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## Table of Contents

	<u>Page</u>
Introduction.....	1-2
Body.....	3-4
Key Research Accomplishments.....	4-5
Reportable Outcomes.....	5
Conclusion.....	6
Figures.....	7-13
Appendices.....	14-52

# Introduction

In this introduction, we summarize the stated goals of the study in the original proposal.

**Projects 1 & 2.** In these studies, we will conduct parallel assessments in two different strains of mice: C57/BL6 mice, representing euglycemic hosts, and db/db mice, representing diabetic hosts. In both cases, we will assess the cutaneous microbiome before wounding to establish a baseline, and after wounding to see how the microbiota are perturbed. We will assess how the microbiome changes as the wound heals, and whether it returns to baseline within 10 days. We will also follow the un-wounded mice during this same period as a control. After these experiments are completed and the results analyzed, we started the intervention studies proposed in project 2. In this case both euglycemic and diabetic mice will be wound and infected with *Staphylococcus aureus* methicillin resistant and a group of animals will be treated and another not to assess the effect of the skin microbiome on healing.

**Project 3.** This study will analyze the cutaneous microbiome in relation to skin abscesses. We have stored specimens of cutaneous swabs from both abscess patients and control patients. For the abscess patients, we obtained swabs from the area adjacent to the abscess site and from the contralateral but unaffected site. We have swabs from the control subject – those unaffected by cutaneous abscesses - the same site in control subjects unaffected by cutaneous abscesses. Prior studies of the cutaneous microbiota show substantial symmetry in healthy subjects. First, by comparison of the unaffected and control specimens, we can ascertain whether there was a substantial and reproducible difference between baseline in abscess patients and controls. Then, by comparison of the unaffected and affected specimens, we can assess whether the presence of the abscess is associated with variation in that person's local cutaneous microbiota. We also can address whether MRSA infections result in any specific microbiome changes, since in some cases the subjects' infections were due to MRSA, and in some cases they were due to other bacteria. From each abscess and control site, up to four specimens were obtained, from each quadrant surrounding the lesion. The specimens were obtained using swabs with detergent (Tween-20). All the analyses have been done and a paper has been submitted and we are waiting for a decision of the editorial board of the Journal of Infectious Diseases.

**Project 4.** We believe the human gut microbiome has a major role in health. We are particularly interested in investigating how the commensal organisms of the colon provide resistance against pathogenic organisms. We seek to understand the methods by which the gastrointestinal tract microbiome resists invasion by pathogens, either wound-related (e.g. *Acinetobacter*) or those commonly encountered under military field conditions (e.g. *Campylobacter*), since these are major problems in military medicine. We hypothesize that perturbation of the gut microbiome may result in increased susceptibility to colonization of the gut by pathogenic bacteria and ensuing infections.

The main goal of this project is to address the role of the mouse GI microbiome in resisting colonization by *Acinetobacter baumannii* and *Campylobacter jejuni*. We have performed several mouse challenge experiments to assess this.

We have performed oral challenge of mice with *C. jejuni* and *A. baumannii* and determined the colonization capabilities of these two organisms. We provide results about the total amount of DNA obtained from the stool samples, the total number of bacteria, and the specific number of *C. jejuni* and *A. baumannii* at various longitudinal time points before and after the oral challenge. We were able to confirm that *C. jejuni* is a better colonizer than *A. baumannii*.

The GI tract microbiota affects the immune status of the host. This microbiome is a critical determinant of host recovery from infections, wounds, and trauma. The ability to manipulate host immunity has largely been exploited in terms of donor-regulation (e.g. corticosteroids and immunosuppressives), but there also is a need to develop immune enhancers. We used our mouse challenge experiments with *Acinetobacter baumannii* and *Campylobacter jejuni* to explore one such potential enhancer: SFBs (segmented filamentous bacteria) are organisms that, when present in mice, affect levels of intestinal T-cells, and in particular up-regulate Th17 cells in the gastrointestinal tract. In year 3 of the project, we have finished our analysis of the first group of experiments and we have a manuscript currently in preparation that will be submit by the beginning of next month.

# Body of the Report

Our fourth and final annual report is short when compared to our three previous years' report; this is as a result of consolidation of our different experiments and in some cases the finalization and preparation of final publications. There is only new experimental work relates to project 2.

## **Project 1. Establish an experimental mouse model of wound healing in relation to microbiota profile.**

We have previously reported all our progress in this project and we are now in the final stages of the analysis and we are starting a draft for publication of our findings.

## **Project 2. Assess the role of CRT in the regulation of the wound microbiome, reduction of wound infection and ameliorating scarring during wound healing.**

We performed our intervention studies during this year. The designs of these experiments are illustrated in **Figures 1** and **2**. We are also including the animal protocol and the intervention drugs (**Figures 3** and **4**).

We started the intervention studies during this last year. We ordered 48 wild type (WT) C57BL/6 mice to assess the effect of calreticulin on wound healing in the presence or not of methacillin resistant *Staphylococcus aureus* (MRSA). The mice were ordered by the end of September and we initiated the study the first week of October. After this study at the beginning of 2015, we ordered 48 *db/db* C57BL/6 mice to assess the effects of treatment and infection as mentioned above. We finished the study and we are now summarizing all the samples collected from both studies in Table 1.

We have documented changes in mouse weight as a result of the different interventions and the results are presented in Figures 5 to 8. We have evaluated the results and performed DNA extraction of samples collected during both experiments and we have performed sequencing analysis and we are finalizing these analyses (Table 2).

Table 1. Number of samples collected for sequencing analysis in the wound experiments

Sample type	C57/BL6 wild type	C57/BL6 db/db
Skin swab	202	206
Fecal pellets	107	116
Cecal samples	47	45
Skin tissue	47	45
Fur	8	8
Total	411	420

Table 2. Preliminary results of the sequencing analysis of the Wound mouse experiment

	Mice strain	
	WT	<i>db/db</i>
Number of samples	417	419
Total counts	7620292	8668553
Median	14883	20107
Mean±SD	18274.1±15566.1	20688.7±8551.7
Range	6-129723	132-59156

**Project 3. Role of the microbiota in MRSA cutaneous abscesses.**

We have previously presented all our progress of this project and now in this report we are included our paper that was published in which we reported the results of the role of the microbiota in MRSA cutaneous abscesses.

Published manuscript: Horton *et al.* J Infect Dis. 2015;211:1898

**Project 4. To understand the resistance of the gastrointestinal tract microbiota to invasion by pathogens.**

We have finished all the analysis of this project and a final manuscript has been prepared which we now are enclosing in this report.

## Key Research Accomplishments

We have accomplished the skin wound experiments in C57BL/6 wild type mice and in the *db/db* mice. We found two distinct gut microbiome populations differing between the WT and the *db/db* mice. In contrast, both mice strains had nearly identical skin microbiome when we assessed by alpha and beta diversity. Despite the similarities in alpha and beta diversity, skin microbiome from WT and *db/db* mice showed difference in the relative abundance of particular species (Project 1).

We have finished the intervention studies to assess the efficacy of wound healing (Project 2).

We have finished our study of the skin microbiome in MRSA cutaneous abscesses by high throughput sequencing and a final manuscript was submitted for publication (Project 3).

Our progress in Project 4 is also encouraging: we have a nearly finished draft of a manuscript that we are planning to submit for publication.

## Reportable Outcomes

1. We have overcome a series of problems in the animal room and we have made significant progress in project 2 in the last quarter.
2. We have finished a manuscript and are preparing a second manuscript on the occurrence of SFB in our mouse model.

# Conclusions

## PROJECT 1

- We were able to perform the skin wound experiments in wild type and obese mice. Our preliminary observations indicate that the gut microbiome is different between the WT and *db/db* mice but not the skin microbiome. No major differences in wound healing were observed between the two groups of animals.

## PROJECT 2

- We have major progress in this project .

## PROJECT 3.

- We determined the skin microbiome composition of the samples from patients with abscess and also in the control group.
- We found that the skin microbiome composition varies in relation to MRSA status of the patient, but also in relation to body location of the sample.
- A manuscript was submitted for publication.

## PROJECT 4

- We investigated gut microbial composition in mice and determining how antibiotic treatment affects this composition, as well as how antibiotic treatment affects SFB composition.
- Gut microbiome composition may also be affected by colonization with *C. jejuni* or *A. baumannii*, and we plan to determine the long-term effect of these pathogens on gut microbiome in the future.
- A manuscript is currently in preparation

# Figures

Figures Project 2:

## US Army Microbiome Project 2

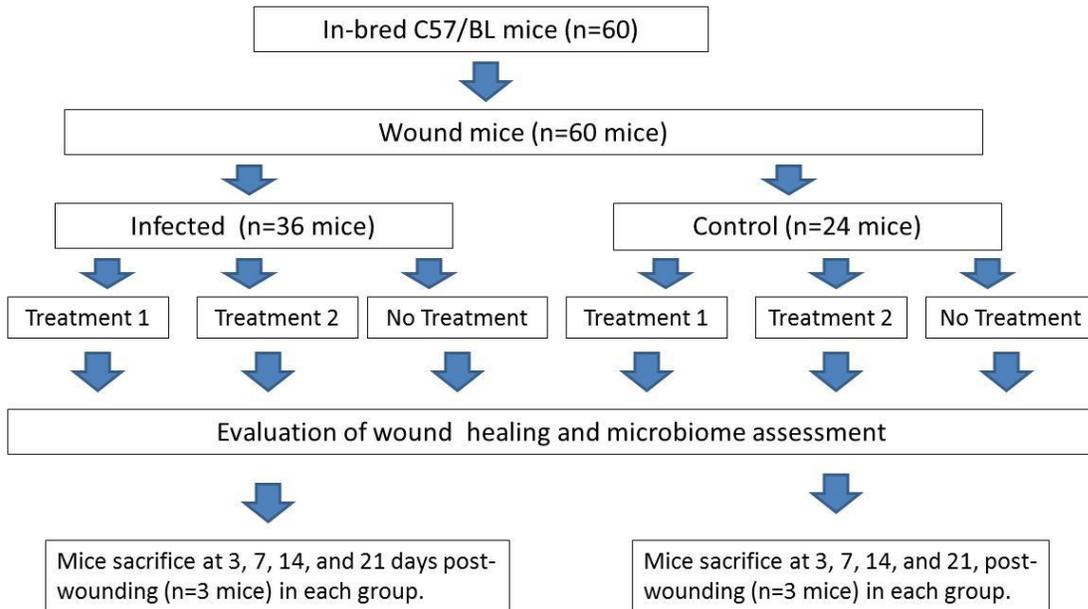
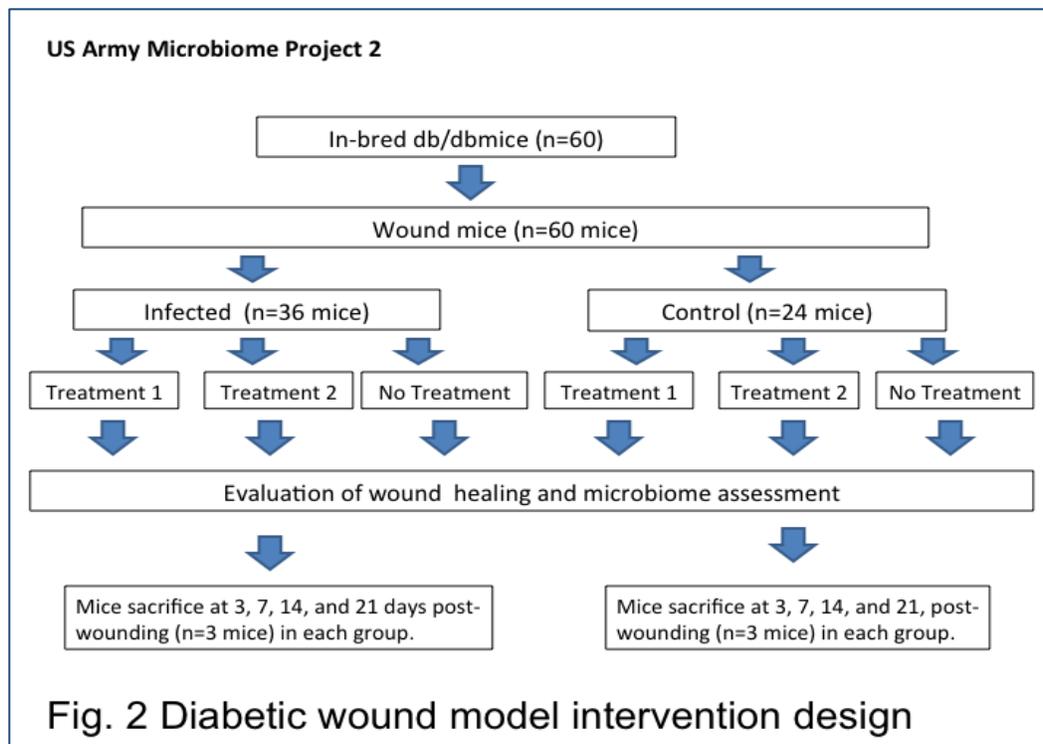


