Oxygen deprivation influences the survival of *Listeria monocytogenes* in gerbils[1]

Jillian Harris,[*] Oindrila Paul,[†] Si Hong Park,[‡,2] Sally J. White,[†] Khemraj Budachetri,[†,3] Daniel M. McClung,[*] Jessica G. Wilson,[§] Alicia K. Olivier,[#] Justin A. Thornton,[*] Paul R. Broadway,[‖] Steven C. Ricke,[‡] and Janet R. Donaldson[†,4]

*Department of Biological Sciences, Mississippi State University, Mississippi State, MS; †Department of Biological Sciences, The University of Southern Mississippi, Hattiesburg, MS; ‡Department of Food Science and Center for Food Safety, University of Arkansas, Fayetteville, AR; §Burnett School of Biomedical Sciences, University of Central Florida, Orlando, FL; #Department of Pathobiology and Population Medicine, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS; and ‖USDA-ARS, Livestock Issues Research Unit, Lubbock, TX

ABSTRACT: *Listeria monocytogenes* is a facultative anaerobic foodborne pathogen capable of surviving harsh environments. Recent work has indicated that anaerobic conditions increase the resistance capability of certain strains to environmental stressors. The goal of the study was to conduct a preliminary study to determine whether exposure to anaerobic conditions prior to infection increases the ability to survive in vivo. Gerbils were inoculated with one of five doses of the *L. monocytogenes* strain F2365 by oral gavage: phosphate-buffered saline (control), 5×10^6 colony forming units aerobic culture (low aerobic), 5×10^8 aerobic culture (high aerobic), 5×10^6 anaerobic culture (low anaerobic), or 5×10^8 anaerobic culture (high anaerobic) dose of F2365. Gerbils inoculated with a high aerobic or anaerobic dose exhibited significant weight loss. Gerbils administered either the low or high anaerobic dose had at least 3 $\log_{10}$ of *L. monocytogenes* present in fecal samples, which contrasted with gerbils that received the low aerobic dose. Animals that received the high anaerobic dose had a significant increase in bacterial loads within the liver. Histologic examination of the *L. monocytogenes* positive livers exhibited locally extensive areas of hepatocellular necrosis, though the extent of this damage differed between treatment groups. Microbial community analysis of the cecum from gerbils infected with *L. monocytogenes* indicated that the abundance of Bacteroidales and Clostridiales increased and there was a decrease in the abundance of Spirochaetales. This study suggests that anaerobic conditions alter the localization pattern of *L. monocytogenes* within the gastrointestinal tract. These findings could relate to how different populations are more susceptible to listeriosis, as oxygen availability may differ within the gastrointestinal tract.

Key words: aerobic, anaerobic, gerbils, *Listeria monocytogenes*, listeriosis, virulence

© The Author(s) 2018. Published by Oxford University Press on behalf of the American Society of Animal Science.
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

Transl. Anim. Sci. 2019.3:304–314
doi: 10.1093/tas/txy110

[1]We acknowledge Dr. Brandy Roberts for her helpful insight into this project. This work was funded by the National Institutes of Health, award numbers P20GM103646 and P20GM103476.

[2]Present address: Department of Food Science and Technology, Oregon State University, Corvallis, OR.

[3]Present address: Department of Veterinary Biosciences, The Ohio State University, Columbus, OH 43210.

[4]Corresponding author: janet.donaldson@usm.edu

Received July 10, 2018.
Accepted October 3, 2018.

INTRODUCTION

Listeria monocytogenes is a foodborne pathogen responsible for causing listeriosis in immunocompromised individuals, in addition to stillbirth or abortion in pregnant women (Farber and Peterkin, 1991; Kathariou, 2002; Ramaswamy et al., 2007; Gould et al., 2013). This Gram-positive, facultative anaerobe causes approximately 1,600 illnesses and 260 deaths each year in the United States alone (Ramaswamy et al., 2007; Scallan et al., 2011; Gould et al., 2013). Individuals can develop listeriosis by consuming contaminated foods, including fruit, cheese, ice cream, and ready-to-eat meats.

Anaerobic conditions have been shown to influence the virulence potential of *L. monocytogenes*. This bacterium, as well as other foodborne pathogens, is exposed to anaerobic conditions during food processing, storage in vacuum sealed containers, and the infectious pathway (Lungu et al., 2009). Research has previously indicated that *L. monocytogenes* have an increase in survival in acidic bile under anaerobic conditions in comparison to aerobic environments (White et al., 2015). In addition, virulence has been shown to increase in *L. monocytogenes* strain Scott A when cultured under oxygen-restricted conditions in a guinea pig model (Bo Andersen et al., 2007). Therefore, the goal of this study was to determine the impact that anaerobiosis has on the virulence of *L. monocytogenes* and what changes the infection elicits on the gut microbiome. Dysbiosis within the intestinal microbiome has been found to be linked to the severity of several infections, including *Clostridium difficile*, and inflammatory bowel disease (Hawrelak and Myers, 2004; Brandt, 2012; Bien et al., 2013). Therefore, this study used the Illumina sequencing platform to study the bacterial profile alteration after being infected with aerobically or anaerobically cultured *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial Cultivation Conditions

Listeria monocytogenes strain F2365 was routinely cultured in tryptic soy broth (TSB) at 37 °C under either aerobic or anaerobic conditions. Anaerobic conditions were achieved through cultivating F2365 in an anaerobic chamber (Coy Lab Products, Grass Lake, MI); oxygen concentration was monitored throughout the incubation period using an oxygen detector present in the anaerobic chamber. Inocula used for the animal challenges were prepared from either aerobically or anaerobically grown cultures. Briefly, overnight cultures of F2365 grown either aerobically or anaerobically at 37 °C in a shaking incubator were washed two times with phosphate-buffered saline (PBS) and resuspended in PBS with 10 mg/mL calcium carbonate to a concentration of 1×10^6 or 1×10^8 colony forming units (CFU)/mL. For selection of *L. monocytogenes*, fecal and intestinal samples were cultured onto *Listeria* selective agar base with modified *Listeria* selective supplement (Oxoid SR0206).

Animals

A total of 29 female Mongolian gerbils aged 5 wk were purchased through Charles River Laboratories. All animal studies were approved by the Mississippi State University IACUC (protocol #15–042). Animals were acclimated to the animal facility for 3 d prior to infection. Animals were provided oral doses in 0.5-mL volume using a bulbous-ended feeding needle: 5×10^6 CFU aerobic or anaerobic culture and 5×10^8 aerobic culture per gerbil (n = 5 per group). The second study involved the high anaerobic dose of 5×10^8 CFU per gerbil (n = 7). PBS was used as the control for both studies (n = 5 for first study, n = 2 for second study). Fecal samples (40 mg) were collected daily from individual gerbils and homogenized in 0.4 mL of PBS. Five days post-challenge, gerbils were euthanized by CO_2 inhalation and gastrointestinal (GI) contents were collected for assessment of *Listeria* viability. Samples were homogenized using a Bead Mill homogenizer (Fisher Scientific, Hampton, NH) with 0.1-mm zirconium beads and subsequently diluted in PBS and plated onto *Listeria* selective media. Plates were incubated at 37 °C for 24 h prior to analysis. Samples for histological examinations were immediately placed in 10% formalin. Cecal contents were also collected for microbial community analysis.

