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CITROBACTER RODENTIUM INFECTION RATES IN C57BL6/J MICE ARE INFLUENCED BY HOST DIET

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ABSTRACT

Citrobacter rodentium causes an infection in mice that models food borne infections of humans with enteropathogenic *Escherichia coli*. Previous experiments conducted in mice on the C57BL6/J background have shown inconsistent *C. rodentium* infection rates. This study assessed what factors affect initial colonization of *C. rodentium* in C57BL6/J mice. Mice were fed one of three diets: chow diet (CD), a laboratory made purified gel diet (PD), or a laboratory made chow gel diet (CGD) and then infected with *C. rodentium*. Agar was used in the PD and CGD for gelatinization. In some experiments, chow mice were switched from CD to CGD one week prior to infection. Diet was an environmental factor that significantly altered *C. rodentium* infection rates in mice. CD fed mice had increased *C. rodentium* infection rates compared to PD fed mice ($p<0.001$). In determining whether agar was the ingredient affecting infection rates, the study showed the CGD promoted weight gain in females ($P <0.0001$), but did not improve *C. rodentium* infection rates in either sex. When comparing infection rates between CD, PD, and CGD fed mice, there was no significant difference, suggesting that perhaps agar is not affecting infection rates, but rather there is another diet-related factor important for infection with *C.rodentium*. These findings suggest that diet can affect infection rates with enteric pathogens.

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Chapter 1

Introduction

Gastrointestinal tract

Gastrointestinal health has become the subject of extensive research in recent years, particularly in disease prevention and control. The gastrointestinal tract is a complex, diverse ecosystem comprised of many microorganisms, with bacteria making up the majority of the gut microbiota. The gut is the main organ that comes into contact with these microorganisms and each bacterial population has a unique role in maintaining host health (1). The gut microbiome are responsible for regulating inflammatory responses, and providing competition for infection with pathogenic organisms (2). The mutually beneficial relationship between host and gut microbiota is regulated by environmental, genetic, and dietetic factors.

Microbiota

Commensal, non-pathogenic bacteria and pathogenic bacteria normally reside in the gastrointestinal tract. Commensal bacteria reside in the lumen layer and in the mucus layer covering the intestinal epithelial layer (3). They protect the gastrointestinal tract through pro-inflammatory and anti-inflammatory mechanisms. Upon infection pathogenic bacteria cause infection by crossing the mucus layer and invading the intestinal epithelial cells (3).

The intestinal mucosa is the first portion of the gut to have direct contact with gut microbiota. The mucus layer covering the intestinal epithelial cells provides a nutrient source for commensal bacteria strains and provides an immune defense barrier against invading pathogens (4). If the mucus layer integrity is compromised, increased susceptibility to enteric pathogens can occur (3). Experiments using germfree animals highlight the importance of the gastrointestinal microflora for maintaining health (5). Vital immune function within the host requires a healthy and balanced gut microbiota, and the diet is an important regulator of the composition of the gut microbiota (6).

Gastrointestinal Disease

Any deviation in commensal gut microbiota from the normal (dysbiosis) can result in inflammatory bowel disease (IBD); such as Crohn's disease and ulcerative colitis (7), which are characterized by chronic gastrointestinal inflammation. Symptoms of IBD in humans include rectal bleeding, fatigue, abdominal pain, lack of appetite, diarrhea, and weight loss (8).

There are large and small animal models useful for studying several aspects of human IBD. Mouse models are used due to their anatomic similarities to humans, genetic tools available to study them and ease in maintenance of animals. Infection with *C. rodentium* models inflammation following infection of the colon that has also been shown to happen in humans with IBD.