Fig.1 Wound model intervention design in 60 mice



**Figure 3**

## Treatment and control groups

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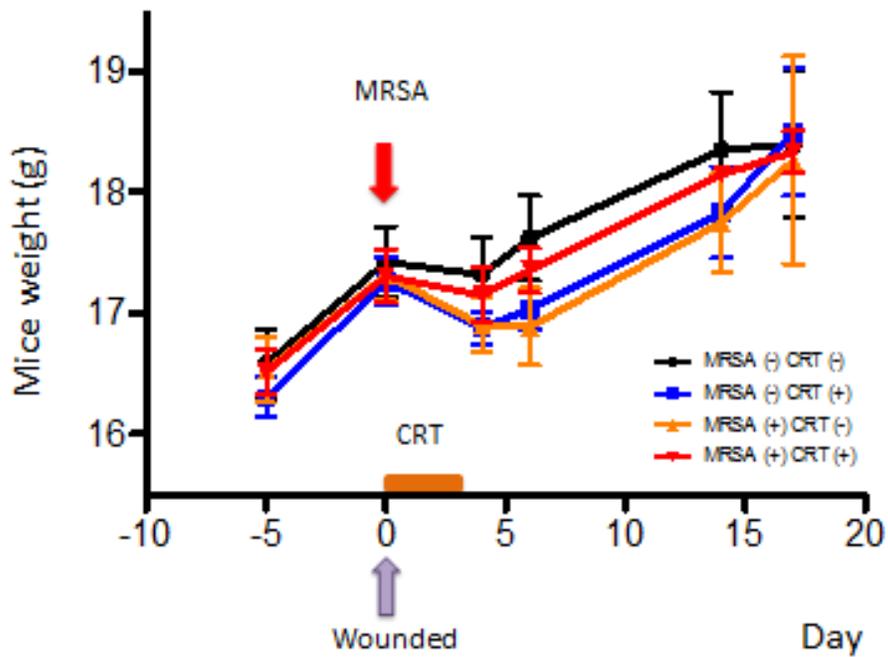
- Control wound: PBS
- Control treatment 1: Calreticulin 2  $\mu$ l
- Control treatment 2: antibiotic
- Inoculation bacterial infection, either:  
*Staphylococcus aureus* (MRSA), *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, or *Acinetobacter baumannii*.

Figure 4

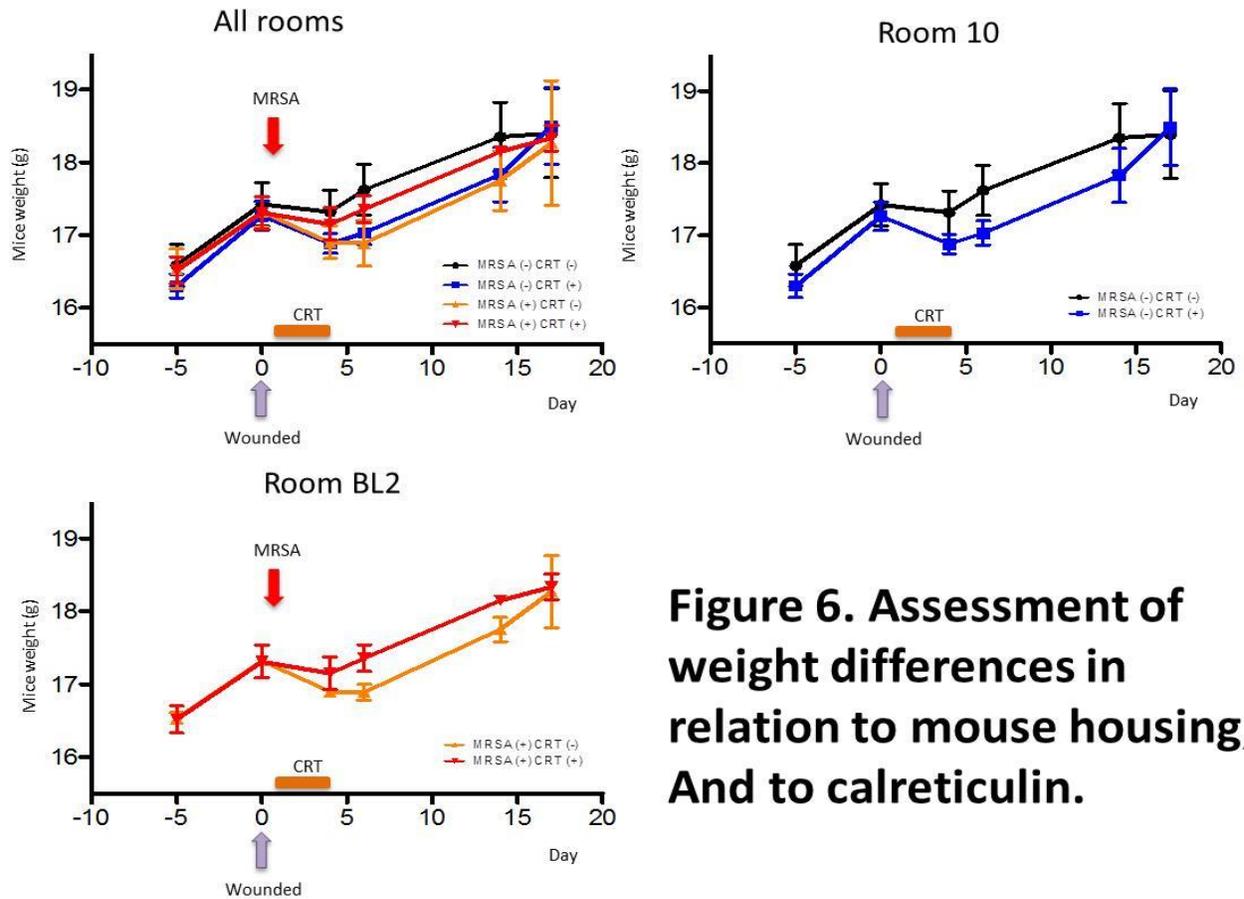
## Animal Protocol

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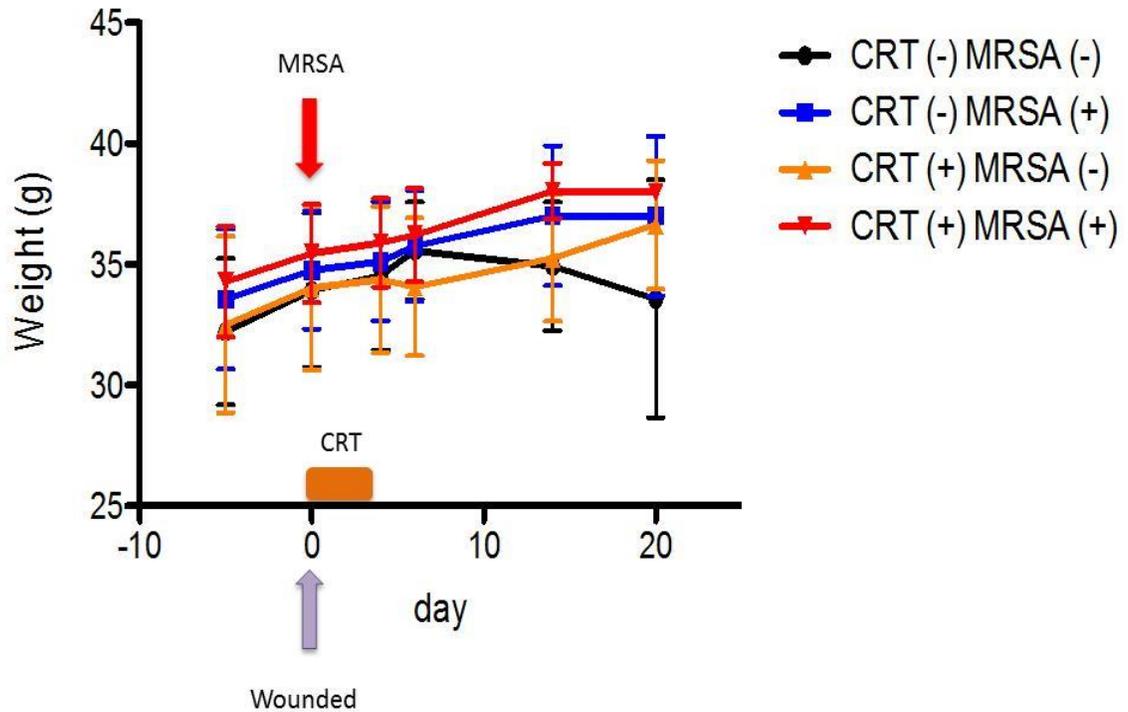
- Mark animals with ear clipper
- Weigh animals every day
- Collect hair samples before wounding
- Collect stool samples
- Collect skin samples every day from unaffected and lesional area
- Photograph of each lesion on days 3, 7, 14, and 21
- Dexa scan after wounding on days 3, 7, 14, and 21
- Obtain blood, skin and cecal samples at sacrifice



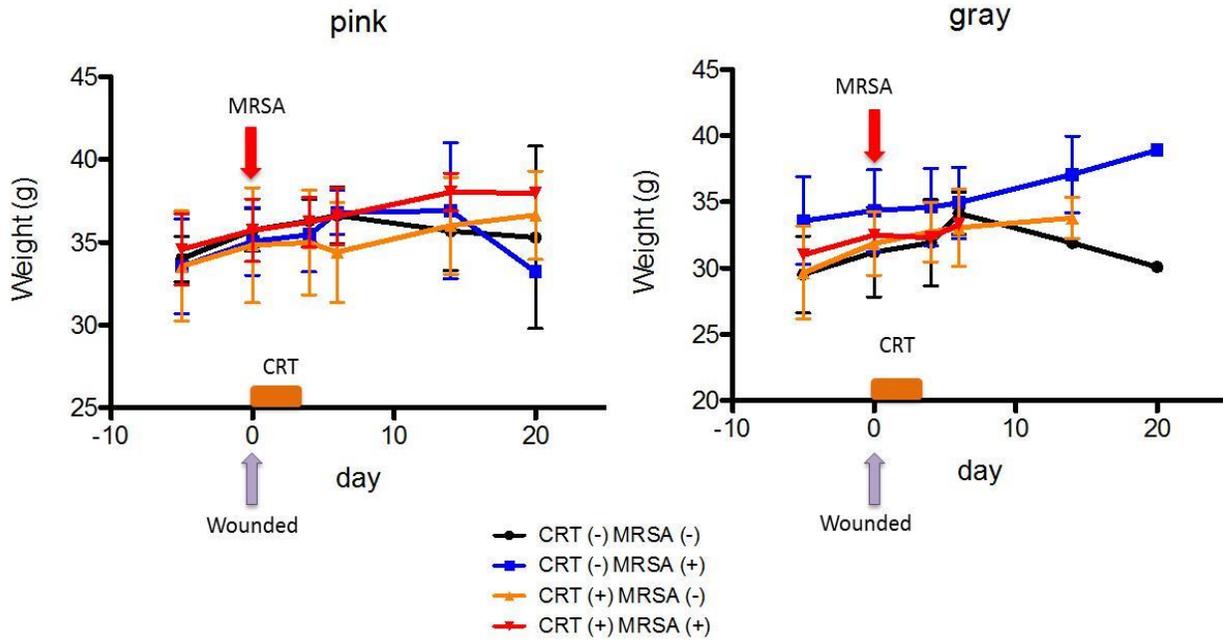
**Figure 5. Changes in weight in wounded C57BL/6 mice with either infection and/or treatment. Treatment was calreticulin (CRT) 5mg/ml given in 10mM Tris and 3mM CaCl<sub>2</sub>**



**Figure 6. Assessment of weight differences in relation to mouse housing, And to calreticulin.**



**Figure 7. Assessment of weight difference in db/db mice with either treatment and/or infection. Mice received calreticulin 5 mg/ml or not and then were challenged with MRSA (or not) at  $3 \times 10^8$  cfu/ml**



**Figure 8. Differences in weight gain among db/db mice with distinct skin phenotypes. The pink phenotype refers to a bright pink skin and the gray refers to a darker skin**

## The Cutaneous Microbiome in Outpatients Presenting With Acute Skin Abscesses

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**Background.** Previous studies have demonstrated an association between antibiotic use and the development of skin abscesses. We tested the hypothesis that alterations in the composition of the cutaneous microbiota may predispose individuals to skin abscesses.