Statistical analyses for bacterial loads were conducted using the GLIMMIX procedure of SAS (v.9.4, SAS Institute, Cary, NC) with the fixed effects of treatment, tissue, time, and their interactions. Data were log transformed prior to analysis to achieve normality. Means were separated using the LSMeans at an alpha of 0.05 utilizing the Tukey option.

Cecal DNA Extraction and Library Construction

DNA was isolated from cecal contents (0.2 g) using a QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) with minor modifications (Park

and Ricke, 2015). Isolated DNA concentration was measured using a Qubit 2.0 Fluorometer (Life Technology, Carlsbad, CA). Libraries were constructed using cecal DNA (10 ng), targeting the V4 region of 16S rRNA as described previously (Kozich et al., 2013). Briefly, DNA was amplified using dual-index primers via PCR and normalized amplicons using a SequalPrep Normalization Kit (Life Technology) according to the manufacturer's recommendation. Normalized samples (5 µL) were combined to generate a pooled library for further assays. Both library concentration and an exact product size were measured using a KAPA Library Quantification Kit (Kapa Biosystems, Woburn, MA) and an Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, CA) prior to being subsequently diluted to 4 nM.

Microbiome Sequencing via Illumina MiSeq Platform

A pooled library (20 nM) and a PhiX control v3 (20 nM; Illumina) were mixed with 0.2 N NaOH and HT1 buffer (Illumina) to produce the final concentration at 12 pM each. The resulting library was mixed with the PhiX control v3 (5%, v/v; Illumina), and 0.6 mL was loaded on a MiSeq v2 (500 cycle) reagent cartridge for sequencing. All sequencing procedures were monitored through the Illumina BaseSpace website. Demultiplexed R1 and R2 sequencing reads (approximately 250 bp) were acquired from the Illumina BaseSpace website, and data were processed using the QIIME pipeline (v. 1.9.0) for alpha diversity (rarefaction curve for OTUs, Chao1, and PD_Whole_Tree) and beta diversity using weighted and unweighted UniFrac distance (Caporaso et al., 2010). The clustered sequences were utilized to construct Operational Taxonomic Units (OTUs) with 97% identity, and representative sequences were classified into the respective taxonomical level based on the Greengene 16S rRNA gene database. Using the QIIME scripts, the core microbiome was estimated at 50% or 90% (i.e., bacteria present in at least 50% or 90% of samples). The multiresponse permutation procedures (MRPP) and principle component analysis (PCA) were estimated using BiodiversityR (vegan package) in R-statistical software package (Kindt and Coe, 2005). The community data sets of bacterial genera were log transformed before running both MRPP and PCA. All FASTAQ files can be found at the following link: https://doi.org/10.6084/m9.figshare.5046955.v1.

Histology

Intestinal samples were fixed in 10% formalin, routinely processed, sectioned at 5 µm, and stained with hematoxylin and eosin. Slides were subsequently evaluated by a boarded pathologist blinded to the origin of samples.

RESULTS

Intestinal Presence of L. monocytogenes *Differed between Gerbils Provided an Aerobic or Anaerobic Dose*

As previous studies indicated anaerobiosis increased the ability of *L. monocytogenes* to resist stressors in vitro (Payne et al., 2013; White et al., 2015), we aimed to conduct an exploratory study to determine whether cultivating *L. monocytogenes* under anaerobic conditions increased bacterial survival within the GI tract in vivo. Gerbils that were inoculated with a low aerobic or anaerobic dose of F2365 had no significant weight loss following the challenge in comparison to day 0 (Figure 1). However, comparing the body weights between the two treatment groups did indicate that there were significant differences ($P < 0.05$). Within 3 d after infection, the body weights were significantly different between gerbils provided an aerobic dose in comparison to those that were provided an anaerobically prepared dose. Gerbils that received a high aerobic dose of F2365 began to have a significant reduction in weight 2 d post-challenge ($P < 0.001$, Figure 1). The reduction was different from that observed for the gerbils that received a low aerobic dose ($P < 0.05$), but were not significantly different than those that received a high aerobic dose ($P > 0.05$) for all days analyzed.

Fecal samples were collected daily to monitor the intestinal load of *L. monocytogenes*. There was an overall effect for the treatments ($P < 0.001$). Interestingly, there was not a significant difference

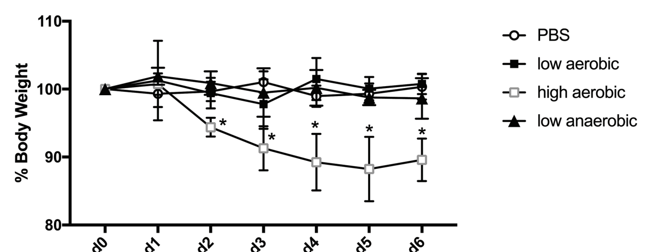

Figure 1. Average weight of gerbils following infection with *Listeria monocytogenes*. Body weights (g) were collected daily (days 0–6) from gerbils challenged with PBS control (○), 5×10^6 aerobically cultured (■), 5×10^8 aerobically cultured (□), or 5×10^6 anaerobically cultured (▲) *L. monocytogenes*. Body weights are presented as a percentage of weight from day 0 prior to beginning of study ($n = 5$ per group), with error bars representing the standard error. *$P < 0.001$.

in an overall comparison between low anaerobic and high aerobic doses, but there was a significant difference overall between the low aerobic and the high aerobic doses. Gerbils that were inoculated with a low anaerobic dose or a high aerobic dose had a greater number of animals still positive with *L. monocytogenes* within the feces 5 d after the infectious dose was administered (Figure 2). Though the dose was the same as that given for the anaerobically grown bacteria, there were less gerbils still positive for *L. monocytogenes* after 5 d of receiving the aerobically cultured bacteria (Figure 2). Gerbils provided either a low or high aerobic dose had significant differences in their bacterial loads on days 3 and 4 ($P < 0.05$). Comparing the bacterial load identified in the feces for gerbils that received a low aerobic or low anaerobic dose was only statistically significant on day 2 ($P = 0.04$), though a trend in differences was evident for the other days tested ($P \leq 0.1$).

