27	2.15 ± 0.014

Table 2 Yields from Altered Protocol Adding a 5 min. Heating Step at 95°C

Another attempt to further increase DNA yields included allowing the samples to gel up after the heating step previously described. A gel extraction kit was then used to purify DNA from the gel. However, the samples produced concentrations of 49ng/µL and 24ng/µL (Table 3), which did not provide any additional advantage over adding the heating step alone.

Sample	Concentration (ng/µL)	260/280
Gel 1	49.25	2.07
Gel 2	24.25	2.08
Avg. Gel Samples	36.75 ± 12.50	2.075 ± 0.0050

Table 3 Yields Using Gel Extraction Kit After Protocol with Additional Heating Step

The greatest increase in DNA yield came from adding the additional heating step along with an added 10-minute centrifugation step. The centrifugation step was added immediately after adding the InhibitEX tablet. The gel-fed samples yielded concentrations ranging from 66-465ng/µL and averaged 221ng/µL. The gel-fed A+ and A- samples yielded average concentrations of 343ng/µL and 92ng/µL respectively (Table 4). The combination of the two protocol changes resulted in a 1,957% increase in DNA yield from the original protocol using gel-fed samples. Additionally, if only looking at the average concentrations for A+ mice, which was 5ng/µL for the original protocol and 294ng/µL for the altered protocol, the new protocol produced a 5,741% increase in DNA yield.

Sample	Concentration (ng/µL)	260/280
A+1	402.3	2.16
A+2	261.9	2.15
A+3	286.3	2.17
A+6	98	2.15
A+7	163	2.17
A+8	464.8	2.14
A+9	384.2	2.15
Avg. A+ Samples	294.4 ± 50.17	2.16 ± 0.00427
A-4	111.4	2.17
A-5	94.4	2.15
A-10	66.5	2.22
A-11	95.7	2.24
Avg. A- Samples	92.0 ± 9.34	2.19 ± 0.0248
Avg. Gel-fed Samples	220.8 ± 43.80	2.17 ± 0.010

Table 4 Altered Protocol Yields with Additional Heating and Centrifugation

There was little observed variation in 260/280 ratios between the samples processed with the original protocol and the altered protocol. Under the original protocol, the gel-fed samples had an average 260/280 ratio of 2.1. Furthermore, the gel-fed samples that underwent only the additional heating step also had an average ratio of 2.1. Finally, the gel-fed samples that underwent the additional heating step as well as the extra centrifuge step had an average 260/280 ratio of 2.2. The 260/280 ratio, measuring extracted DNA quality, did not appear to change between protocols.

Vitamin A Status did not show differences in microbial populations

Real-time PCR was run on the DNA samples obtained from altered isolation protocol described above. The rtPCR data was analyzed using the delta delta CT method. The sample sizes for the A+ and A- groups were 11 and 7 respectively. The PCR results were compared to the corresponding unibac control values to calculate the delta delta CT value. With the exception of the unibac group, the LOG delta delta CT values were used in order to obtain a normal distribution of the data. Results were analyzed using an unpaired t-test ($\alpha = 0.05$). However, the Bacteroides and Gammaproteobacter groups failed the Shapiro-Wilk normality test and were therefore analyzed using a Mann Whitney test. Additionally, three A+ samples in the Firmicutes group that did not amplify were not included in the calculations.

No significant differences were observed in any of the phyla specific groups between A+ and A- mice. The Firmicutes group did not show any significant differences in expression between the groups; the observed p-value was 0.9 (Figure 1b). The Firmicutes group also had three samples that did not amplify, which reduced the number of A+ samples to six whereas the

other groups had a n=9 for A+ mice. The Bacteroides group were also not significantly different in A+ and A- feces with a p-value of 0.6 (Figure 1c). Furthermore, both the Actinobacter and Gammaproteobacter groups showed no significance difference between A+ and A- mice with p-vales of p=0.5 (Figure 1d) and p>0.9999 (Figure 1e) respectively.