Citrobacter rodentium

C. rodentium is an opportunistic gram-negative extracellular enteric bacteria in the Proteobacteria phylum (7). This bacteria includes enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC), both which cause disease in humans. *C. rodentium* is a mouse-specific pathogen that causes colonic hyperplasia, in mice (7). *C. rodentium* causes intestinal attaching and effacing lesions, characterized by adherence of the bacteria to host epithelial cells via pedestal like extensions (9). Attaching and effacing lesions are essential for pathogenicity in small animal models (9). *C. rodentium* shares several properties with EHEC and EPEC, which are also attaching and effacing pathogens; however, EHEC and EPEC do not infect mice (7). C. *rodentium* can be introduced experimentally. The infection peaks between 10-14 days post-infection, and clears within 28 days post-infection (10). C. *rodentium* infection is characterized by development of colonic hyperplasia, thickening of the colon, and changes in gut epithelial permeability (9). Since C. *rodentium* and EHEC and EPEC have shared properties, *C. rodentium* is a beneficial model organism that can be used to study some aspects of infection with these human pathogen types in mice.

Factors affecting susceptibility to *C. rodentium* infection

Intestinal bacteria composition varies between individuals; due to differences in diet, genetics, and environment (9). Previous research by Ghosh et al have shown that hosts display varying degrees of susceptibility to enteric pathogens in part because of differences in intestinal microbiota composition (11). Lower numbers of bacteria from the Bacteroidetes phylum corresponded to increased susceptibility to *C. rodentium* as a result of a diet low in fiber (4). Diet

composition shapes the gut microbiota and susceptibility to gastrointestinal pathogens. Several studies point to diet as having a major influence on the gut microbiota composition.

Statement of Problem

The Cantorna laboratory have had *C. rodentium* infection rates ranging from 20-100%. Because of this issue, experiments take more time to complete and use more resources. The purpose of this study was to determine what look at several potential variables that could be affecting the *C. rodentium* infection rates in C57BL6/J mice. This was done by compiling data from already completed experiments over the last 12 months and looking at infection rate. Diet and, in particular, agar was identified as a possible factor affecting infection rates and was tested to determine the effect of diet and agar on *C. rodentium* infection rates.

Chapter 2

Materials and Methods

Mice

All experimental mice were on the C57BL6/J background, 8-10 weeks old, and bred for experiments at the Pennsylvania State University (University Park, PA). Mice were initially analyzed from retrospective studies completed by Lindsay Snyder and Yang-Ding Lin in the Cantorna lab. Experiments carried out between the diet and genotype were compiled to evaluate possible factors affecting *C. rodentium* infection rates. Genotypes evaluated for infection rates from historical experiments included wildtype (WT), T dominant negative retinoid acid receptor (dnRAR), villin dnRAR, B vitamin D receptor (VDR) knockout (KO), cyp27B1 ko/+ and Cyp27B1 KO mice all on the C57BL/6 background (Table 1). Additional factors considered in the retrospective analyses of infection rates included room where the mice were housed, diet PD or CD, and age at the time of infection.

In addition, experiments were done that used 8 wk old WT mice, split equally between males and females and fed CD prior to the experiments (n=13). For experiments mice were either kept on CD (n=6) switched to CGD (n=7 one week prior to infection. Mice were weighed after one week on their respective diets (CD or CGD), all prior to infection.

Experimental procedures were approved by the Office of Research Protection Institutional Animal Care and Use Committee at The Pennsylvania State University (University Park, PA).

Diet

Three diets were used in the experiment: the standard chow diet (CD, Laboratory Rodent Diet 5001, LabDiet, Quakertown, PA), purified gel diet (PD) made in the laboratory (12), and chow gel diet (CGD), made in the laboratory. Nutritional composition of CD and PD are shown in Table 1. The CGD and PD diets both contained agar while the CD diet had no agar. The composition of the CDG diet was the same as CD but contained agar. WT mice were fed the PD, CD and CDG diets for 1 week prior to *C. rodentium* infection.

Chow Diet	
Nutrition Facts	
Protein	24 %
Fat	5.0 %
Carbohydrates	
Starch	32 %
Sucrose	3.7 %
Lactose	2.0 %
Fructose	0.3 %
Glucose	0.2 %
Fiber	5.1 %
Minerals	
Calcium	1.0 %
Phosphorus	0.7 %

Purified Diet	
Nutrition Facts	
Protein	19 %
Fat	5.0 %
Carbohydrates	
Glucose	44 %
Sucrose	22 %
Fiber	3.0 %
Minerals	
Calcium	0.9 %
Phosphorus	0.5 %

Calories	
Protein	29 %
Fat	14 %
Carbohydrates	58 %

Calories	
Protein	20 %
Fat	12 %
Carbohydrates	69 %

Table 1. Nutritional composition of chow diet (CD and CGD) and purified gel diet (PD) (12).