The duodenum, cecum, colon, liver, and spleen were harvested after the study (day 6) and cultured for the presence of *L. monocytogenes*. *Listeria* was isolated from the duodenum from three of the five gerbils challenged with a high aerobic dose (5×10^8 CFU/dose). However, *L. monocytogenes* was isolated from only one gerbil inoculated with the low (5×10^6 CFU/dose) aerobic and anaerobic doses (Figure 3). Infecting gerbils with a low anaerobic dose resulted in a slight trend in an increase in the presence of *L. monocytogenes* in the cecum in comparison to the gerbils infected with the low aerobic dose ($P = 0.09$; Figure 3). Interestingly, *L. monocytogenes* was only isolated from the liver in gerbils inoculated with *L. monocytogenes* cultivated aerobically, but not anaerobically (Figure 3). *Listeria* was only identified from the spleen of one gerbil infected with a low anaerobic dose (data not shown).

Sections of the livers, gallbladders, spleens, and ilea of challenged and control gerbils were processed after the study for histological examination. Histologically, there was no significant difference in the ileum when challenged with *L. monocytogenes.* Challenged gerbils had multifocal to locally extensive hepatocellular necrosis with infiltration of neutrophils (Figure 4). Gram-positive bacteria were detected by Gram stain within the areas of necrosis. There were no significant histologic changes noted in the gallbladder or spleen of infected gerbils.

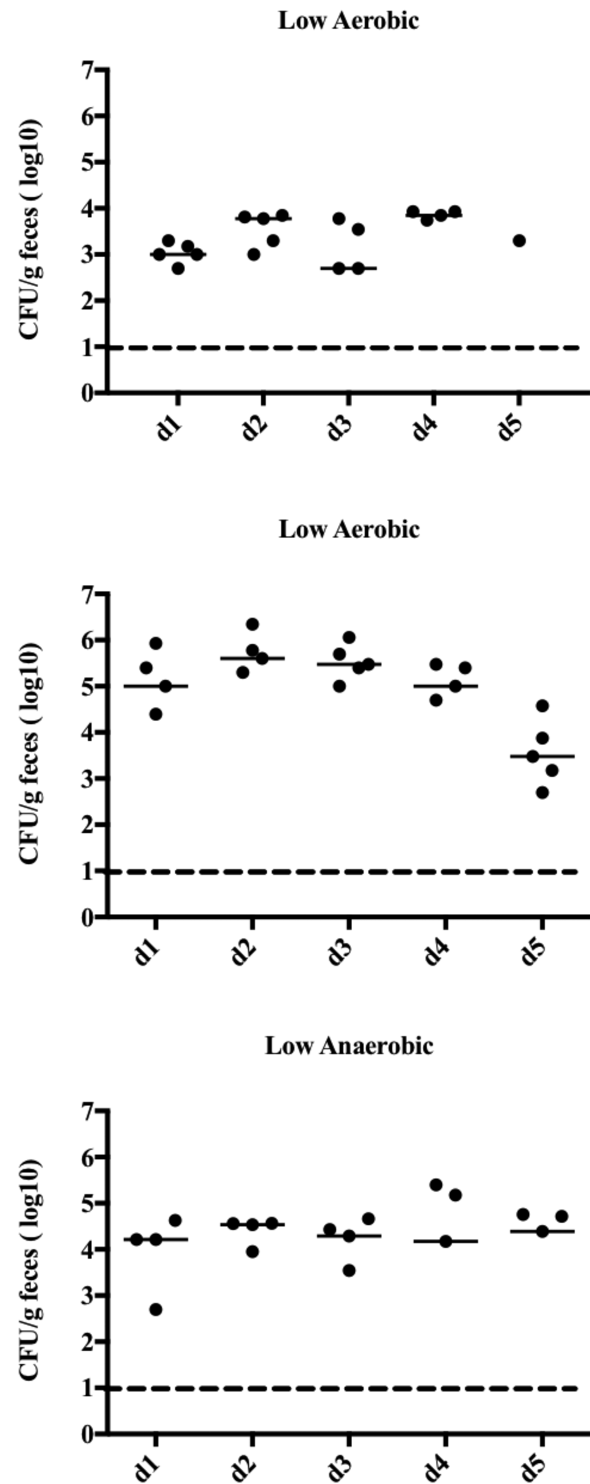

Figure 2. Fecal shedding of gerbils infected with *Listeria monocytogenes*. Each graph represents the individual CFU/g feces ($\log_{10}$) collected from five gerbils from day 1 to the conclusion of the study on day 5 for each treatment group. Lines represent the median of *L. monocytogenes* for each group; dotted lines indicate limit of detection.

Gerbils Challenged with a High Anaerobic Dose of **L. monocytogenes** *Exhibited Greater Morbidity and Mortality*

As the data presented above indicated exposure to anaerobic conditions prior to infection enhanced the ability of *L. monocytogenes* to survive within the cecum, an additional exploratory study to determine the impact that a high anaerobic dose had on gerbils was performed. Two gerbils, however, did not survive the study (Table 1). Body weights and fecal samples were collected daily from