The Unibac group showed a trend illustrating increased total bacteria in A- mice (Figure 1a). The p-value was 0.0505, which is not significant at the 0.05 level; however, the values indicated that a larger sample size may have produced significant results.
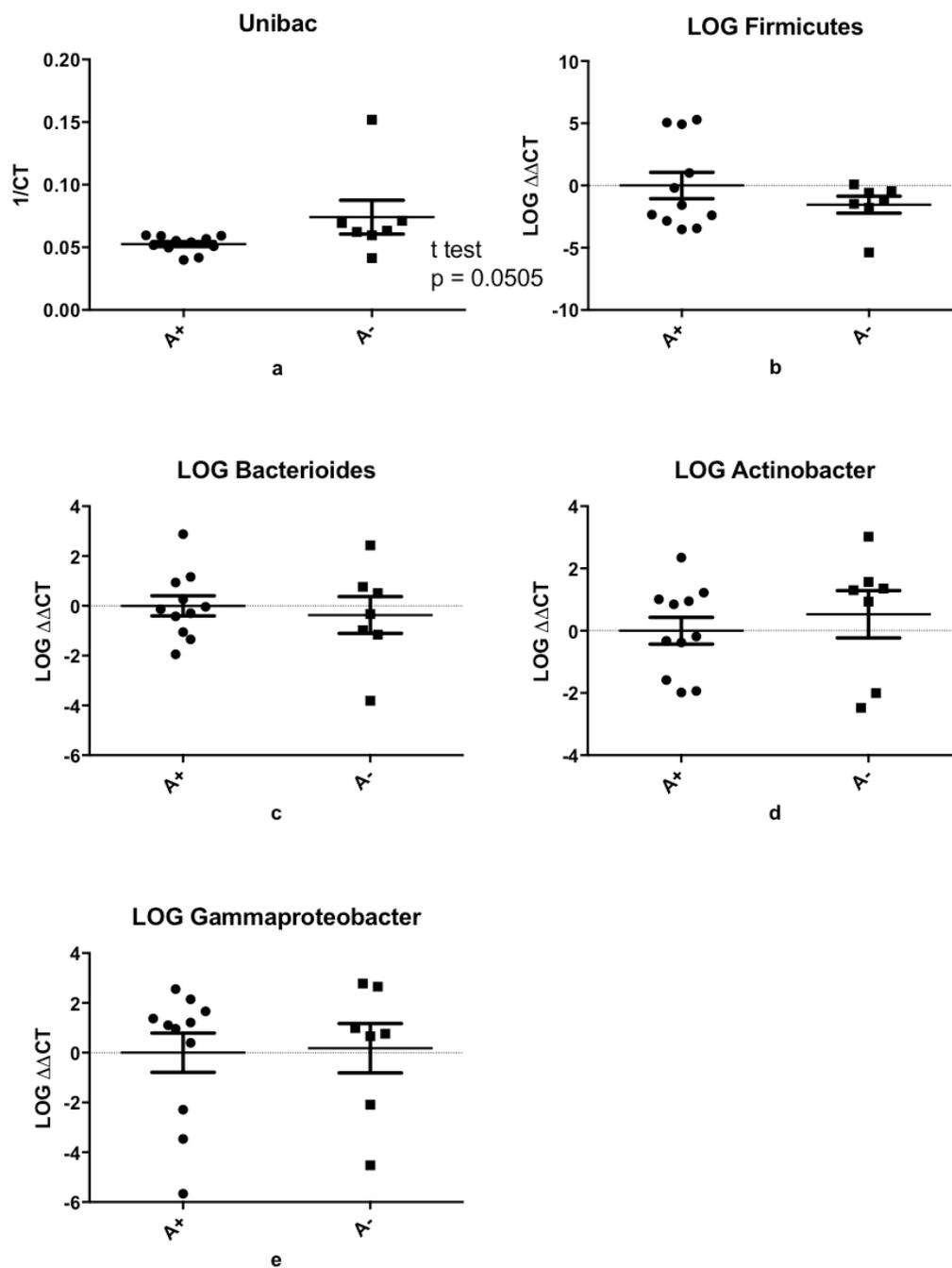
Figure 1 Change in Relative Expression of Phyla Specific Primers Between A+ and A- mice using rtPCR a) The data was transformed to compare 1/CT values between A+ and A- mice using Unibac 16S primer b) LOG ΔΔCT Firmicutes values were compared between A+ and A- groups; three A+ samples did not amplify and were not included) c) LOG ΔΔCT values were compared for Bacteroides between A+ and A- groups d) LOG ΔΔCT values for Actinobacter were compared between A+ and A- groups e) A+ and A- groups for Gammaproteobacter were compared. Unpaired t-tests and Mann Whitney tests were used to determine differences in A+ and A- groups for a, b, c, d, and e.