**Methods.** We studied 25 patients with skin abscesses and 25 age-matched controls, who each completed a questionnaire. Skin swab samples were obtained for DNA analysis from 4 sites around the abscess site (hereafter, "peri-abscess specimens") and from similar sites on the patient's contralateral side and on healthy control subjects. DNA was extracted and analyzed by quantitative polymerase chain reaction (qPCR) and high-throughput sequencing. The purulent abscess drainage was sent for culture.

**Results.** Fifteen patients with abscess were infected with *Staphylococcus aureus*. Use of *muc* qPCR to quantitate *S. aureus* revealed a significantly greater frequency of positive results for peri-abscess and contralateral skin samples, compared with control skin specimens. Analysis of community structure showed greater heterogeneity in the control samples than in the peri-abscess and contralateral samples. Metagenomic analysis detected significantly more predicted genes related to metabolic activity in the peri-abscess specimens than in the control samples.

**Conclusions.** The peri-abscess microbiome was similar to the contralateral microbiome, but both microbiomes differed from that for control patients. Host characteristics affecting microbial populations might be important determinants of abscess risk.

**Keywords.** skin infection; MRSA; skin microbiome; abscess.

Patients commonly present to emergency departments with skin abscesses, and the incidence appears to be increasing [1]. While many factors contribute to the development of infection with community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA), such as participation in contact sports, incarceration, injection drug use, and, for men, having sex with men, the strongest risk factors are having a household contact with MRSA infection and having a history of recent antibiotic use [2]. Systemic antibiotics, as well as topical agents, including antibacterial soaps and cleansers,

can suppress or eliminate normal skin bacteria [3]; antibiotic use may alter the indigenous microbiota for several weeks or longer [3]. Among patients presenting to the emergency department with skin abscesses due to MRSA, antibiotic use within the prior month has been associated with an increased risk [2].

The composition of the normal cutaneous microbiota is complex; analysis of skin bacteria indicates multiple species, with particular compositions depending on body site [4–10]. The skin microbiome can be divided into dry, moist, and sebaceous sites [6]. As at other sites, the residential bacteria may play a role in defenses against pathogenic bacteria [3]. Our hypothesis is that the perturbation of the normal protective bacterial population predisposes individuals to contracting MRSA and developing skin abscesses. We tested this hypothesis by comparing the microbiota in patients with skin abscesses to the microbiota in control patients without abscesses. We hypothesized that, compared with controls, individuals who developed skin abscesses would

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Figures and documents Project4:

**Effect of antibiotic pre-treatment and pathogen challenge on the  
intestinal microbiota in mice**

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## **Abstract**

In models using C57/BL6 mice, we explored the effects of pathogen challenge, and antibiotic treatment on the intestinal microbiota. Mice were treated with either ciprofloxacin, penicillin, or water (as control) for a 5-day period followed by a 5-day washout period prior to oral challenge with *Campylobacter jejuni* or *Acinetobacter baumannii* to assess antibiotic effects on colonization susceptibility. Mice were successfully colonized with *C. jejuni* more than 118 days, but only transiently with *A. baumannii*. These challenges did not lead to any major effects on the composition of the gut microbiota. Mice acquired Segmented Filamentous Bacteria (SFB) in our facility at different time points, with essentially simultaneous acquisition within a cage,

acquisition of SFB also did not affect microbiota composition. Although antibiotic pre-treatment did not modify pathogen colonization, it affected richness and community structure of the gut microbiome. However, the dysbiosis produced by antibiotics was significantly reduced by pathogen challenge. We conclude that despite gut microbiota disturbance, susceptibility to gut colonization by these pathogens was unchanged. The major gut microbiome disturbance produced by antibiotic treatment may be reduced by colonization with specific taxa.

## Introduction

The mammalian intestine hosts a complex and diverse microbial community (1, 2). This ecosystem interacts extensively with its host, with substantial physiological and pathological effects (3). For example, the gut microbiota is crucial to the host's ability to resist colonization by pathogens (4, 5), although the mechanisms involved are incompletely characterized (6).

The clinical use of antibiotics has become massive in recent decades (7). Their use increases susceptibility to acquired pathogen, although the underlying mechanisms are not well-understood (8). Antibiotics change the composition of microbiota in the GI tract (9), affecting metabolic, hormonal, and immunological interactions between community and host, as well as intra-community interactions (10, 11, 12). Separately, or together, these effects may increase host susceptibility to infection by introduced pathogens.

The commensal bacteria in the gut are important to immunological maturation (13, 14), for example, affecting the balance of IL-17-producing T helper (Th17) cells (15). Commensal bacteria are required for induction of Th17 cells; acquisition of Segmental Filamentous Bacteria (SFB) by mice leads to Th17 cell differentiation and to a diminished number of Treg cells (16). This illustrates how commensal organisms could affect resistance to pathogens colonizing the intestinal tract.

*Campylobacter jejuni* are gram-negative, microaerophilic, curved rods, that commonly cause diarrheal illnesses, and can affect previously healthy hosts (17, 18, 19). *Acinetobacter baumannii*, non-fermentative gram-negative cocobacilli, have become increasingly common nosocomial pathogens, especially in intensive care units (ICUs) (20, 21). The high prevalence of intestinal *A. baumannii* colonization in ICU patients points suggest that the colon may be an important reservoir (22).

In this study, we developed mouse models involving colonization with these human pathogens to address three questions germane to colonization resistance; (i), what is the extent to which pathogens such as *C. jejuni* or *A. baumannii* colonize the GI tract of mice; (ii), how does such colonization affect the gut microbiota; and (iii), does pre-treatment with antibiotics change microbiota compositions and affect susceptibility to colonization by these pathogens?

## MATERIALS AND METHODS

**Study design.** Three related experiments were conducted in which control (untreated) mice were compared to experimental mice that were challenged by a pathogen, either alone, or in conjunction with antibiotic pre-treatment. In experiment #1, seven week-old mice were challenged with either *A. baumannii* or *C. jejuni* (**Supplemental Figure 1**, panel A). In experiment #2, six week-old mice were challenged with one of three strains of *C. jejuni* that varied based on their mouse-passage histories (**Supplemental Figure 1**, panel B). In experiment #3, mice were exposed first to an antibiotic regime of either penicillin or ciprofloxacin or neither (control), and then were challenged with either *A. baumannii* or *C. jejuni*, or remained unchallenged (**Supplemental Figure 2**).

**Mice.** Female C57BL/6NJ mice were obtained from Jackson Laboratories at ~ 5-6 weeks of age and allowed to adjust to the NYU animal facility for 1 week. The animals then were used in experiment #1 (**Supplemental Figure 1**, panel A). In addition, animals originally received from Jackson Laboratories were used for breeding at the NYU animal facility, and the offspring females were used for experiments #2 and 3 (**Supplemental Figure 1**, panel B). In experiment #3, 10 days prior to bacterial challenge, mice were given water containing penicillin VK (1.67 mg/ml; Sigma Aldrich, St Louis MO), or ciprofloxacin (0.13 mg/ml; Acros Organics, Geel, Belgium), or no antibiotic (control) for five days. Water containers were changed twice during these five days to supply fresh antibiotics. The protocols for the mouse experiments included in this study were approved by the New York University School of Medicine Institutional Animal Care and Use Committee (IACUC).

**Bacterial strains used for mice inoculation.** *C. jejuni* strain 81-176, that was originally isolated from a milkborne outbreak of human campylobacteriosis (23), and has been used in human volunteer studies (24), was used in all three experiments. For experiment #2, we used

two additional *C. jejuni* strains that were recovered from mice experimentally inoculated with strain 81-176 in experiment #1. *C. jejuni* strain MP-10 was isolated from mouse stool 42 days after colonization, and *C. jejuni* strain Cecum J1 was isolated from the cecum of a mouse 119 days after colonization. All *C. jejuni* strains were cultured for 48 hours on Skirrow agar (Becton Dickinson, Franklin Lakes NJ) under microaerobic conditions at 37°C. Cultures then were resuspended in phosphate-buffered saline (PBS; pH 7.2) and adjusted to a concentration of 10<sup>8</sup> CFU (by OD<sub>600</sub>) in 400µl, which was introduced via oral gavage to test mice. Control mice received an oral gavage of 400 µl of PBS.

*A. baumannii* strain 11-1, used in experiments #1 and 3, was a recent clinical isolate obtained from the New York University Langone Medical Center (NYULMC) Clinical Microbiology Laboratory. *A. baumannii* was cultured for 24 h using Columbia sheep blood agar (BD) and CHROM agar Acinetobacter Base (DRG International, Springfield NJ) under aerobic conditions at 37°C. *A. baumannii* was resuspended in PBS and adjusted to a concentration of 1.3×10<sup>11</sup> CFU (by OD<sub>600</sub>) per 400µl to create the oral gavage inoculum.

**Fecal specimen collection, culture and DNA extraction.** In all experiments, fecal specimens were collected from mice before and after pathogen challenge (**Supplemental Figures 1 and 2**), and were either immediately cultured or frozen at -20°C. About 20 mg of feces were resuspended in 1 ml of PBS and vortex-mixed for 30s at room temperature. From this stock suspension, 10-fold dilutions were made in PBS. Aliquots (100µl) of the 10<sup>0</sup>, 10<sup>-2</sup>, and 10<sup>-3</sup> dilutions were plated on CHROM agar for *A. baumannii* and on Skirrow agar for *C. jejuni*. Plates were incubated under the conditions indicated above and colony counts were reported as CFU / mg stool. Fecal DNA also was extracted from a 20mg aliquot of mouse feces using the PowerSoil DNA Isolation Kit (MOBIO, West Carlsbad CA), according to the manufacturer's

protocol. The concentration of extracted DNA was determined by Nanodrop 1000 (Thermo Scientific, Waltham MA), and DNA was stored at -20°C until used.

**Quantitative PCR.** Sets of qPCR primers (**Table 1**) were used to quantitate bacterial populations, based on the universal bacterial 16S rRNA sequences (25), *C. jejuni luxS* (26), *A. baumannii oxa51* (27), and *CTL5-6* of segmented filamentous bacteria (SFB) (28). qPCRs were performed using 3.5 mM MgCl<sub>2</sub>, 0.4 ng/μl bovine serum albumin, 0.2 mM of each deoxynucleoside triphosphate, 10 pmol of each primer, 0.625 U *Taq* DNA polymerase (Qiagen, Valencia CA), and 2 μl extracted DNA in a final 20-μl volume of SYBR green master mix. qPCR conditions included 5 min at 94°C and 45 cycles of 10s at 94°C, 10s at 60°C (*C. jejuni* and *A. baumannii*) or 56°C (total bacteria), and 20s at 72°C. qPCR conditions for SFB included 10 min at 95°C and 40 cycles of 10s at 95°C, 20s at 62°C, and 20s at 72°C. All assays were performed using a Light Cycler 480 (Roche Diagnostic Corporation, Indianapolis IN). Bacterial numbers were determined using standard curves based on serial dilutions of cloned PCR products. Each sample was tested at least twice, and the results were analyzed using the Rotor-Gene 3000 v.6.1.81 software.