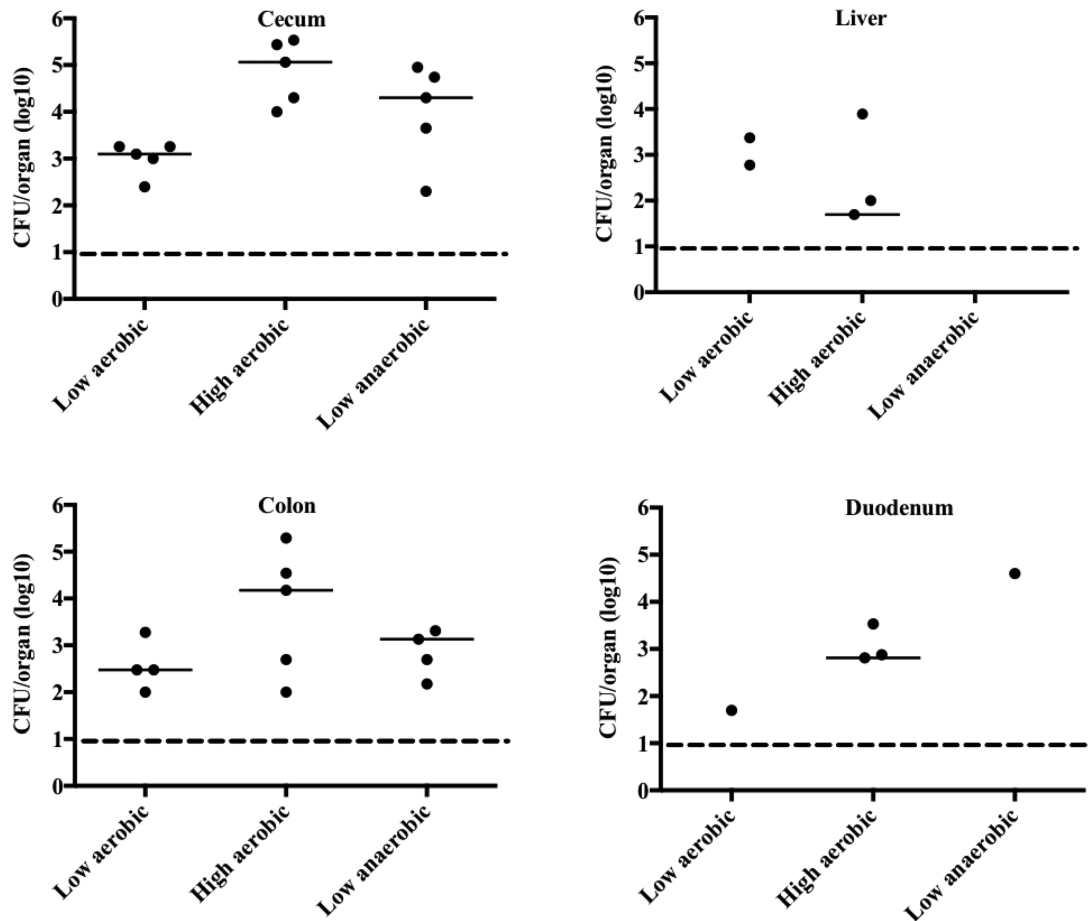

Figure 3. Bacterial load of *Listeria monocytogenes* in the duodenum, cecum, colon, and liver. Intestinal samples were collected from each gerbil from each treatment group. Each graph represents individual intestinal samples ($n = 5$ per group). Lines represent the median for each group; dotted lines indicate limit of detection.

gerbils inoculated with 5×10^8 CFU of *L. monocytogenes* cultivated anaerobically (Figure 5). A significant decline in body weight was observed within 2 d post-challenge (Figure 5). The decline in weight observed by day 2 for the gerbils provided the anaerobic high dose was more severe than that observed by animals provided the aerobic dose ($P = 0.001$). Bacterial load detected in the feces remained, on average, at 5 $\log_{10}$ 5 d post-infection. This contrasted with animals provided the equivalent aerobic dose, which had a 2 $\log_{10}$ decline in the population by day 5. However, the overall bacterial load by day 5 within the feces was significantly greater for animals provided the high anaerobic dose in comparison to the other treatments previously provided. Comparing the body weights of animals provided high aerobic to those that received a high anaerobic dose was not statistically significant.

Analysis of the intestinal content indicated that anaerobic cultivation increased the ability of *L. monocytogenes* to survive in the cecum, colon, and liver. On average, the bacterial loads detected in the cecum, colon, and liver were all greater in gerbils that received an anaerobic dose of 5×10^8 CFU in comparison to those that received an aerobic dose of the same amount (Figure 3 vs Figure 5). There were overall effects observed due to the treatments ($P < 0.001$). Interestingly, there was not a significant difference between low anaerobic and high aerobic doses, but there was a difference overall between the low aerobic and the high aerobic and the high anaerobic doses. Significant differences were also observed between high anaerobic treatments compared with low anaerobic ($P = 0.009$) and low aerobic ($P = 0.004$). Liver loads were significantly increased in animals provided a high anaerobic dose in comparison to all other treatments provided.

Histological examination of one gerbil challenged with the high anaerobic dose had a necrosuppurative typhlitis with large numbers of bacteria within the lumen of the cecum and extended into the mesentery (Figure 6). However, it is important to keep in mind that two of the seven gerbils that were challenged with the high anaerobic dose did not survive to the completion

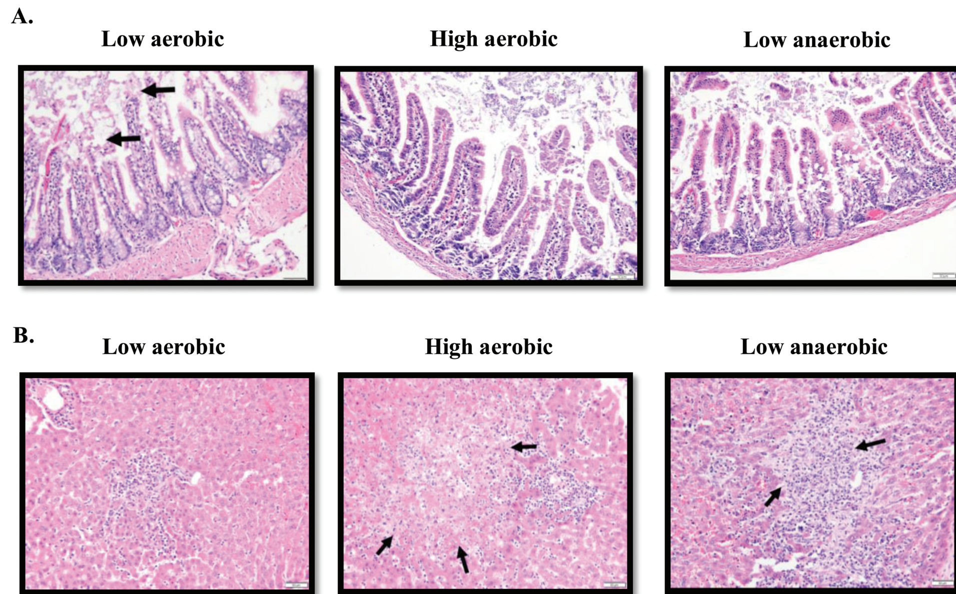

Figure 4. Images of liver histology from gerbils inoculated with a low aerobic, high aerobic, and low anaerobic dose of *Listeria monocytogenes*. Arrows indicate necrosis and neutrophil infiltration. Images in (B) are higher magnifications of regions depicted in (A).

Table 1. Survival of gerbils following challenge with various doses of F2365

Challenge dose CFU/gerbil	Number of deaths within 6 d/number of inoculated
PBS	0/7
Aerobic 5×10^6 (low)	0/5
Aerobic 5×10^8 (high)	0/5
Anaerobic 5×10^6 (low An)	0/5
Anaerobic 5×10^8 (high An)	2/7

of the study. This indicates that the anaerobic cultivation of *L. monocytogenes* increased the ability of the bacteria to survive within the intestinal tract and potentially increased the virulence capability of the bacteria.

Community Sequencing Suggested a Possible Shift in Microbiota between Challenged and Control Gerbils

As the cecum appeared to be the most heavily *L. monocytogenes* populated area of the intestine (Figure 3 and 5), cecal samples (19 total) were harvested after the study for microbial community analysis to understand how a *L. monocytogenes* infection influences the normal microbiota in the intestinal tract (Figure 7). Sequencing and data processing were accomplished by Illumina MiSeq and QIIME pipeline software (Caporaso et al., 2010). Sequencing reads (1,083,000 total reads; 57,000 per sample) were obtained after the necessary curation and filtering of low sequence count samples of FASTQ files. The relative abundance of bacterial genera was identified in at least 50% of samples across the ceca of control and challenged gerbils (Figure 7). Overall, the four major phyla that were identified included Bacteroidetes, Firmicutes, Spirochaetes, and Verrucomicrobia (98% of total reads).