The ∆∆CT values were plotted in Figure 3 to demonstrate relative abundance of each

phyla for both A+ and A- mice. It was observed that the ∆∆CT values for Firmicutes were the

least abundant of the four phyla with a mean of 1.4 for A+ and 0.3 for A- mice. Bacteroides

showed a mean of 72 for A+ mice and 40 for A- mice. Actionbacter was observed to have a large

difference in mean with averages of 24 for A+ mice and 163 for A- mice. However, this

difference was accompanied by a large standard error. Gammaproteobacter also showed large

variation, but again, this was accompanied by a large standard error. The A+ mice had an
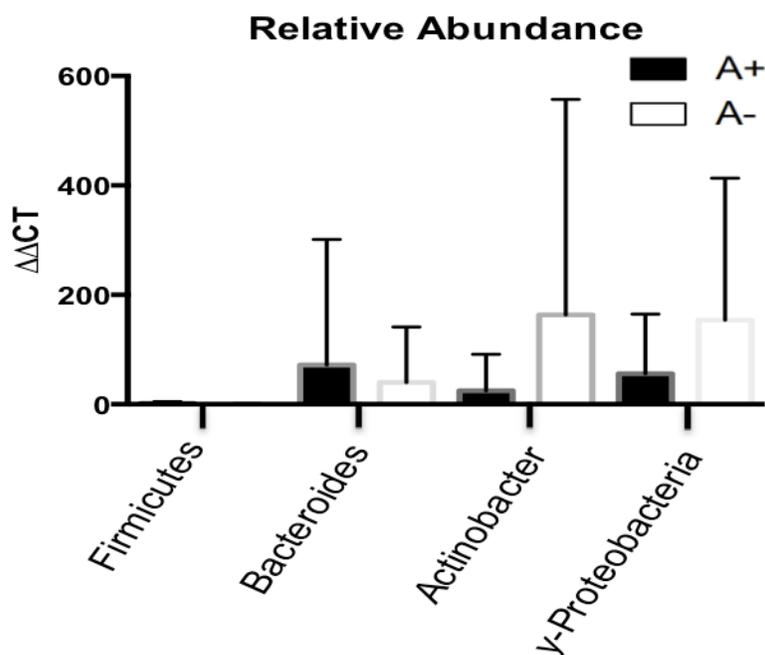
average of 56 and the A- mice averaged 154.

Figure 2 Relative Abundance of Four Phyla for Both A+ and A- Samples

Discussion

This study was successful in altering the existing DNA extraction protocol from the Qiagen Kit[7] to work with fecal samples taken from gel-fed mice. This study was unable to be performed initially due to the samples gelling up in the middle of the process. This led to either low DNA yields or the inability to complete the protocol with the gelled sample. The three alterations to the protocol successfully increased the average DNA yield recovered from gel-fed fecal samples by close to 2,000%. Additionally, looking at only the A+ samples, the new protocol had increased DNA yields that were 5,741% higher. The average DNA yields of the altered protocol were close to the yields observed from the original protocol on chow samples. In fact, the average A+ yield was slightly higher than that recovered from the chow diet; however, including the A- samples in the average moved the mean slightly lower than the chow yields.