***C. rodentium* infection**

The *C. rodentium* strain ICC169 was a gift from Dr. Gad Frankel (London School of Medicine and Dentistry, London, United Kingdom). C. *rodentium* ICC169 was cultured

overnight in Luria-Bertani (LB, EMD Chemicals, Inc., Gibbstown, NJ) broth containing 50 µg/ml nalidixic acid (Sigma-Aldrich). Mice were infected (8-10 wks of age) by oral gavage with 5 x 10^9 colony forming units (CFU) in 100 µl phosphate-buffered saline (PBS) of C. *rodentium* strain. An aliquot of the infectious dose was serially diluted in PBS and plated on LB agar containing 50 µg/mL nalidixic acid to confirm C. *rodentium* of the inoculum. For all experiments mice were housed one per cage to prevent transmission from mouse to mouse. Fecal samples were collected and homogenized in PBS (1 ml PBS per 0.1g feces) and serial dilutions were plated in triplicate on LB agar plates containing nalidixic acid and cultured at 37°C to enumerate colonies to determine fecal shedding. Successful infection was defined as at least 60 CFU/g feces on d3 post-infection. Failure of infection was defined as < 60 CFU/g feces on d3 post-infection. Infection rates were calculated as follows: number of mice infected / number of mice inoculated x 100%.

Statistical Analysis

Statistical analyses were completed using GraphPad Prism software (GraphPad, LaJolla, CA). Fischer's exact test for independence was used to determine the significance of diet and agar (CDG) on whether or not mice were successfully infected with *C. rodentium*. For these experiments infection was one of two values (nominal value). For mouse weights a 2-tailed t-test was used to analyze mouse weights after one week on their respective diets. For all analyses, $p \leq 0.05$ was used as the limit for significance. $P \leq 0.05$ is denoted by *, $P \leq 0.001$ is denoted by **, and $P \leq 0.0001$ is denoted by ***.

Chapter 3

Results

Evaluation of experiments done over the last six months (Table 2) looked at six genetically different types of mice all on the C57BL6/J background and the infection rate. Examples of very high (79%) and very low (0%) infection rates were seen in WT mice and even T-dnRAR mice there was a big range (69-100%) in the infections rates recorded (Table 2). Since there was such high variability in the infection rates of WT mice in the historical data it was concluded that genotype was not a factor affecting infection rates.

Mice were next evaluated for the diet they were fed (CD or PD) irrespective of genotype. Based on the data in Table 2 the mice were pooled regardless of genotype. There was a difference (P=0.02) in infection rates between mice fed CD and mice fed PD diets (Table 3). CD fed mice (n=60) had an 82% infection rate while PD fed mice (n=92) had a 63% infection rate (P=0.2, Table 3). To determine if age affected infection rates, mice were analyzed according to age at the time of infection. Because Table 3 showed that diet affected infection rates the data on age at the time of infection was evaluated separately for CD and PD fed mice. When comparing CD fed mice infected at 9.5 versus 10 wks , there was no difference in infection rates (Table 4). When comparing PD fed mice 8, 9, or 10 wks of age, there was no difference in infection rates (Table 4). Age at the time of infection was not a factor affecting infection rates in mice.

Genotype	Infected	Inoculated	Infection ate
WT	26	33	79%
WT	4	10	40%
WT	7	11	64%
WT	0	3	0%
WT	0	3	0%
WT	26	33	79%
TdnRAR	9	13	69%
TdnRAR	9	9	100%
TdnRAR	9	9	100%
TdnRAR	18	22	82%
VillindnRAR	7	11	64%
B VDR KO	5	5	100%
CYP27B1-ko/+	0	2	0%
CYP27B1-KO	1	4	25%
CYP27B1-KO	1	4	25%

Table 2. Comparing C. *rodentium* infection rates by genotype. WT = wild type. TdnRAR = T cell specific dominant negative retinoic acid receptor. VillindnRAR = Villin dominant negative retinoic acid receptor. B VDR KO = B vitamin D receptor knock out. Cyp27B1 KO = Cyp21B1 knock out.