The Bacteroidetes phylum increased approximately 2-fold in the cecum of gerbils provided the high anaerobic dose of *L. monocytogenes* in comparison to those that were provided PBS. Bacteroidetes were detected at a mean of 20.8% in PBS-treated gerbils; this increased slightly to 24.2% and 24.4% in gerbils provided the low or high aerobic dose, respectively. However, the abundance of Bacteroidetes increased to 33.6% and 40.6% in the cecum of gerbils provided either the low or high anaerobic dose of *L. monocytogenes*, respectively. The abundance of Firmicutes was 32.5% of the reads from gerbils provided PBS. This decreased slightly to 28.6% and 26.6% in gerbils provided either a low or high aerobic dose, respectively. Surprisingly, the abundance of Firmicutes reads decreased to 23.3% in gerbils provided the low anaerobic listerial treatment, but increased to 41% in gerbils treated with the high anaerobic dose. The abundance of Spirochaetes (*Treponema* genus) sequence reads was 27.9% in gerbils provided PBS; this increased slightly in the low aerobic dose, but increased to 47.9% in the high aerobic dose. For the anaerobic dose, there was a decrease to 17.2% and 2.7% for the low and high doses, respectively. The abundance of Verrucomicrobial reads (*Akkermansia* genus) was 16.8% in gerbils

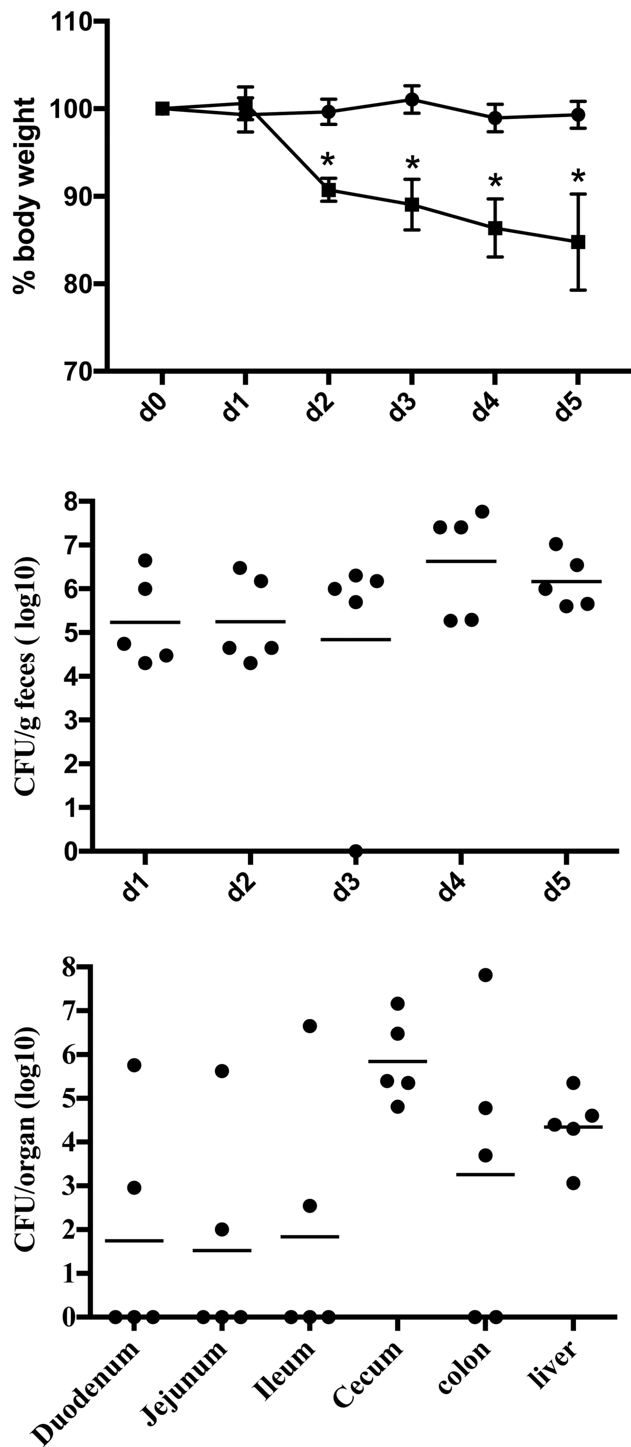

Figure 5. Body weights, fecal loads, and intestinal presence from gerbils challenged with a high dose of anaerobically cultured *Listeria monocytogenes.* Gerbils (n = 7) were challenged by oral gavage with 5×10^8 CFU cultured anaerobically. Body weight and fecal samples were collected daily from day 0 to day 5, with intestinal samples collected at day 6. One gerbil was euthanized on day 4, and a second gerbil was euthanized on day 5. Graphs represent individual gerbils, with lines representing the geometric mean. Intestinal data represent samples collected from animals that survived to day 6.

provided PBS or the low aerobic dose. However, this decreased to 0.1% abundance in gerbils provided the high aerobic dose. For the gerbils provided the anaerobic doses, the abundance of Verrucomicrobial reads was 22.7% and 13.8%, respectively (Figure 7).

At the genera level, the abundance of *Bacteroides* reads was 0.6% in gerbils provided PBS; the abundance did not change when gerbils were provided either dose of the aerobic culture. An unknown genus was detected within the Bacteroidetes that increased from 19.6% to 22.8% and 24% when provided an aerobic low or high dose, respectively. However, the abundance increased to 32% and 30.1% in gerbils provided the low and high anaerobic dose, respectively (Figure 8). Within the Proteobacteria, the abundance of *Desulfovibiro* was 0.8% in gerbils provided PBS, but increased to 2% and 1% in low and high aerobic infections, respectively. For the low anaerobic doses, the abundance increased to 2.5%. Within the Firmicutes, the abundance of *Lactobacillus* reads was 12.2% in gerbils provided PBS. This abundance decreased to 6% (low aerobic), 10.9% (high aerobic), 11.5% (low anaerobic), and 10.4% (high anaerobic). An unknown genus from the Firmicutes phylum and Clostridiales order increased to 13.8% and 12.4% in low and high aerobic listerial-infected ceca compared with that of 9% in PBS-infected gerbils. This increased to 19.6% in gerbils provided the high anaerobic dose.

The principle coordinate analysis of the bacterial profiles across the differential treatments indicated that the high dose of anaerobic *L. monocytogenes* clustered differently when compared with the other treatments (Figure 9). Three-dimensional PcoA plot showed 15.77%, 10.22%, and 7.12% of the total variation based on axes 1, 2, and 3, respectively, as estimated by an unweighted unifrac distance matrix (Figure 9).