The 260/280 ratios did not vary much from the original protocol to the altered one. The total variation from both protocols was from 2.1 - 2.2. Since slightly higher values are not believed to be indicative of a lower DNA quality, the observed 260/280 values did not demonstrate any decreased quality of extracted DNA.

The success of the altered DNA isolation protocol has widespread applicability in the Cantorna lab as well as other labs that use gel diets for their mice. Since most of the mice in the Cantorna laboratory are fed a purified gel diet, this new protocol allows for further bacterial studies to be performed, which were previously more difficult to accomplish.

This study also observed that vitamin A status did not significantly alter the gut microbiota populations in mice at the phyla level. For the four phyla tested, none showed significant differences between A+ and A- groups. However, total bacteria levels, observed using 16s primers, showed that A- mice tend to have more total bacteria in their feces. Because of the failure to see any significant relationship between microbial populations and vitamin A status, this study concluded that altered microbial populations might not be the cause of the inability for vitamin A deficient mice to clear *Citrobacter rodentium* infections, as seen in previous research.[8] Comparisons of abundance between the four phyla were also inconclusive. Although Actinobacter and Gammaproteobacter were more abundant in A- mice, the standard error was too high to make conclusive arguments. However, the Firmicutes abundance was much lower than expected, as Firmicutes is normally more abundant than the other three phyla.[11] This discrepancy could indicate a problem with the Firmicutes primers used in the study.

One of the limitations of the study was the use of only four phyla-specific primers. Of the thirty different bacterial phyla, this study was only able to detect changes in four. The phyla that were studied are representative of the four dominant phyla found in the mouse and human gut. However, microbial population shifts due to vitamin A deficiency may be subtle and occur in the less abundant phyla. Additionally, microbial shifts may also have occurred at the species level. This study was not able to detect at the species level and therefore could not detect more subtle changes that may have been caused by altered vitamin A status.

The results from this study did not support the findings of the previous experiment that found shifts in bacterial populations at the phyla level.[9] The previous study found lower bacterial numbers for *E. Coli,* Enterococci, Clostridium, Lactobacilli, and Bacteroides in A- mice.[9] The previous study examined changes at the genus and species level and this study focused on the

phyla level. The changes in microbiota due to vitamin A deficiency may be subtle and not able to be detected at the phyla level. The previous study also observed a significant change in Bacteroides, which was not supported by this study. [9] This inconsistency may be due to the small sample size with only 11 A+ mice and 7 A- mice. Replicating this study using a larger sample size may result in a significant difference between A+ and A- groups.

In addition to the sample size, there are many factors that could have led to varying results between this study and previous ones. Microbiota studies are very erratic and can have widely different results based on several factors. One major factor is the environment in which the mice live. Different laboratory mouse colonies have very different populations of microbiota that can lead to varying results in studies performed at different institutions. Additionally, it has been shown that diet can have a major effect on the types of bacteria present in the gut. The mice in the Cantorna laboratory are fed a purified gel diet, whereas the mice in the previous study were fed a standard chow diet. [9] A simple change in diet could be a major determinant in the composition of bacteria in the gut. Different conditions in this study and the previous study may have caused the composition of bacteria in the gut to be inconsistent and not controlled for between the two studies. This inconsistency may have led to the different results observed in each respective study.

Microbial composition is also known to differ based on gender. [12] This study did not control for gender and used five females and six males for the A+ group, whereas the A- group used four females and 3 males. Using mixed numbers of each gender in the A+ and A- groups may have been a confounding variable. However, figure 3 in the appendix illustrates the distribution of males and females for the four phyla, which does not appear to illustrate any

trends in regards to gender. Although there does not seem to be a gender bias in this study, using only one gender may have provided more consistent results.

Future studies could look at all bacterial phyla to detect changes in some of the less abundant phyla present in the gut. This would ensure a greater coverage of a wide range of bacterial types. More specifically, future studies could also examine changes at the species level to detect even smaller variations in microbial populations. Changes detected at the species level would also give a greater knowledge of what specific bacterial populations were being altered. Changes detected at the phyla level provide less detail about the actual microbial changes occurring in the gut. Finally, deep sequencing could also be used to sequence the DNA extracted from fecal samples and detect even smaller differences in bacterial composition.