**Library preparation for High-Throughput Sequencing (HTS).** All samples were amplified and barcoded for multiplex pyrosequencing using primers targeted to the V4 region of the bacterial 16S rRNA gene under uniform PCR conditions that included 3 min at 94°C and 45 cycles of 45s at 94°C, 60s at 50°C, and 90s at 72°C with final extension for 10 min at 72°C (29). We used forward primer (AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT ATG GTA ATT GTG TGC CAG CMG CCG CGG TAA) that includes a 5' Illumina adaptor, forward primer pad, 2bp linker and the 515F 16S rRNA primer, and reverse primer (CAA GCA GAA GAC GGC ATA CGA GAT NNNNNNNNNNNN –AGT CAG TCA G-CC-GGA CTA CHV GGG TWT CTA AT) that includes the Illumina 3' adapter with 12-nt error-correcting Golay barcode, reverse primer pad, 2bp linker

and the 806R 16S rRNA primer. We ran PCR in triplicate using 0.2 $\mu$ M of the primers, 1 $\mu$ l of template and 1X HotMasterMix (5 PRIME, Gaithersburg MD), and cleaned the products using a PCR Purification Kit (Qiagen) after pooling. Cleaned PCR products were quantified using the Qubit dsDNA HS Assay Kit (Invitrogen™, Eugene OR), then adjusted to an optimal molarity as described. Sequencing was performed using the Illumina MiSeq platform in the NYULMC Genome Technology Core.

**Taxonomic and ecological analyses.** We analyzed all sequence data using the QIIME software (version Mac Qiime 1.8.0) (30). After filtering procedures, similar sequences were clustered into operational taxonomic units (OTUs) using an open reference approach with UCLUST (31) against the Greengenes Core set. A representative sequence was then aligned using PyNAST, and FastTree created phylogenetic trees. Rarefaction analysis used Chao-1 and whole PD to measure alpha diversity. Unweighted UniFrac distances were calculated to assess beta diversity; the unweighted paired group method with arithmetic mean (UPGMA) was performed for UniFrac-based jackknifed hierarchical clustering. Principal coordinates analysis (PCoA) of UniFrac distance matrices provided graphical representation using a KING, ANOVA was used to compare OTU and genus-level abundances, and Linear discriminant analysis (LDA) effect size (LEfSe), a tool that can compare differences of relative abundance between  $\geq 2$  biological conditions (32), also was used for analysis.

## RESULTS

**Quantifying bacteria in fecal DNA.** Assessing total DNA concentrations using Nanodrop and total bacterial log<sub>10</sub> copy number/ng DNA by qPCR, we found that they were similar between the control animals and those treated with either after antibiotic or pathogen challenge (**Supplemental Figure 3**, panels a to j). Thus, neither the antibiotic treatments nor the pathogen challenge affected the overall population size of the intestinal microbiota.

**Assessment of mouse intestinal colonization after challenge.** Although we were able to colonize mice with *A. baumannii* (**Figure 1**, left column), as evaluated by both culture and qPCR, colonization was transient and at low density. In contrast, we could achieve persistent mouse colonization with *C. jejuni* strain 81-176 (for ≥15 weeks), as confirmed by both culture and qPCR, until the experiment ended (**Figure 1**, middle and right columns). Thus, the *A. baumannii* and *C. jejuni* strains used differed greatly in their ability to colonize the murine gut.

**Detection of SFB colonization.** We next evaluated the presence of SFB in mice purchased from Jackson Laboratories. As expected (15), JAX mice were not SFB-colonized upon entering NYU facilities at 6 weeks of age, and remained so for at least 3 weeks. However, all mice in the facility for more than 4 weeks eventually became SFB-positive with acquisition occurring either at 10-12 weeks of age or later (20 weeks of age). Every mouse within each cage became positive for SFB at the same time (**Figure 2**), developing levels of 10<sup>4</sup> to 10<sup>5</sup> SFB 16S rRNA copies/μg DNA, declining to 10<sup>2</sup> to 10<sup>3</sup> copies over the next 12 weeks. While some mice showed consistently decreasing SFB levels, SFB levels oscillated reproducibly in others (**Figure 2**). Overall, we observed high reproducibility in our SFB detection; with independent runs by two investigators, results were nearly identical (**Supplemental Figure 4**). Among the mice challenged with *A. baumannii* and kept only for 8 weeks, only one of 10 became SFB-positive. (**Supplemental Figure 5**).

**Assessment of gut microbiome changes associated with *C. jejuni* challenge and cage effects.** We then assessed the gut microbiota in mice followed prospectively from 6.5 to 23.9 weeks (**Figure 3**). In the initial pre-challenge samples, the bacterial communities were nearly identical in their community structure (Column A). However, over time, the communities differentiated, based on the cage in which they were housed, and independently based on *C. jejuni* challenge or not. Next, assessing the species richness of the gut microbiota, all three cages were similar, although at the final time point, the *C. jejuni*-challenged group showed greater richness than controls (Column B). All three groups were similar in relative abundance at the phylum level (Column C) except for late changes in *Firmicutes* abundance in cage 3.

**Assessment of gut microbiome changes associated with SFB status and bacterial challenge.** To determine the extent of SFB colonization, in this experiment, we compared the determinations by specific qPCR and the HTS relative abundance. The qPCR and HTS results for SFB were highly consistent (**Supplemental Figure 6**, panel A). Taxa relative abundances showed only minor and not consistent differences across the time of SFB acquisition (**Supplemental Figure 6**, panel B). Thus, SFB acquisition had little if any effect on the overall microbiota composition.

**Quantitative differences in specific taxa.** We next analyzed the HTS results using LEfSe, identifying specific taxa that showed significant differences between mice at the beginning (6.5 weeks of life) and end (23.9 weeks) of the experiment (**Supplemental Figure 7**). Multiple taxa within *Firmicutes*, *Bacilli*, and *Tenericutes* were significantly increased at the early time point in the three groups of mice with only cage-related minor variations. In contrast, taxa within *Bacteroides* and *Verrucomicrobia* were significantly higher at the later time point in all groups. Animals in cage 3 showed significantly increased *Proteobacteria*, consistent with our finding of

persistent *C. jejuni* colonization. *Proteobacteria* were not increased in cage 2 (control) as expected, nor in cage 4 after *C. jejuni* colonization had spontaneously ceased.

We also compared specific taxa between the three cages at the same time points (**Supplemental Figure 8**). As expected, only minor differences were observed before challenge (at 6.5 weeks). After challenge, specific taxa were differentially elevated: *Tenericutes* at 8.3 and 23.9 weeks in cage 2, *Verrucomicrobia* in cage 3, and *Erysipelotrichi* in cage 4 at 8.3 and 23.9 weeks, and *Proteobacteria* in cage 3 at 23.9 weeks. There were no consistent differences between cage 2 (control) and the two cages in which mice had been *C. jejuni* challenged.

**Detection of *C. jejuni* after mouse passage.** Next, we investigated whether three *C. jejuni* strains with different passage histories varied in their abilities to colonize the mouse gastrointestinal tract after oral challenge (**Supplemental Figure 9**). All three *C. jejuni* strains showed similar kinetics; culture (panel A) and qPCR results (panel B) were consistent. Despite some variations in the kinetics of colonization, all three *C. jejuni* strains colonized the mouse gut to similar degrees.

**Detection of SFBs in mice bred at NYU facilities.** All 19 mice born and raised at NYU for our experiments were positive for SFB by 6 weeks of age. The SFB kinetics in the mice challenged with different *C. jejuni* strains and in controls varied substantially. Among the controls, in mice colonized with the original 81-176 strain, or with MP-10, SFB levels ranged between  $10^1$  to  $10^4$  copies/ug DNA (**Supplemental Figure 10**, column A). In the MP-10 colonized mice, SFB levels oscillated, but by the experiment's end, SFB levels in these three groups were similar to those present at 6 weeks, with same degree of variation. In the group challenged with the Cecum J1 strain, SFB colonization gradually declined from  $10^4$  to  $10^1$  (**Supplemental Figure 10**, column B). Thus, SFB colonization patterns varied.

**Effect of antibiotic treatment on mouse intestinal colonization.** We next studied the effect of pre-treatment of the mice with penicillin or ciprofloxacin, two antibiotics often used in clinical practice, on gut colonization with *C. jejuni* or *A. baumannii*. The antibiotics used to pre-treat the mice had no significant influence on intestinal colonization with either pathogen (**Figure 4**). As in previous experiments, *A. baumannii* only transiently colonized the mice.

**Effect of antibiotic pre-treatment on SFB colonization.** We next assessed the effect of penicillin and ciprofloxacin on SFB colonization (**Supplemental Figure 11**). In the no antibiotic (control) groups, SFB levels at baseline were  $\sim 10^3$  SFB copy number / total bacterial DNA. Once the animals were treated with PBS or ciprofloxacin (column B), they showed little or no decreases in SFB levels, and SFB were detectable throughout the experiment. In contrast, in mice treated with penicillin (column C), SFB levels immediately dropped. The further courses were variable, with loss of detection (in control), or continued lower levels (with pathogen challenge). In contrast, ciprofloxacin treatment had no significant effect on SFB copy number began increasing after *C. jejuni* challenge..

**Assessment of gut microbiota changes associated with bacterial challenge and antibiotic pre-treatment.** We assessed the gut microbiome in mice followed for nearly nine weeks (**Figure 5, Panel A**). In (PBS control) mice that did not receive antibiotic treatment, the bacteria communities were stable, with all groups nearly identical in community structure. The groups of mice treated with penicillin showed distinct differences in community structure, but gradually recovered in those challenged with *C. jejuni*, but not in the controls. The mice receiving ciprofloxacin had minor effects on community structure (**Supplemental Figure 12**). In an early assessment of beta-diversity of the gut microbiota in relation to antibiotic treatment and pathogen challenge (**Figure 5A**) using Multivariate Analysis of Variance, PERMANOVA (Adonis), we observed that at time1 (4.3 week) there are small differences, which are not

significant. At time 2 (5.4 week) there are significant differences between the groups. Since no intervention other than antibiotic pre-treatment has been performed on the mice, we assessed that the difference may be related to a cage effect in all time points. Intergroup variability at time 2 allows us to estimate the magnitude of the cage effect between the challenged and not challenged group within each antibiotic treatment. We used this fact to determine whether challenging with bacterial pathogens results in difference in intergroup variability at later time points. If the intergroup variability (challenged vs. unchallenged mice) is significantly different in a paired analysis after a pathogen challenge is introduced (time 3 and onward) then the effect of the challenge on the community structure is greater than that of the cage effect. We determined intergroup phylogenetic distance between groups of mice treated or not with antibiotics and challenged with either *A. baumannii* or *C. jejuni* (**Supplemental Figure 13**). We did not observe statistical differences in the community structure of the groups challenged with either *A. baumannii* or *C. jejuni* without antibiotic pre-treatment (control groups). In contrast, community structures of longitudinal time points treated with antibiotics and challenged with *A. baumannii* were significantly different that community structures before challenge. Similar results were observed with *C. jejuni* challenge with the exception of time point 5. From these studies, we confirmed that community structure of the gut is affected by antibiotic pre-treatment and pathogen challenge.

In mice without antibiotic pre-treatment, alpha-diversity was highest in the *C. jejuni*-challenged group (**Figure 5, Panel B**), due to the short follow up of the *A. baumannii* group, we could not assess changes. We consistently observed that in animals treated with penicillin alpha-diversity was significantly increased in those challenged with *C. jejuni*, ciprofloxacin did not affect richness.

Finally, the relative abundance of taxa in the control mice (not pre-treated with antibiotics) and challenged with *A. baumannii* or *C. jejuni* (**Figure 6**, column A) differed little from the mice with no pathogens. There was a major effect on taxa abundance in mice pre-treated with penicillin, but this was significantly reduced with *A. baumannii*, or *C. jejuni* challenge (column C). In contrast, ciprofloxacin had much smaller effects (column B).