DISCUSSION

The GI tract is designed to not only digest food and absorb the resulting nutrients for nutritional value, but also protect the body from harmful pathogens. Pathogens that are ingested encounter harsh conditions including low pH in the stomach, bile secreted by the gallbladder, and varying oxygen levels in the intestines. *Listeria monocytogenes* can survive these environments by regulating genes related to stress response mechanisms. This exploratory study specifically focused on the pathogenicity of *L. monocytogenes* F2365 in an anaerobic environment. The hypothesis was that oxygen deprivation influences localization of *L. monocytogenes* in vivo. We utilized gerbils for this model as the gerbil has been proposed to be a more appropriate animal for studying listeriosis due to possession

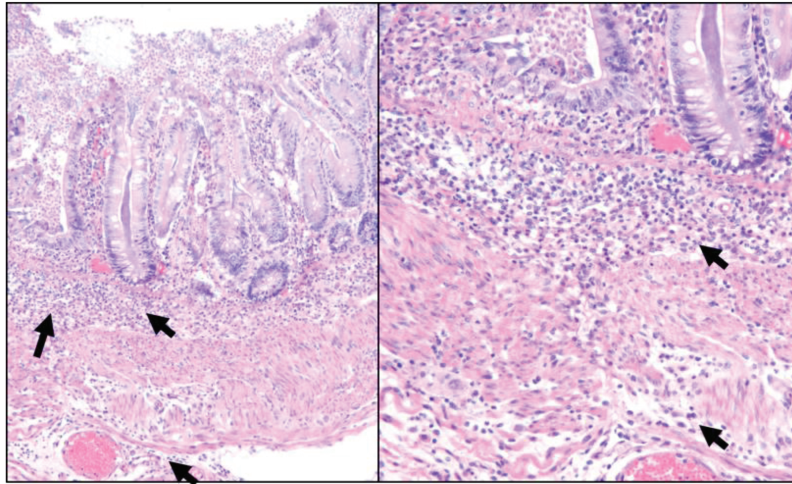

Figure 6. Cecal damage induced in gerbils challenged with a high dose (5×10^8 CFU/dose) of anaerobically cultured *Listeria monocytogenes*. Transmural inflammation was observed that extended into the mesentery, with infiltration of neutrophils, eosinophils, lymphocytes, and macrophages. Image on right is higher magnification of region depicted on left.

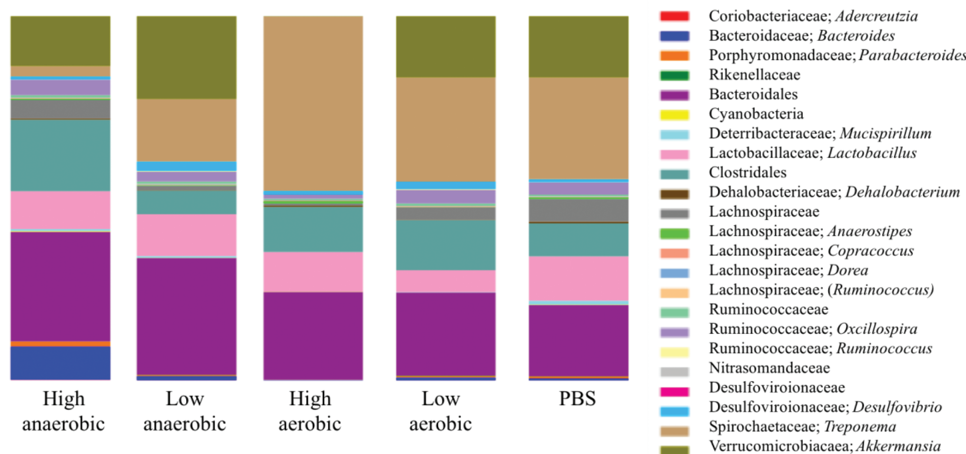

Figure 7. The bacterial taxa (genera) abundances in the cecum of gerbils challenged with low or high doses of aerobically or anaerobically grown *Listeria monocytogenes*. Ceca were collected and microbial communities were analyzed by 16S rRNA gene sequences using the Illumina platform. SH85 represents the gerbil that died on day 4; SH87 represents the gerbil that died on day 5. The core bacterial genera were estimated at 90% (OTUs that are present in at least 90% of samples) and averaged based on the treatment groups.

of receptors for both Internalin A and Internalin B (D'Orazio, 2014). Though natural variations can occur within the presence of such internalins in *L. monocytogenes*, such as the truncated InlB in F2365 used in this study (Nightingale et al., 2007), this study was nevertheless informative to the role that oxygen deprivation has on dissemination of *L. monocytogenes*.

Oxygen availability throughout the GI tract varies from high oxygen concentrations found in the stomach to low oxygen concentrations in the large intestines (Albenberg et al., 2014). Previous research has suggested exposure to low oxygen can affect the virulence capability of enteric pathogens, including *Salmonella*, *Shigella*, and *Vibrio cholera* (Burkholder et al., 2009). This current study indicated that localization of *L. monocytogenes* F2365 differed in gerbils when exposed to anaerobic conditions prior to challenge, which may affect the virulence potential.

Genes that are regulated in oxygen-deprived environments are speculated, but remain largely unknown. Bo Andersen et al. (2007) suggested oxygen deprivation resulted in an upregulation of InlA, therefore increasing colonization of *L. monocytogenes*. In addition, Burkholder et al. (2009) also showed evidence of increased expression of *Listeria* adhesion proteins, which increased adhesion and translocation of *L. monocytogenes* in oxygen-deprived conditions in both in vitro and in vivo models. It is possible that environmental stressors, including low oxygen, upregulate virulence genes that are needed to survive in other stressors including low pH and bile salts. This could

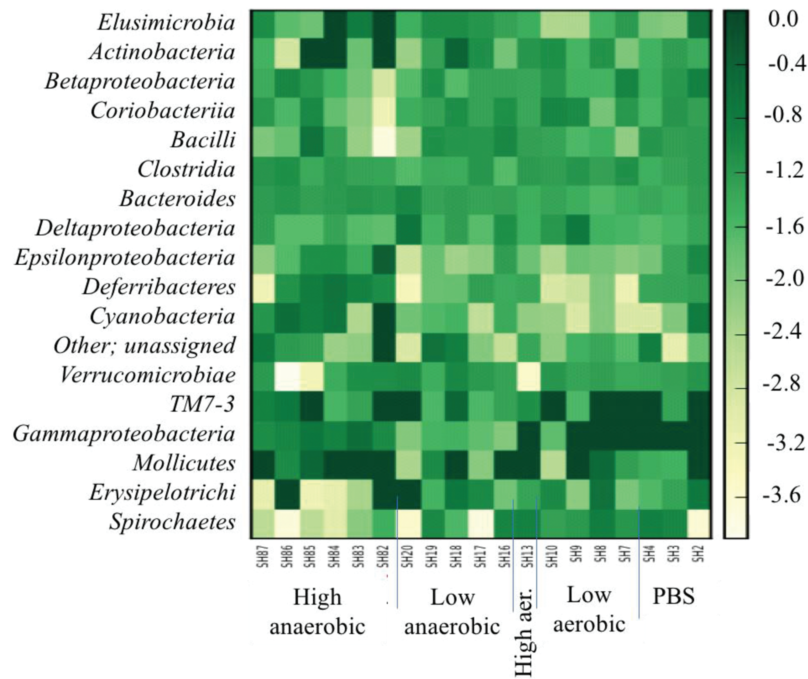

Figure 8. The heat map representing bacterial OTU abundance profile across the low or high aerobic and anaerobic *Listeria monocytogenes* challenged gerbils' ceca. OTUs that are present in at least 50% of the samples were used in abundance estimation. The OTU table was clustered based on UPGMA hierarchical clustering.