This study was successful in creating an altered version of the Qiagen DNA Isolation Kit[7] protocol that can extract sufficient amounts of DNA from gel-fed fecal samples. No significant relationship was found between vitamin A status and altered gut microbiota populations. However, it was observed that A- mice tend to have a higher abundance of total bacteria in their feces. Additionally, the results from previous research indicating that vitamin A status does affect gut microbiota[9] was not supported by this study.

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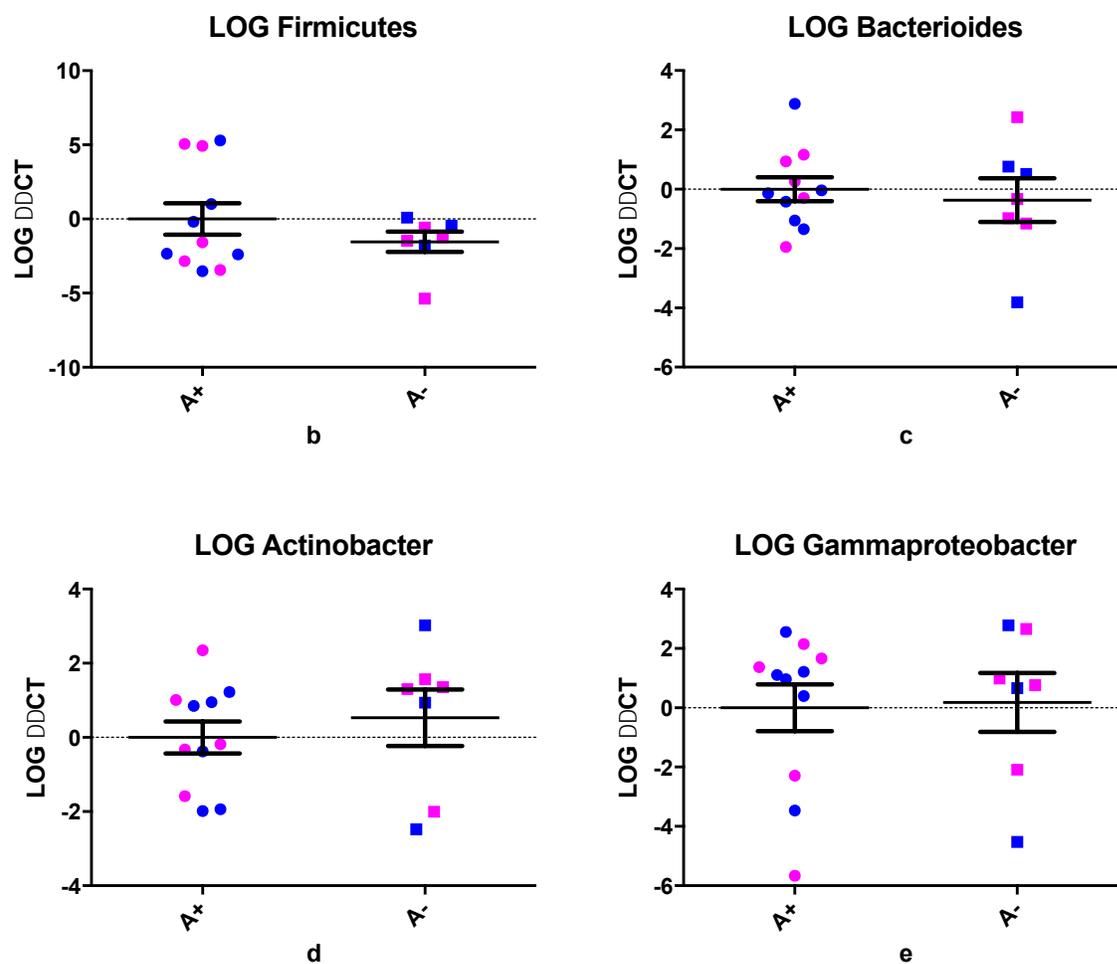
Appendix

Figure 3 Gender Differences Among Firmicutes, Bacteroides, Actinobacter, and Gammaproteobacter. Pink dots indicate samples from female mice and blue dots indicate samples from male mice.