Diet	Infected	Inoculated	Infection Rate	Fischer's Exact P-Value
PD	58	92	63%	P =0.02
CD	49	60	82%	

Table 3. Comparing C. *rodentium* infection rates by diet

Diet/Age	Infected	Inoculated	Infection Rate
CD 9.5 wks	18	24	75%
CD 10 wks	15	18	83%
PD 8 wks	21	30	70%
PD 9 wks	7	8	88%
PD 10 wks	11	21	52%

Table 4. Comparing C. *rodentium* infection rates by diet and age

Effect of room on infection rate

Different rooms in animal facilities can result in mice that have different microbiota. In the Cantorna laboratory, mice are housed in two different rooms (Room 23 or in Room 19) in the Henning animal facility at the Pennsylvania State University. To determine if room affected colonization rates, the mice were pooled regardless of genotype or diet and analyzed based on whether the mice were housed in Room 23 or in Room 19 (Table 5). There was a difference

(P=0.001) in infection rates between the two rooms. Room 23 (n=24) had a 71% infection rate while Room 19 (n=111) had a 77% infection rate.

Location	**Infected**	**Inoculated**	**Infection Rate**	**Fischer's Exact**
Room 23	10	24	71%	P =0.001
Room 19	85	111	77%	

Table 5. Infection rates between room 23 and 19 in the Henning Animal Facility.

When analyzed based solely on room, there appeared to be a room effect until diet was taken into account, so PD versus CD diets were compared. There was an effect (P=0.03) of diet type on infection rates. Mice fed PD in Room 19 (n=51) had a 63% infection rate and mice fed CD in Room 19 (n=51) had an 82% infection rate (Table 6). Analysis of diet types in Room 23 could not occur because no experiments using C. *rodentium* mice on CD were conducted. Although it initially appeared that room was a factor determining infection rate, further analysis confirmed that the effect was likely due to a difference in diet. Diet is a factor in C. *rodentium* infection rates.

Diet	Infected	Inoculated	Infection Rate	Fischer's Exact
PD	32	51	63%	P =0.03
CD	42	51	82%	

Table 6. Infection rates between PD and CD in Room 19 in the Henning animal facility

CD fed mice have higher *C. rodentium* infection rates

There are a few differences between the CD and PD composition. CD has a higher protein content (29%) compared to PD (20%), and has a lower carbohydrate content (58%) than PD (69%) (Table 1). However, CD has more carbohydrate sources, including starch, sucrose, lactose, fructose, and glucose. Starch is the main source of carbohydrates in CD, added at 32% of the total diet. In PD, glucose and sucrose are the main carbohydrate sources at 44% and 22%, respectively. Another major difference between the CD and PD composition is the addition of agar in PD.

To determine if it was the composition of the diet that affected infection rates, the mice were pooled regardless of genotype, room or age at infection and analyzed solely based on whether the mice were fed CD or PD. The diet had an effect ($P<0.0001$) on C. *rodentium* infection rates. Mice on CD had a 90% infection rate and mice on PD had a 51% infection rate (Table 7). Diet does contribute to C. *rodentium* infection rates, with mice on CD exhibiting increased infection rates when compared to mice on PD.

Diet	Infected	Inoculated	Infection Rate	Fischer's Exact P-Value
CD	69	77	90%	P < 0.0001
PD	44	87	51%	

Table 7. *C. rodentium* infection rates based on diets fed to experimental mice.

C. rodentium shedding kinetics are not affected by diet modifications

The kinetics of C. *rodentium* shedding was determined in mice that were successfully infected on the PD versus CD diets. All infected experimental mice (n=8) had colonies in their feces by d3 post infection (p.i), reached peak infection by d10 p.i., and cleared the infection by d35 p.i (Figure 1). If experimental mice do become infected, diet does not affect outcome or fecal shedding.