We assessed the longitudinal changes in intergroup distances (in beta diversity) based on data in **Figure 5A**. In the *C. jejuni*-challenged mice, both the control and ciprofloxacin groups were almost identical with low differences in unweighted unifrac distances over course of the experiment (**Supplemental Figure 14**). However, the distances increased dramatically after penicillin treatment, and then gradually decreased after the *C. jejuni* challenge.

## Discussion

In these studies, persistent colonization was achieved with *C. jejuni*, an intestinal pathogen, but not with *A. baumannii*, consistent with prior reports (33, 34). Mice purchased from JAX laboratory were free of SFB, as reported (15) and they acquired SFB after several weeks in NYU facilities. When one member of a cage acquired the organism, they all did, essentially simultaneously. That cages did not all convert simultaneously, reflects differences in SFB transmission within the facility. The similar qPCR and HTS relative abundances for SFB cross-

validate the two detection methods. From these experiments, we can assess the effects of on the microbiota of exposure to exogenous bacteria.

Longitudinal assessment of bacterial gut community structure indicates that at early time points, essentially all mice were identical. However, as the experiments progressed, community structures varied, in relation to cage effects, and independently in relation to antibiotic treatments, *C. jejuni* challenge, or SFB-acquisition.

However, the community richness in the gut microbiome was relatively constant, unaffected by cage effects, SFB exposure, or antibiotic effects. However, *C. jejuni* challenge increased richness, which was unaffected by penicillin pre-treatment. Variation of relative genus abundance was minor; the differences present were not associated with either bacterial challenge or SFB acquisition. Mouse-adapted strains of *C. jejuni* were not better than a parental strain in mouse gut colonization. *C. jejuni* colonization of the mouse gut was not affected by the presence of SFB. The mechanism for the increased richness are not clear, but could include *C. jejuni* competitions with dominant strains, permitting the blooming of more minor taxa.

Antibiotic pre-treatment showed no effect on the capabilities of *C. jejuni* and *A. baumannii* to colonize the mice. Mice treated with penicillin or ciprofloxacin showed transient colonization with *A. baumannii*, similar to the control group. No variation in gut colonization was observed in relation to the presence of SFB or not; however, conversely SFB levels were significantly affected by antibiotic use. Stable community structure was observed in mice for nearly 10 weeks whether challenged or not. Ciprofloxacin induced minor community-wide disturbances but penicillin induced major disturbances (**Figure 6**).

In the absence of antibiotic pre-treatment, pathogen introduction did not affect relative abundances of the colonizing taxa. However, penicillin caused strong disturbances in taxa

abundances and community structure. That the effect of penicillin was so much greater than ciprofloxacin, probably results from its anti-anaerobic activity (35, 36), compared to the lack of ciprofloxacin activity (37). Surprisingly, the introduction of *A. baumannii* or *C. jejuni* ameliorated the disturbances in the relative abundances (community structure), and facilitated the recovery to normality. We speculate that pathogen introduction affected either host responses that led to stereotypic changes, or to altered competition dynamics in the gut favoring status quo ante.

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## Figure Legends

**Figure 1. Quantitation of *A. baumannii* and *C. jejuni* intestinal colonization. Upper panels: Detection by culture. Lower panels: Detection by qPCR.** Quantitation of *A. baumannii* is shown for weeks 7 to 8.5.. Quantitation of *C. jejuni* is shown for weeks 7 to 12, and for weeks 7 to 26.

**Figure 2. Assessment of SFB enumeration in individual mice and by group.** SFB density was assessed by qPCR and results expressed according to cage. Cage 2 represents a control group. Cages 3 and 4 housed mice challenged with *C. jejuni*. SFB enumeration of individual mice (panel A) and the mean per cage (panel B), respectively.

**Figure 3. Assessment of change in gut microbiome following *C. jejuni* challenge.** Mice were challenged with either PBS (cage 2), or *C. jejuni* (cages 3 and 4) at 7 weeks of age. Fecal pellets were collected serially, DNA extracted, and HTS performed on the Illumina Miseq platform. Beta-diversity (column A) alpha-diversity (column B). and relative taxon abundance at the phylum level (color-coded, column C) are shown. Colors: light blue indicates control group (cage 2); dark blue indicates *C. jejuni* group with early SFB acquisition (cage 3); red indicates *C. jejuni* group with late SFB acquisition (cage 4).

**Figure 4. Quantitation of *A. baumannii* and *C. jejuni* in fecal pellets. Panels A and E: culture detection. Panels B-H: qPCR assessment.** Mice received five days of PBS (panels B and F), ciprofloxacin (panel C and G), or penicillin (panel D and H) and then were pathogen-challenged. The blue box indicates the period of antibiotic exposure. The yellow arrow indicates inoculation date of challenge with either *A. baumannii* (panel A-D) or *C. jejuni* (panel E-H).

**Figure 5. Assessment of longitudinal changes in microbial diversity associated with antibiotic treatment and bacterial challenge.** Each row shows a different time point and each column indicates the treatment groups. The pathogen challenges are color-coded. Mice challenged with *A. baumannii* was studied only up to 7.6 weeks of age. **Panel A:** Beta-diversity. PCoA of the unweighted UniFrac distance of microbial 16S rRNA sequence (V4 region) in fecal samples is presented longitudinally from 4.3 to 13 weeks of age. **Panel B:** alpha-diversity by whole PD metric associated with antibiotic treatment and bacterial challenge. Alpha-diversity was calculated using the whole PD evenness metric. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , by ANOVA.

**Figure 6. Relative abundance at phylum level of individual mice followed prospectively.** Each column indicates treatment groups and the boxes indicate time points of antibiotic treatment or not (PBS control). Each row represents the bacterial challenge and the arrows indicate time of challenge. The study period was from 4.3 to 13 weeks of age, except the groups challenged with *A. baumannii* were studied only to 8.9 weeks o

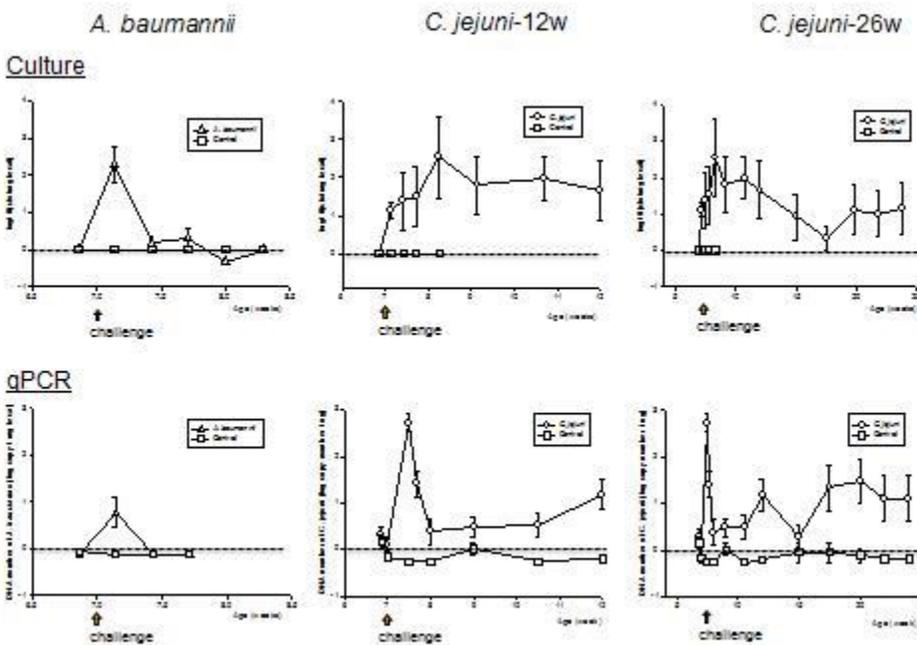


Figure 1.

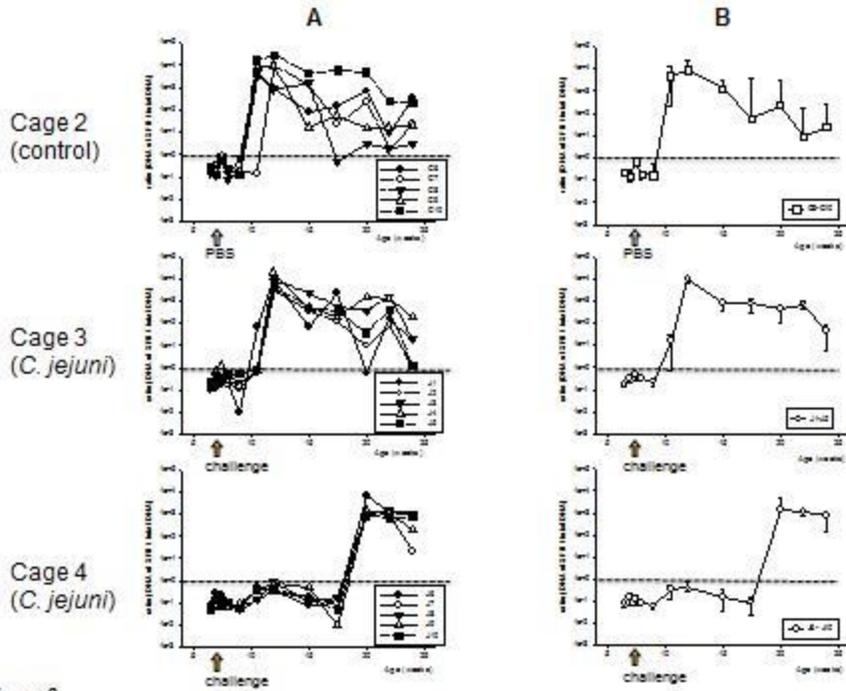


Figure 2.

*A. baumannii*

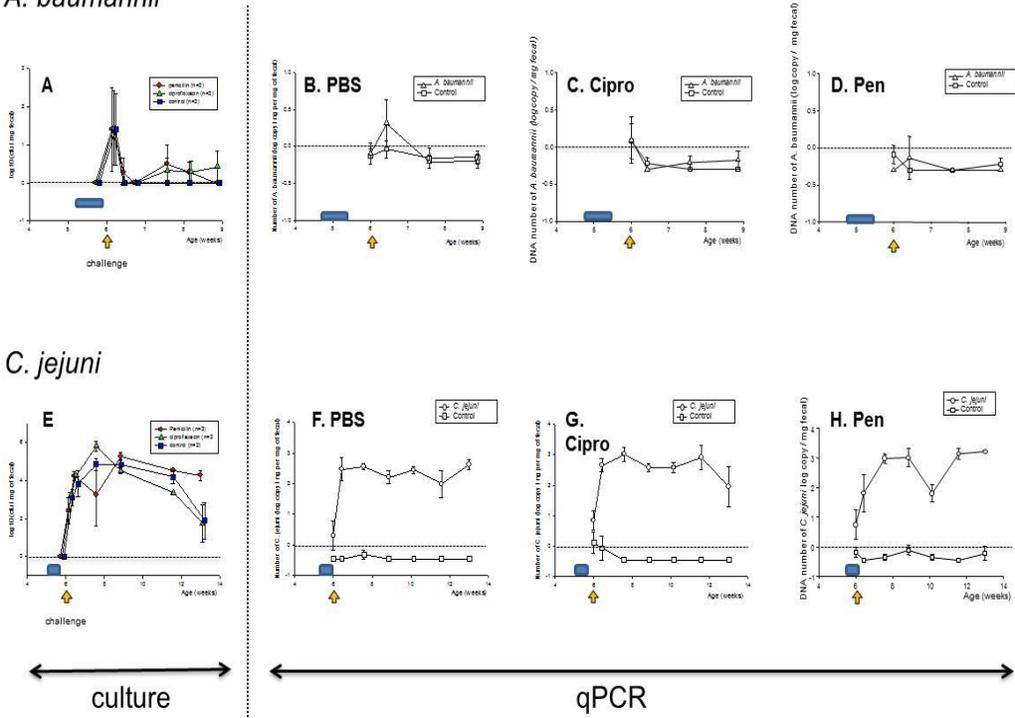
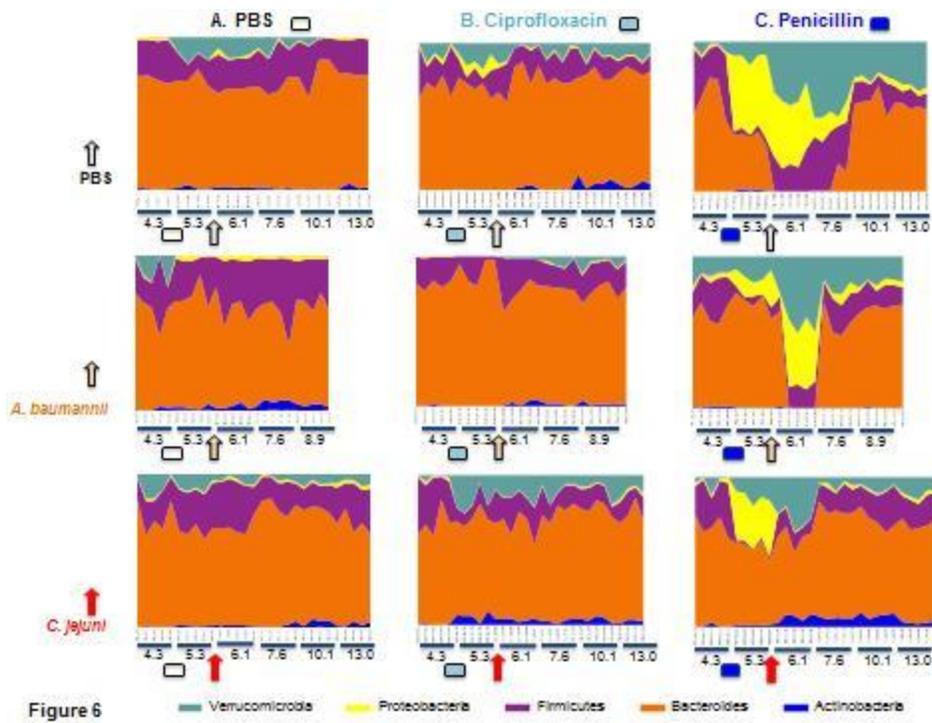