Academic Vita

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EDUCATION

Pennsylvania State University, Eberly College of Science University Park, PA
Schreyer Honor's College Class of May 2016
B.S. in Biology
Psychology Minor

RELEVANT EXPERIENCE

Independent Research **University Park, PA**
Dr. Margherita Cantorna Lab *January 2014 - Present*
- Conduct research on Vitamin A status effects on microbiota populations in the gut using a mouse model
- Learned and practiced how to prepare, execute, and analyze experiments.

Shadowing Physicians **Doylestown, PA and Anderson, IN**
Observer *June 2012-Present*
- Over 100 hours of shadowing physicians and scrubbing into the operating room in the fields of orthopedic surgery, ophthalmology, neurosurgery, otolaryngology, and family practice.

Child Life Program **Indianapolis, IN**
Riley Children's Hospital *April 2014-Present*
- Assist children going through chemo and recovery from surgery in activities as part of therapy.
- Engage patients in games, puzzles, and other activities to reestablish cognitive and motor functions following treatment.

Global Medical Brigades **University Park, PA**
Volunteer *September 2012- Present*
- Established a medical clinic in Darien, Panama for a week to provide healthcare to individuals who did not have access.
- Shadowed Panamanian physicians, worked in the Pharmacy, took vital signs, and assisted with tooth extractions.

LEADERSHIP POSITIONS

Schreyer Honors College **University Park, PA**
Scholar's Assistant *May 2013 – May 2014*
- Created events and educational opportunities for Schreyer Scholars as the student leader of the Honor's College
- Worked alongside the Associate Dean to create an enriching honors experience for scholars.

Circle –K **University Park, PA**
Preferred Charity Chair *September 2012 – May 2013*
- Organized service events, raised money for Circle-K's affiliated charities, and spread awareness for the causes.

Schreyer Leadership Jumpstart Program **University Park, PA**
Student *August 2012 – December 2012*
- Completed a semester-long leadership program and learned the theories behind successful leadership.
- Organized an event to bring more students to Penn State Women's Ice Hockey games for their first year as a Division-I team.

Sigma Chi **October 2012 – December 2012**
Ritual Chair *August 2013 – May 2014*
- To establish and maintain the traditions and core values among all active members of the Alpha Chi chapter through various brotherhood and alumni events.

ACTIVITIES

Varsity Athlete **University Park, PA**
Penn State Cheerleading *April 2014 - Present*
- Lead the crowd and student section in cheers at Penn State games, including football, basketball, volleyball, and hockey.
- Communicated and spoke in front of distinguished Alumni.
- 2016 UCA National Championship Finalists, and ranked 5th in the country.

THON Hospitality Committee **University Park, PA**
Committee Member *September 2012- Present*
- Worked all year to help provide meals and beverages to the dancers and Four Diamonds families during THON weekend every February.
- $13,026,653.23 was raised by THON last year to support children battling pediatric cancer at the Hershey Medical Center.

WORK EXPERIENCE

Johnson and Johnson **Fort Washington, PA**
Microbiology Laboratory Intern *May 2015- Present*
- Tested various raw materials, equipment, and finished product to ensure that all aspects of the manufacturing process for consumer products were biologically safe.
- Assisted in investigations to discover the cause of excessive bio-burden results.

DAA Athletic Association **Doylestown, PA**
Certified Baseball Umpire *June 2007- August 2013*
- Officiated intramural, travel, and Connie Mack baseball games for ages 12-18. Gained experience in dealing in peacefully resolving situations in which coaches disagree. Also gained leadership skills, as I was solely responsible for ensuring the game operates smoothly.