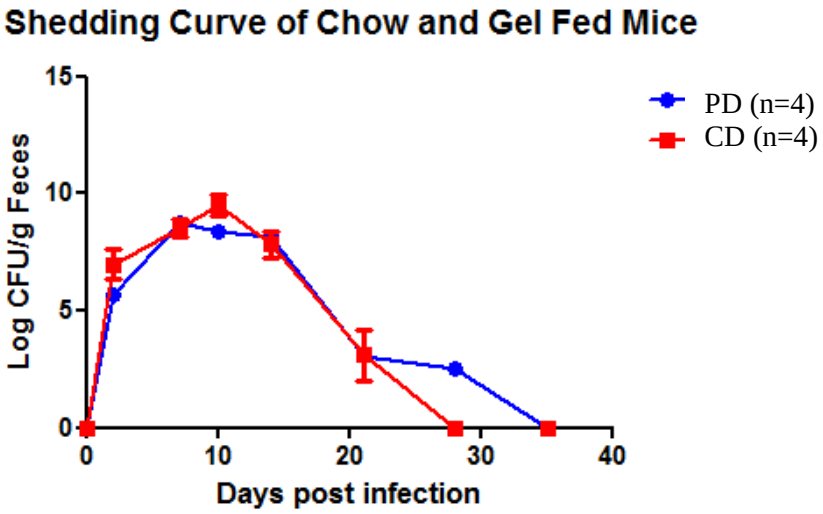

Figure 1. Shedding kinetics were similar once infected with *C. rodentium*. CD n=4; PD n=4.

CGD and CD fed mice have similar infection rates

Diet has an effect on C. *rodentium* infection rates. One difference between the CD and PD is the use of agar in PD. Besides the composition differences between CD and PD diets (Table 1), the PD diets also contain agar added to solidify the diets. To determine if the agar in the PD diet was the ingredient affecting infection rates, the CD diets were powdered and then agar was added to gelatinize the diet. The nutritional requirements for one mouse per day is as follows: 4 grams of powdered PD, 0.1 g agar, and 6 mL of water. A food processor was used to grind the CD pellets into a powder. In the first attempt, the PD diet recipe was followed but substituted powdered CD for the PD powder. The resulting CGD would not solidify and did not have the correct consistency. In the second attempt, the amount of agar and water and doubled but kept the powdered CD amount the same. The resulting CGD was similar to the PD and did solidify. The third attempt used the following recipe: kept the powdered CD diets the same and used 1.5X the agar and water. The resulting CGD solidified but was softer than desirable. For the actual experiment the CGD was made with doubled agar and water.

To determine if the agar in the PD was the ingredient affecting infection rates, WT mice fed CD their entire lives either remained on CD or were switched onto CGD one week prior to infection and results were compared and analyzed. CD and CGD groups were not different and exhibited 67% and 72% infection rates, respectively, (Table 8). Female mice fed the CGD weighed more than female mice fed CD after one week. Females on CGD weighed more ($P<0.0001$) than female mice on CD. There was no effect of diet on the weights of the male mice (Figure 2). Agar does not seem to be a major contributing factor in C. *rodentium* infection rates in mice but does result in increased female mouse weights.

Diet	Infected	Inoculated	Infection Rate	Fischer's Exact P-Value
CD (male n=3, female n=3)	4	6	67%	P= 1
CGD (male n=3, female n=4)	5	7	72%	

Table 8. Infection rates of mice by CD and CGD.