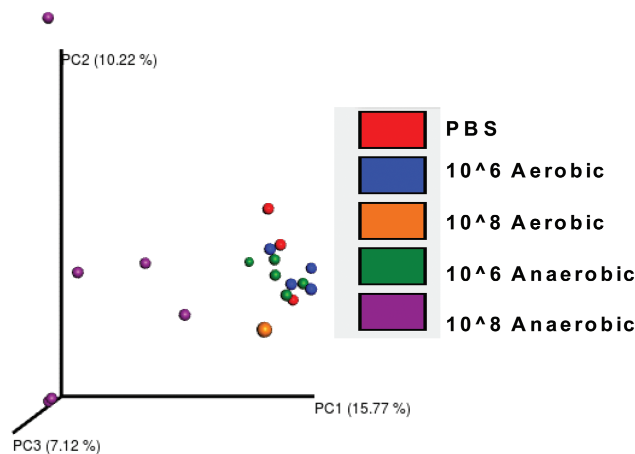

Figure 9. Principle coordinate analysis of bacterial OTUs across the low or high aerobic and anaerobic *Listeria monocytogenes* challenged gerbils' ceca. The ordination plot (PCA) was estimated from distance matrix of unweighted OTUs across the experimental groups.

possibly explain why Sewell et al. (2015) observed higher acid tolerance and survival in *L. monocytogenes* J0161 in anaerobic conditions. Gene regulation of most virulence factors are regulated by the transcriptional factors *prfA* and σ^B. It is possible that a single stressor upregulates additional virulence factors associated with expression from *prfA* and σ^B transcription. In addition, our laboratory observed strain-specific bile resistance in oxygen-restricted conditions (White et al., 2015). Resistance was associated with differential protein expression including proteins involved in the SOS response leading to DNA repair, in addition to proteins involved in invasion and metabolism (Wright et al., 2016). Further evaluation is needed to understand and determine specific genes involved in increased infectivity and resistance of *L. monocytogenes* in oxygen-deprived conditions.

Significant weight loss was associated with gerbils that were infected with the high aerobic or anaerobic dose of F2365. Though the decline in weight was similar between the two infected groups, the presence of *L. monocytogenes* in the feces differed. With animals provided the aerobic dose, a decline in fecal presence began to occur after day 4. However, the presence increased at day 4 and continued to remain elevated at day 5 post-challenge for gerbils infected with anaerobically cultured *L. monocytogenes*. Interestingly, though no significant weight loss was observed with the lower anaerobic dose, the fecal presence remained steady throughout the study. This suggests that even though an elevated infectious dose is required to significantly influence weight loss, the prior exposure to anaerobic conditions can influence the survival of *L. monocytogenes* within the host.

A significant difference was observed in the bacterial loads within the liver for animals that were provided the high anaerobic dose. Proliferation of *Listeria* in the liver is critical for the initiation of listeriosis (Vazquez-Boland et al., 2001). After invasion in the liver, *L. monocytogenes* may then

disseminate to the secondary target organs, including the brain and the uterus. As the loads within the GI tract and feces were also higher in this treatment group, these results suggest that the high anaerobic dose may prime the bacteria to survive the GI tract stressors and cross to the liver.

An important aspect of our study was to examine the impact an infection had on the normal GI tract microbiota. Understanding how *L. monocytogenes* influences the microbiome could shed light on the relationship between GI tract ecology and pathogenesis of this organism. The role of the natural GI microbiota has been shown to be vital for protecting a wide range of hosts against potentially harmful pathogens. Shifts in the GI tract microbiome can be caused by host diet, antibiotics, or infection and can potentially lead to long-term diseases (Fujimura et al., 2010). Our results indicated that *L. monocytogenes* exposed to anaerobic conditions prior to infection, such as what occurs within a food processing environment or packaging, enhances the probability for *L. monocytogenes* to resist the stressors encountered within the GI tract.

To determine the impact that *L. monocytogenes* had on the gut flora, cecal samples were collected and analyzed. The cecum had an approximate 1 $\log_{10}$ increase in the presence of *L. monocytogenes* in animals provided an anaerobic dose in comparison to an aerobic dose. Therefore, the community of the cecum was analyzed to determine the changes that were occurring within the community structure. We reported Bacteroidetes reads to be slightly increased in the ceca of aerobic dosed gerbils, whereas highly increased in anaerobic dosed gerbils (Figure 7, dark blue). Patients with inflammatory bowel disease frequently have *L. monocytogenes* reported in the microbiota and also have a reduced Bacteroidetes abundance (Bien et al., 2013). The increase observed in the abundance of Bacteroidetes and Parabacteroidetes in anaerobically dosed gerbils probably was in response to the digestion of complex glucose molecules (Martens et al., 2009). *Akkermansia*, belonging to Verrucomicrobia, is a Gram-negative, strictly anaerobic, non-motile, nonspore-forming bacterium and was found to remain similar in PBS controls and in gerbils provided either low dose; however, the abundance was nearly depleted in both high aerobic and anaerobic dosed gerbils (Figure 7 olive green, and Figure 9). The *Akkermansia* were reported to be important in degradation of intestinal mucin (Derrien et al., 2004). Therefore, the reduction in these bacterial abundances suggested that mucin degradation was not needed. The *Bacteroides* and *Rumminococcus* were reported to cluster as enterotypes 1 and 3 and functionally assist the host in synthesizing biotin (vitamin B7) and heme. The human microbiome is dominated by phyla Firmicutes; this was observed in the ceca of the gerbils in this study. *Clostridium* genera reads increased with high doses of anaerobic *L. monocytogenes* (Figures 5 and 7 teal green). The dominance of the *Clostridium*, *Bacteroides*, *Akkermansia* (strict anaerobe), *Lactobacillus* (facultative anaerobe), and *Treponema* (facultative anaerobe) in gerbils indicated that anaerobiosis is a discrete parameter that influenced the multidimensional scaling plot (Figure 8).

This study indicated that anaerobically cultured *L. monocytogenes* varied in the localization patterns in comparison to those animals provided aerobically cultured bacteria. In addition, the increase in morbidity observed suggests that exposure to anaerobic conditions prior to ingestion can increase the pathogenic potential of *L. monocytogenes*. Analyzing how the microbiome shifts observed influence the outcome of disease requires further research. Furthermore, understanding how *L. monocytogenes* influences the microbiota of individuals at various ages could provide valuable additional information on the virulence of this pathogen.