Figure 4.



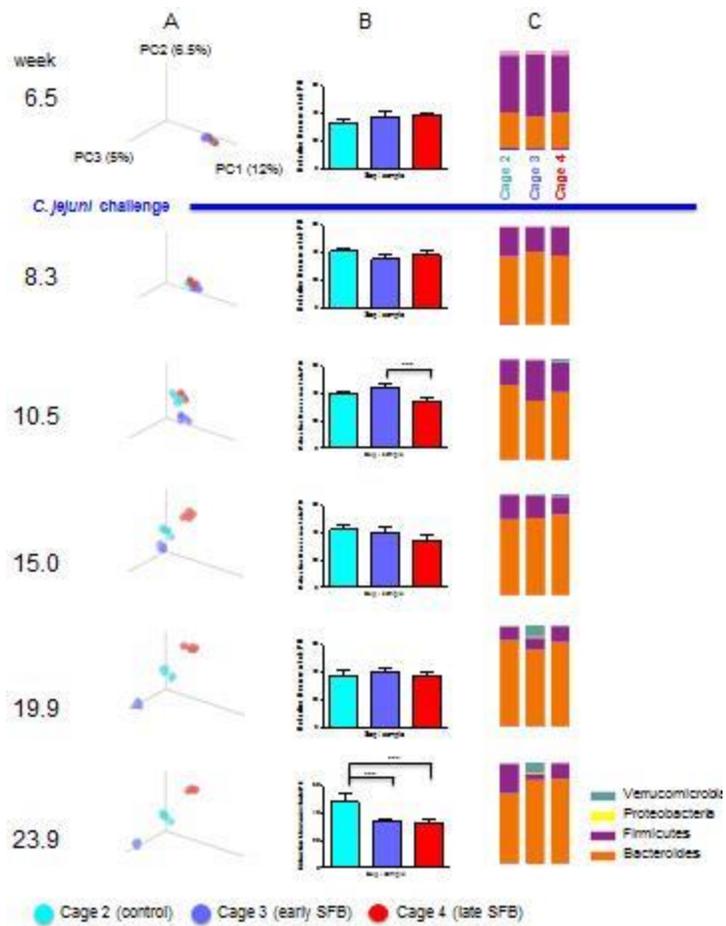


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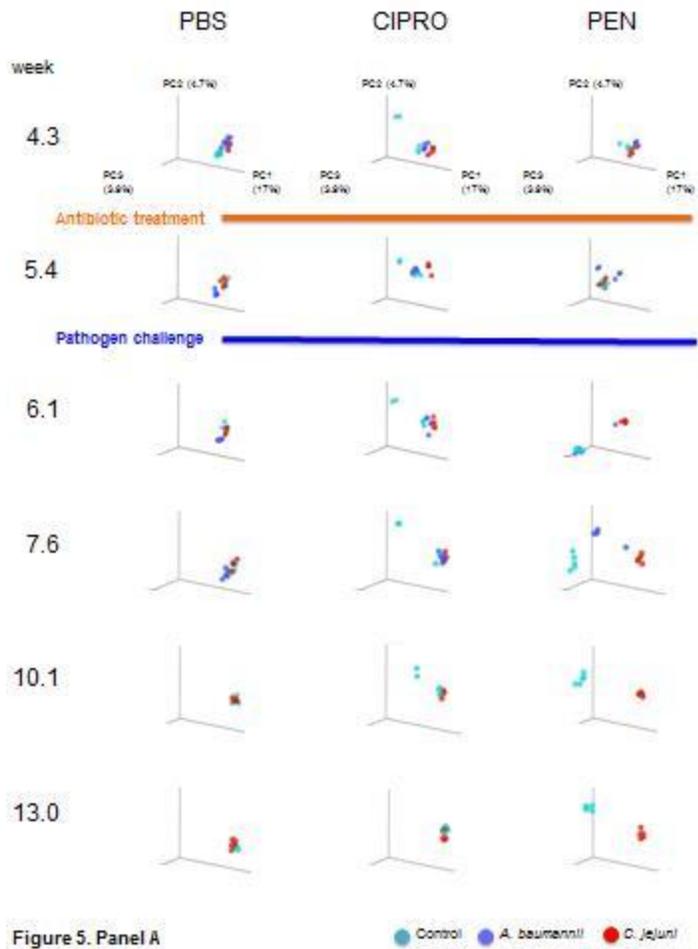


Figure 5. Panel A

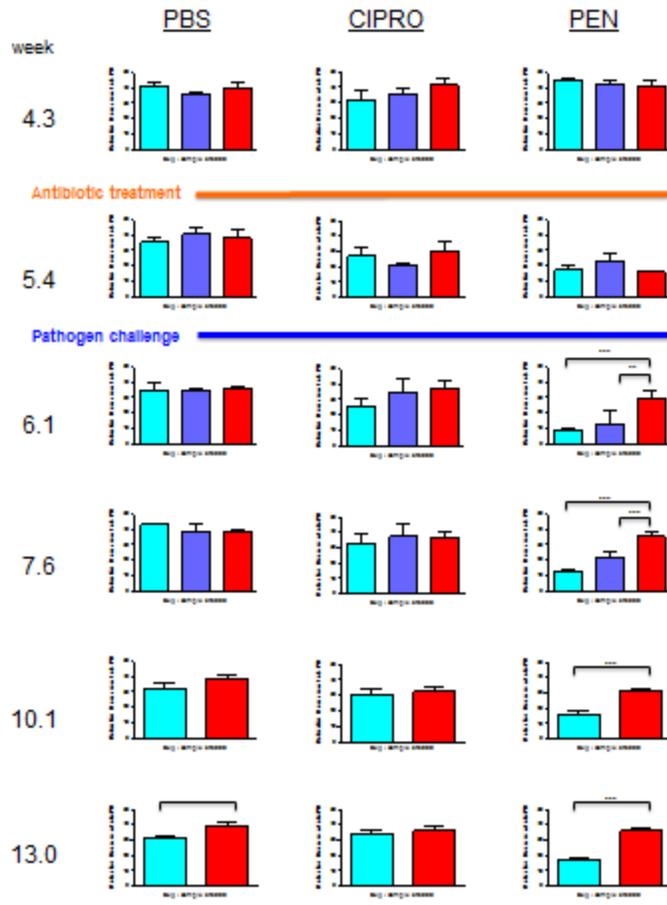
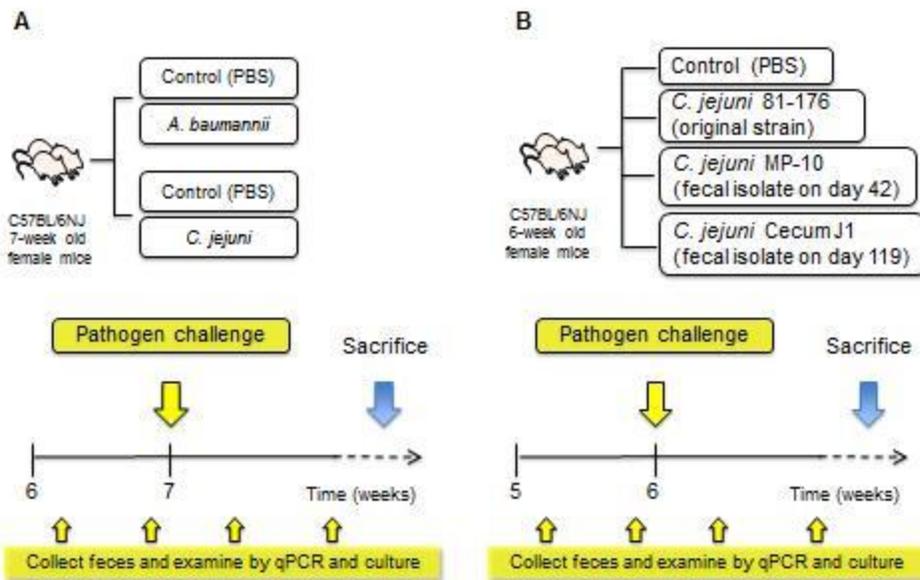
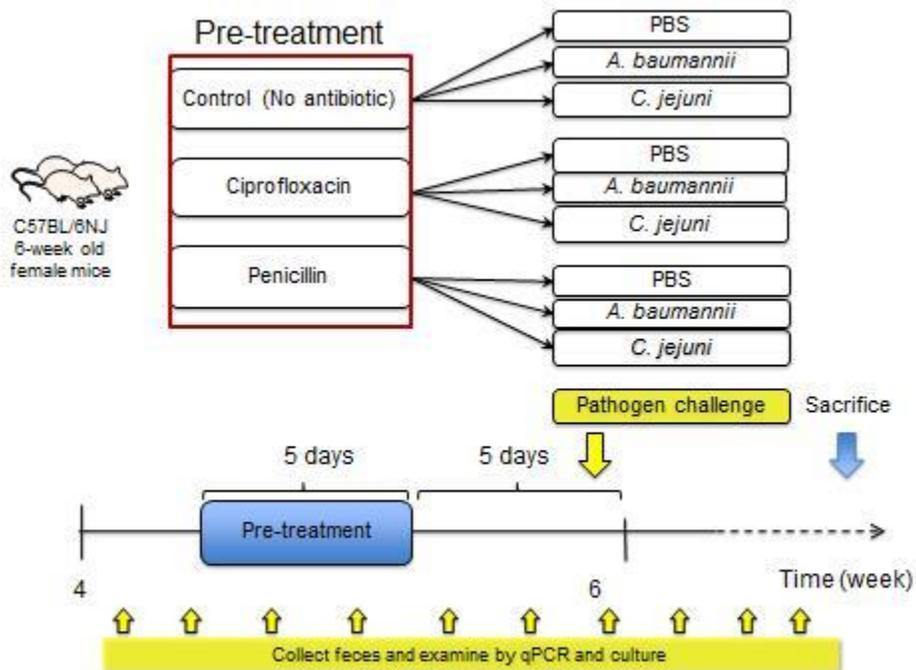