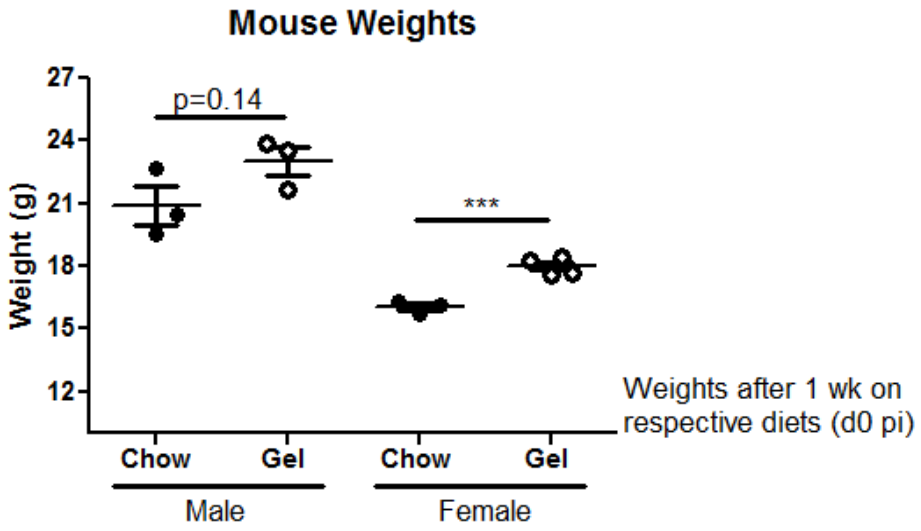

Figure 2. Mouse weights after one week on CD or CGD diet. Mice were weighed after one week on their respective diets . n=3-4/group, analyzed using 2-tailed students' t-test (***=P<0.0001).

Chapter 4

Discussion

There are different environmental factors that contribute to variation in infection rate with C. *rodentium*. This study was successful in investigating some factors that influence initial infection with C. *rodentium* in C57BL6/J mice. Prior to this study, it was unclear what the causes were the variable infection rates from experiment to experiment. Experiments could not be properly compared and analyzed because mice were not getting infected with C. *rodentium*. The analyses of environment, including room, age, and diet, showed that there was an effect of diet but not room or age on infection rates with C. *rodentium* in C57BL6/J mice.

The study found that diet type significantly altered infection rates. CD fed mice had increased infection rates when compared to PD fed mice. Within room 19, CD fed mice had significantly higher infection rates than PD fed mice. It has also been shown that diet can have a major effect on gut microbiota composition (12, 13). Previous studies have shown that dietary changes alter the intestinal bacterial composition, and that changing the diet can alter the microbial community in a single day in an individual mouse (4). WT CD fed mice had significantly higher infection rates than WT PD fed mice, suggesting that diet type is a factor in infection rates with C. *rodentium*.

This study observed that genotype (at least for the genotypes tested) did not significantly alter the infection rates or shedding kinetics once infected. For the genotypes analyzed, all on the C57BL6/J background, none showed significant differences when comparing infection rates

Mice fed CD diets (WT and other genotypes) had higher infection rates than the same genotypes of mice fed PD diets, suggesting that diet has an effect on infection rates. The shedding kinetics were not altered, exhibiting that once infected, peak infection was reached by d10 p.i. and clearance occurred by d35 p.i, which is consistent with what has been published in the literature (10, 14).

Different rooms in the Henning Animal Facility at the Pennsylvania State University were initially considered as a contributing factors to C. *rodentium* infection rates. This could be due to each room having different microbial populations in the air and bedding, resulting in shifts in bacterial populations in mice, which were not studied in these experiments, but previous studies showed that different rooms within the same facility do have different microbiota (1, 4, 11). There did appear to be a room effect until diet was taken into account, which was supported by a study conducted by Ooi et al (12). They found that discrepancies between gut microbial compositions of the same species may be due to diet (12). Room, genotype or age did not affect infection rates with *C. rodenitum*.

Though it was not addressed in this study, fiber source, content, and availability of carbohydrates to gut microbiota of each diet may alter C. *rodentium* infections between mice. In mice, the CD was consistent with recommended dietary fiber intake for mice at 5% fiber, while the PD was just below the recommended dietary fiber intake at 3% fiber. Mice on PD exhibit a protective effect against C. *rodentium* compared to CD fed mice. This may be because the PD has lower available fiber than the CD, with PD having 3.0% soluble fiber, all from cellulose, while CD has 5.1% insoluble fiber, from cellulose, hemi-cellulose, and lignin (12). Soluble fiber from cellulose may contribute a protective effect against C. *rodentium* infection rates. A recent study found that a diet low in microbiota accessible carbohydrates results in progressive loss of

microbiota diversity (16). Since the microbial community is also affected by the availability of carbohydrates for the gut microbiota, further studies could investigate effects of the individual ingredients on the microbiota. Though our diets were not considered low fiber, the diet's fiber source, its accessibility to gut microbiota, and the resulting change in microbiota diversity may affect initial colonization of C. *rodentium*, which could be further studied.