LITERATURE CITED

Albenberg, L., T.V. Esipova, C.P. Judge, K. Bittinger, J. Chen, A. Laughlin, S. Grunberg, R.N. Baldassano, J.D. Lewis, H. Li, et al. 2014. Correlation between intraluminal oxygen gradient and radial partitioning of intestinal microbiota. Gastroenterology 147:1055–1063.e8. doi:10.1053/j.gastro.2014.07.020

Bien, J., V. Palagani, and P. Bozko. 2013. The intestinal microbiota dysbiosis and clostridium difficile infection: is there a relationship with inflammatory bowel disease? Therap. Adv. Gastroenterol. 6:53–68. doi:10.1177/1756283X12454590

Bo Andersen, J., B.B. Roldgaard, B.B. Christensen, and T.R. Licht. 2007. Oxygen restriction increases the infective potential of *Listeria monocytogenes* in vitro in caco-2 cells and in vivo in guinea pigs. BMC Microbiol. 7:55. doi:10.1186/1471-2180-7-55

Brandt, L.J. 2012. Fecal transplantation for the treatment of clostridium difficile infection. Gastroenterol. Hepatol. (N. Y.) 8:191–194.

Burkholder, K.M., K.P. Kim, K.K. Mishra, S. Medina, B.K. Hahm, H. Kim, and A.K. Bhunia. 2009. Expression of LAP, a seca2-dependent secretory protein, is induced under anaerobic environment. Microbes Infect. 11:859–867. doi:10.1016/j.micinf.2009.05.006

Caporaso, J.G., J. Kuczynski, J. Stombaugh, K. Bittinger, F.D. Bushman, E.K. Costello, N. Fierer, A.G. Peña, J.K. Goodrich, J.I. Gordon, et al. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7:335–336. doi:10.1038/nmeth.f.303

Derrien, M., E.E. Vaughan, C.M. Plugge, and W.M. de Vos. 2004. *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int. J. Syst. Evol. Microbiol. 54:1469–1476. doi:10.1099/ijs.0.02873-0

D'Orazio, S.E. 2014. Animal models for oral transmission of *Listeria monocytogenes*. Front. Cell. Infect. Microbiol. 4:15. doi:10.3389/fcimb.2014.00015

Farber, J.M., and P.I. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. 55:476–511.

Fujimura, K.E., N.A. Slusher, M.D. Cabana, and S.V. Lynch. 2010. Role of the gut microbiota in defining human health. Expert Rev. Anti. Infect. Ther. 8:435–454. doi:10.1586/eri.10.14

Gould, L.H., K.A. Walsh, A.R. Vieira, K. Herman, I.T. Williams, A.J. Hall, and D. Cole; Centers for Disease Control and Prevention. 2013. Surveillance for foodborne disease outbreaks – United States, 1998–2008. MMWR. Surveill. Summ. 62:1–34.

Hawrelak, J.A., and S.P. Myers. 2004. The causes of intestinal dysbiosis: a review. Altern. Med. Rev. 9:180–197.

Kathariou, S. 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. J. Food Prot. 65:1811–1829.

Kindt, R., and R. Coe. 2005. Tree diversity analysis: a manual and software for common statistical methods for ecological and biodiversity studies. Nairobi, Kenya: World Agroforestry Centre.

Kozich, J.J., S.L. Westcott, N.T. Baxter, S.K. Highlander, and P.D. Schloss. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq illumina sequencing platform. Appl. Environ. Microbiol. 79:5112–5120. doi:10.1128/AEM.01043-13

Lungu, B., S.C. Ricke, and M.G. Johnson. 2009. Growth, survival, proliferation and pathogenesis of *Listeria monocytogenes* under low oxygen or anaerobic conditions: a review. Anaerobe 15:7–17. doi:10.1016/j.anaerobe.2008.08.001

Martens, E.C., N.M. Koropatkin, T.J. Smith, and J.I. Gordon. 2009. Complex glycan catabolism by the human gut microbiota: the *Bacteroidetes* sus-like paradigm. J. Biol. Chem. 284:24673–24677. doi:10.1074/jbc.R109.022848

Nightingale, K.K., S.R. Milillo, R.A. Ivy, A.J. Ho, H.F. Oliver, and M. Wiedmann. 2007. *Listeria monocytogenes* F2365 carries several authentic mutations potentially leading to truncated gene products, including inLB, and demonstrates atypical phenotypic characteristics. J. Food Prot. 70:482–488.

Park, S.H., and S.C. Ricke. 2015. Development of multiplex PCR assay for simultaneous detection of *Salmonella* genus, *Salmonella* subspecies I, *Salm*. Enteritidis, *Salm*. Heidelberg and *Salm*. Typhimurium. J. Appl. Microbiol. 118:152–160. doi:10.1111/jam.12678

Payne, A., T.B. Schmidt, B. Nanduri, K. Pendarvis, J.R. Pittman, J.A. Thornton, J. Grissett, and J.R. Donaldson. 2013. Proteomic analysis of the response of *Listeria monocytogenes* to bile salts under anaerobic conditions. J. Med. Microbiol. 62:25–35. doi:10.1099/jmm.0.049742-0

Ramaswamy, V., V.M. Cresence, J.S. Rejitha, M.U. Lekshmi, K.S. Dharsana, S.P. Prasad, and H.M. Vijila. 2007. *Listeria* – review of epidemiology and pathogenesis. J. Microbiol. Immunol. Infect. 40:4–13.

Scallan, E., R.M. Hoekstra, FJ. Angulo, R.V. Tauxe, M.A. Widdowson, S.L. Roy, J.L. Jones, and P.M. Griffin. 2011. Foodborne illness acquired in the United States – major pathogens. Emerg. Infect. Dis. 17:7–15. doi:10.3201/eid1701.091101p1

Sewell, D., S.C.H. Allen, and C.A. Phillips. 2015. Oxygen limitation induces acid tolerance and impacts simulated gastro-intestinal transit in *Listeria monocytogenes* J0161. Gut Pathog. 7:11. doi:10.1186/s13099-015-0058-0

Vazquez-Boland, J.A., M. Kuhn, P. Berche, T. Chakraborty, G. Domínguez-Bernal, W. Goebel, B. González-Zorn, J. Wehland, and J. Kreft. 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. 14:584–640. doi:10.1128/CMR.14.3.584-640.2001

White, S.J., D.M. McClung, J.G. Wilson, B.N. Roberts, and J.R. Donaldson. 2015. Influence of pH on bile sensitivity amongst various strains of *Listeria monocytogenes* under aerobic and anaerobic conditions. J. Med. Microbiol. 64:1287–1296. doi:10.1099/jmm.0.000160

Wright, M.L., K. Pendarvis, B. Nanduri, M.J. Edelmann, H.N. Jenkins, J.S. Reddy, J.G. Wilson, X. Ding, P.R. Broadway, M.G. Ammari, et al. 2016. The effect of oxygen on bile resistance in *Listeria monocytogenes*. J. Proteomics Bioinform. 9:107–119. doi:10.4172/jpb.1000396