Figure 5. Panel B

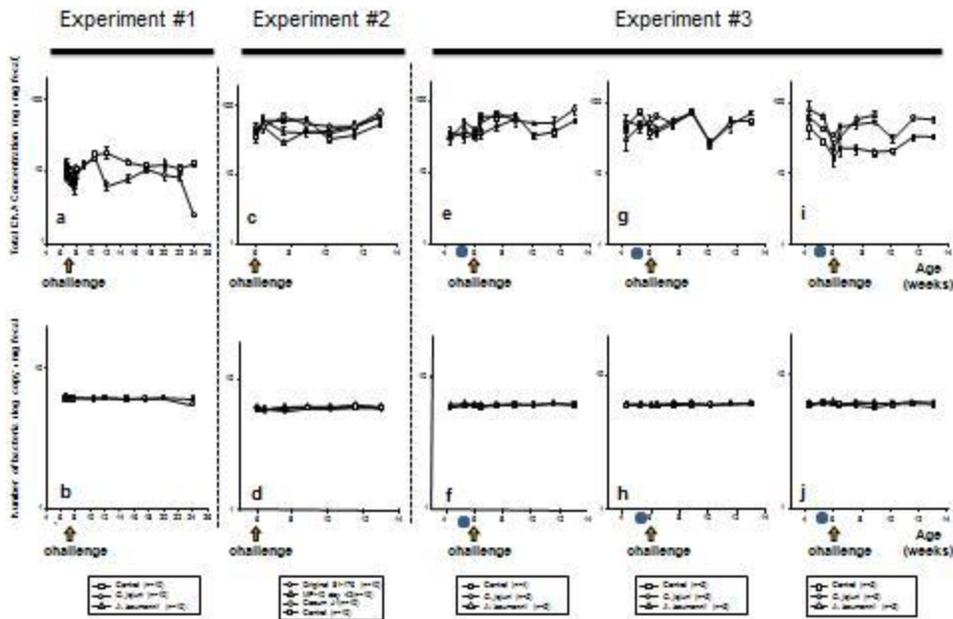
● Control ● *A. baumannii* ● *C. jejuni*



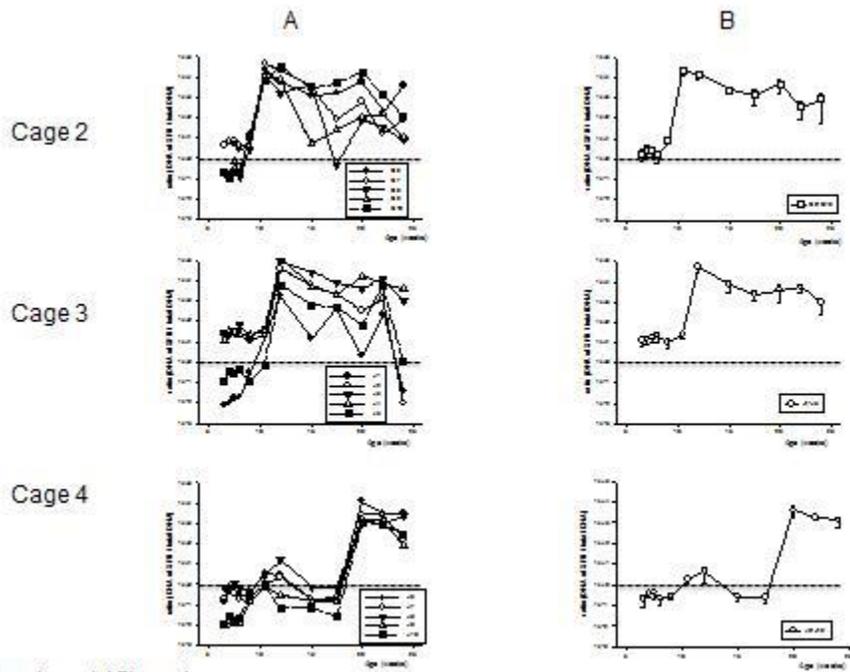
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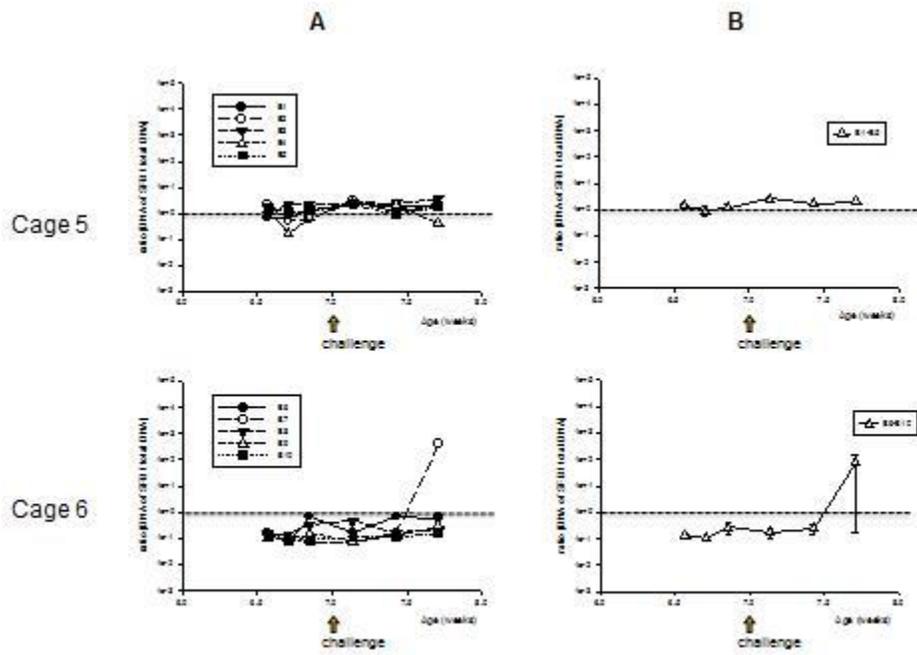
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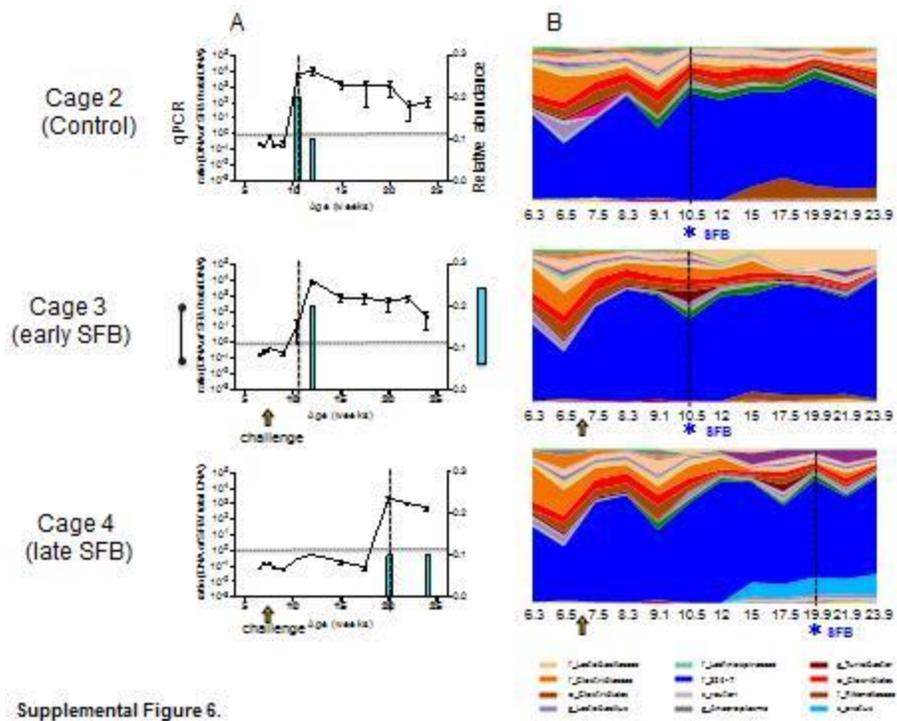
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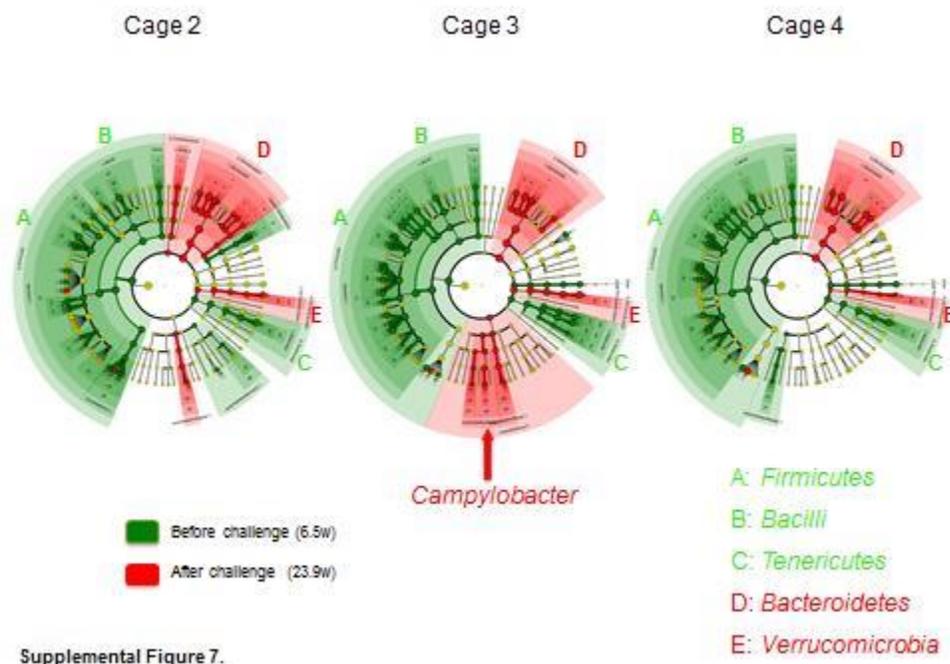
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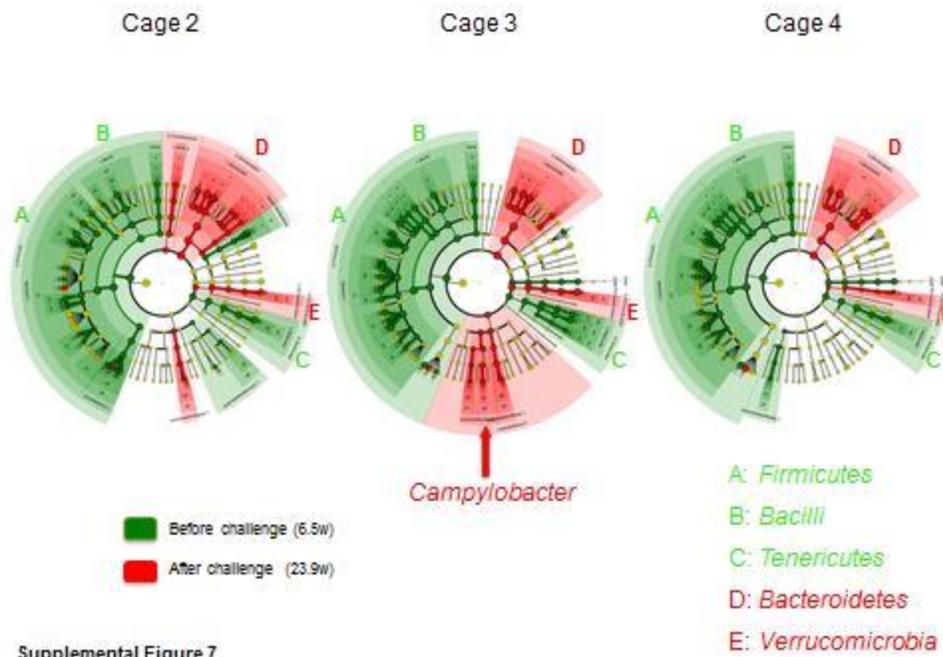
Supplemental Figure 5.