Agar was thought to be a possible factor affecting infectivity, as CD is not made with agar, while PD requires agar for gelatinization, so the CGD was created to account for agar use. After one week on their respective diets, the *C. rodentium* infection rates were not significantly different in the CD and CGD fed mice. A limitation of this finding, however, is that in creating the CGD, water and agar was doubled to allow proper gelatinization. When comparing CD and CGD fed mice and infection rates, there was no significant difference in initial colonization rates, suggesting that perhaps agar is not preventing infection, but rather another factor is affecting infection rates.

The CGD may not have been a contributing factor in *C. rodentium* infection rates, but it did result in the increase in female mouse weight after one week of the CGD diet. Females on the CGD weighed significantly more than females on CD. Males also showed a trend towards increased weight when fed CGD than on CD alone. The increase in weight for both sexes may be due to the increased agar and water content in the CGD, since to create the CGD, agar and water had to be doubled to allow for proper gelatinization. This is a limitation of the study, since the CD is not the same as CGD, even when attempting to account for agar use. The new CGD contained 38.2g agar compared to PD's 19.1g agar and to CD's 0g agar. Additionally, this increase in body weight is not seen in PD fed mice. If the CGD fed mice are eating more due to

the increase in agar, they are increasing their caloric intake, which may explain their increased weight gain.

This study was successful in investigating the factors that affect initial colonization of C. *rodentium* in C57BL6/J mice. The cause of C. *rodentium* infection rates in C57BL6/J mice were not due to genotype effects. Instead, it appears that there is a dietary effect on the initial infection rates of mice with C. *rodentium*. Mice fed CD had significantly higher infection rates than PD fed. . Further studies could investigate ingredient differences between CD and PD; specifically, carbohydrate source and its affect on infection rates could be investigated Overall these findings demonstrate that diet composition can affect infection rates with enteric pathogens, and that further studies should be conducted to determine precisely what dietary component affects infection rate.

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- Learned and practiced how to prepare, execute, and analyze experiments.

Yorktown Animal Hospital, Yorktown, VA
Veterinary and Kennel Assistant May 2015-Present
- Shadowed veterinarian with clinical work, dental procedures, spays and neuters, and vaccinations.
- Assisted in client interaction, patient handling, clinic upkeep, and kennel and boarding maintenance
- Filed patient documents and completed administrative work

Red Cross Administrative Volunteer, Fort Eustis, VA
June 2014-August 2014
- Implemented and improved document filing system
- Created and maintained computer backup of files using Adobe Acrobat and Microsoft Office

Independent Research, Newport News, VA
Virginia Living Museum – Herpetology Department October 2012-July 2013
- Monitored animal behavior and contributed to habitat construction and animal enrichment
- Performed clinic upkeep, quarantine procedures, and general animal husbandry
- Designed and executed independent research project to explore the effects of ultraviolet lights on the American Bullfrog in captivity.

LEADERSHIP POSITIONS

Penn State Women's Rugby, University Park, PA
Secretary December 2016-January 2017
- Liaison between parents and players for game information.
- Maintained records and aided coach with administrative work.
- Worked directly with the coach and other executive members in organizing community service events, communicating with Alumni, and creating a budget for the team.

ACTIVITIES AND AWARDS

Penn State Women's Rugby University Park, PA
Athlete, January 2014-Present
- Driven member of overall 11x National USA Rugby Champions, 4x Big 10 Rugby Champions, Ranked #1 in the nation.

Dean's List, University Park, PA Spring 2015 – Spring 2016