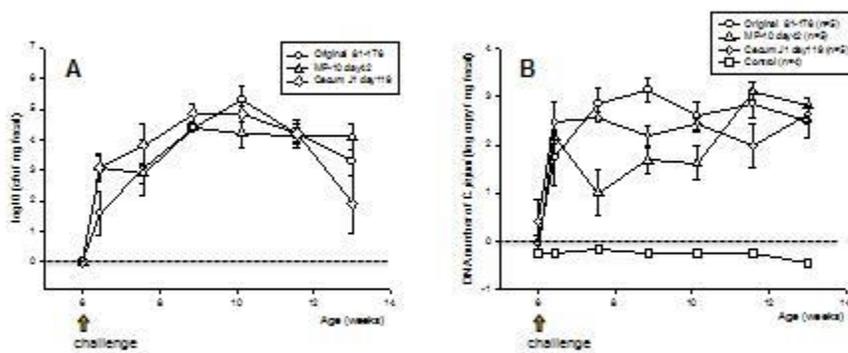
Supplemental Figure 6.



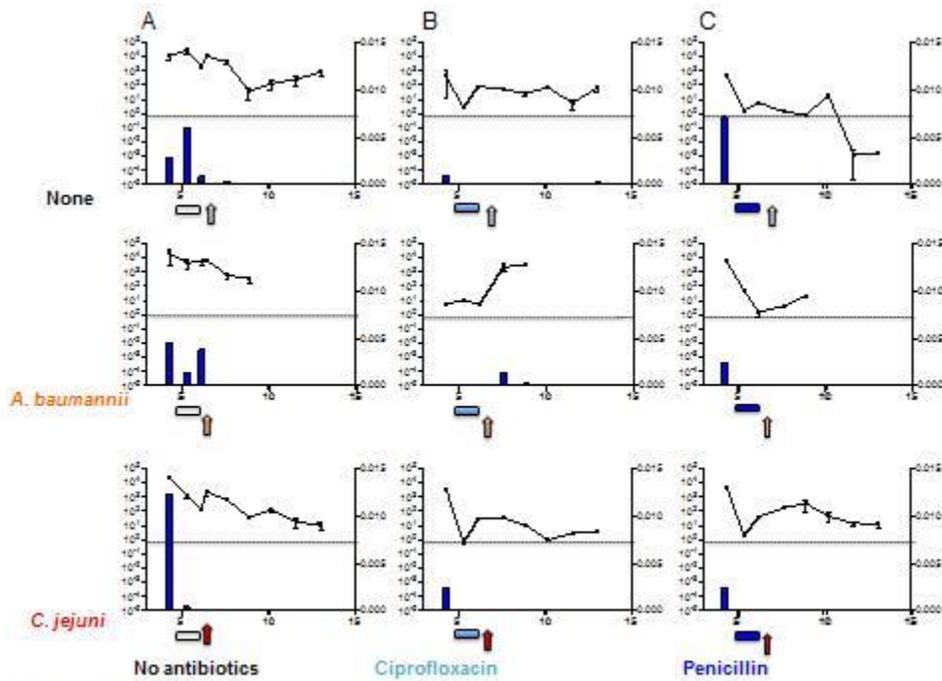
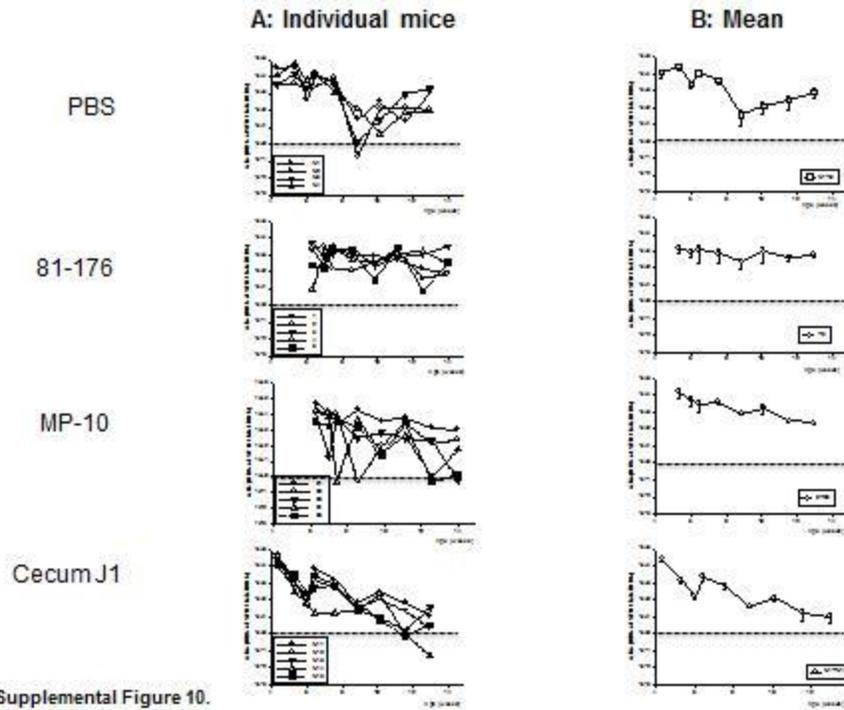
Supplemental Figure 7.

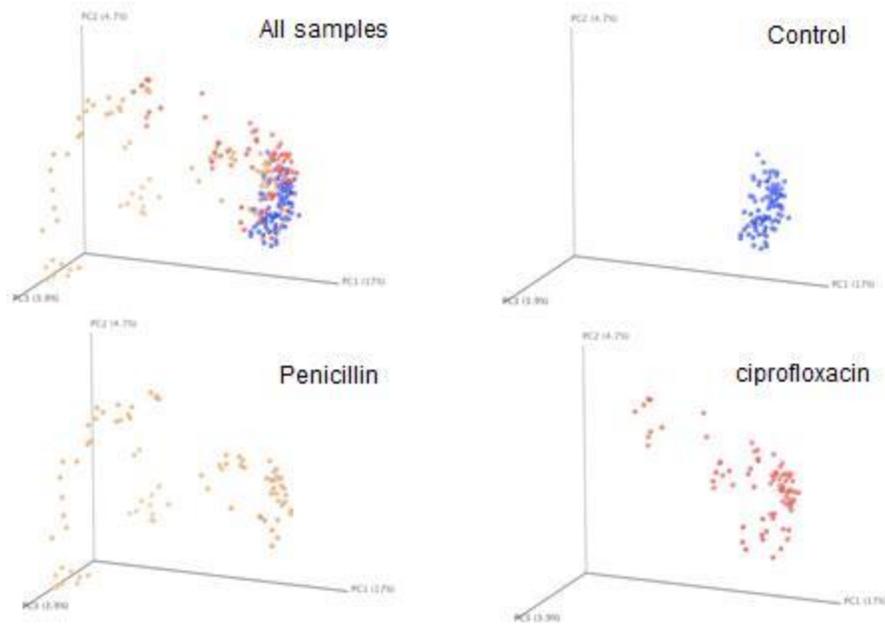


Supplemental Figure 7.

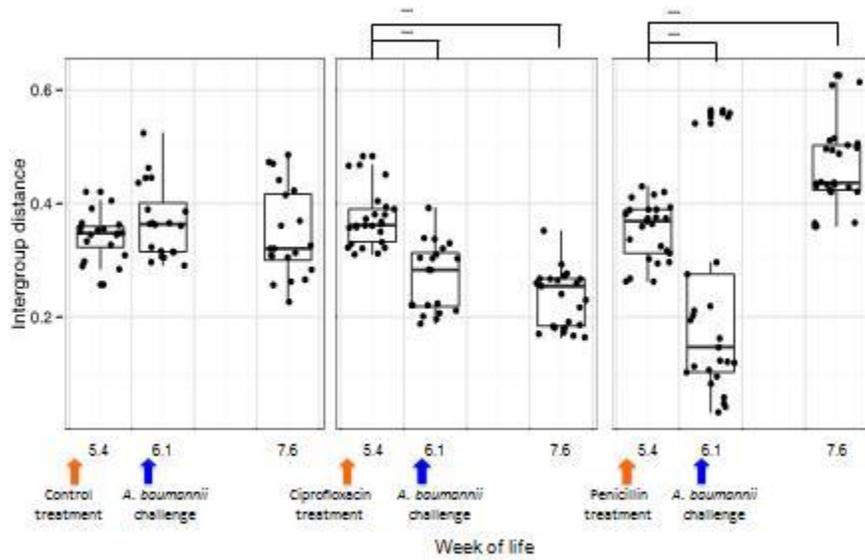


Supplemental Figure 9.

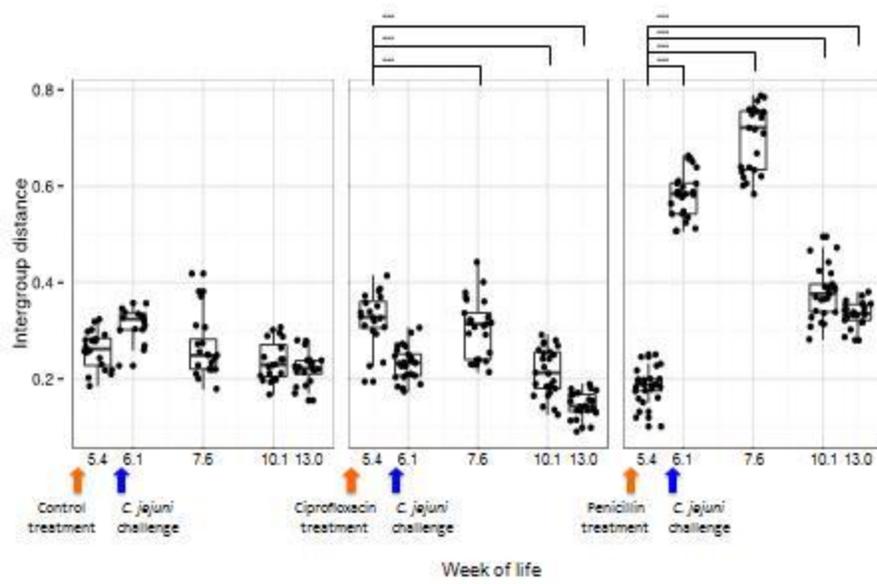




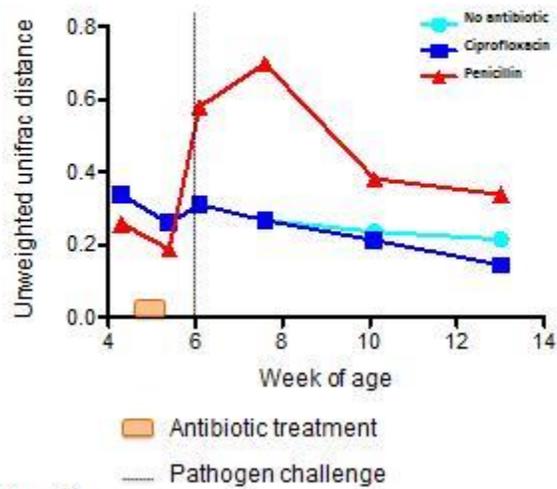
Supplement Figure 12.



Supplement Figure 13. Panel A



Supplement Figure 13. Panel B



Supplement Figure 14.

**Table 1. Primers used for PCR in this study**

Target	Primer designation	Primer sequence <sup>b</sup>	Ref
Total bacterial 16S rRNA	519F	GGACTACCVGGTATCTAAKCC	25
	785R	CAGCAGCCGCGGTRATA	
<i>C. jejuni luxS</i>	luxS-F	AGCGATCAAAGCAAATTCC	26
	luxS-R	GGCAATTTGTTTGGCTTCAT	
<i>A. baumannii oxa-51</i>	oxa-51-F	TTTAGCTCGTCGTATTGGACTTGA	27
	oxa-51-R	GCCTCTTGCTGAGGAGTAATTTTT	
SFB <sup>a</sup> CTL5-6	SFB736F	GACGCTGAGGCATGAGAGCAT	28
	SFB844R	GACGGCACGGATTGTTATTCA	

a: SFB; Segmented filamentous bacteria

b: V=A+C+G, K=G+T, R=A+G