The growth response of *Campylobacter jejuni* to stress catecholamines

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Veterinary Microbiology

Program of Study Committee:
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Iowa State University

Ames, Iowa

2019

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ACKNOWLEDGMENTS

First and foremost, I would like to give my sincere thanks to my research supervisor Dr. Mark Lyte, who gave me the wonderful opportunity to work on this project and always being accessible to answer my questions. His insightful guidance, comments, and suggestions are indispensable for the fruitful outcome of this project. I would also like to thank Dr. Torey Looft and Dr. Yuko Sato for serving on my POS committee and for providing valuable comments on my thesis.

I would like to thank my co-workers: Daniel, Karrie, Sharon, and Ashley, for helping me using their expertise and making the lab a wonderful place to work in. I would also like to thank Dr. Matthew Sylte for his many revisions and insightful comments on the manuscript included in this thesis. I am also grateful to Dr. Zhang, Dr. Sahin, and Nada for their excellent advise on working with *Campylobacter jejuni*, which helped me greatly to start this project.

Finally, I would like to thank my parents for their continuous love and support. My gratitude is beyond words.

ABSTRACT

Campylobacter jejuni is the leading food-borne bacterial pathogen, which causes campylobacteriosis in human. The bacterium is usually carried asymptomatically in the intestinal tract of food-producing animals such as chicken, cattle, and porcine, which contributes to its transmission in the environment. Interventions in the colonization of this bacterium in food-producing animals is one of the promising ways to control for food-borne illness in humans. However, up to date, there has not been an effective measures developed that can successfully reduce the colonization level of this bacterium in food-producing animals. Microbial endocrinology is an interdisciplinary field of research that studies the interaction between gut microbiota and host through neurotransmitters. Studies in this field have provided novel understandings on the pathogenesis of many other food-borne bacteria such as pathogenic *Escherichia coli*, *Salmonella* spp., and *Vibrio* spp. Compared to them, little is known of *C. jejuni*, and the response of this bacteria to a major intestinal neurotransmitter, dopamine (DA), has not, to our knowledge been previously examined. The results of this study demonstrated that intestinal catecholamine neurotransmitters norepinephrine (NE) and DA can significantly enhance the growth of 3 strains of *C. jejuni* in a strain-dependent manner. Furthermore, the study identified a key factor, pyruvate, which involves in the DA-mediated but not NE-mediated growth stimulation in *C. jejuni*.

CHAPTER 1. GENERAL INTRODUCTION

1.1 Introduction

Campylobacter infection (Campylobacteriosis) is the leading bacterial food-borne illness worldwide. In the United States, it is estimated that *Campylobacter* infections affects more than 1.3 million people every year [1]. Among patients with Campylobacteriosis, *Campylobacter jejuni* is the most frequently isolated species [2]. *Campylobacter jejuni* infection in human is characterized by self-limiting gastroenteritis symptoms that are similar to infections caused by other food-borne bacteria such as *Salmonella* and *Shigella* species [3]. On rare occasions, *C. jejuni* infections can lead to a serious post-infectious complication, Guillain-Barré syndrome, an autoimmune disorder that leads to acute or subacute neuromuscular paralysis [4]. Unlike in humans, *C. jejuni* colonization in the gastrointestinal tract of food-producing animals is mostly asymptomatic, which contributes to its transmission through environmental sources that may be contaminated by the fecal shedding of *C. jejuni* by positive animals [5]. Thus, understanding what contributes to the growth of *C. jejuni* in the gastrointestinal tract of human and food-producing animals is important for developing effective control and prevention methods against campylobacteriosis.

Stress, whether it is physiological or psychological, is known to contribute to the development of gastrointestinal infections, as shown by numerous *in vivo* findings on both human [6, 7, 8]and food-producing animals [9, 10, 11, 12, 13]. One of the ways stress can contribute to the development of gastrointestinal infections is through the release of catecholamines during stress from enteric nervous system (ENS) nerve endings into the intestinal lumen, where the catecholamines can interact with enteric bacteria. Catechoalmines are biogenic amines that possess a 3,4-dihydroxyphenyl (catechol) moiety. Three catecholamines, including epinephrine (EPI), norepinephrine (NE), and dopamine (DA), are found *in vivo*, where they serve as neurotransmitters and hormones that are

important for stress responses [14, 15]. Considerable amount of progress has been made on understanding the effect of catecholamines on bacteria in both *in vivo* and *in vitro* studies, where catecholamines were shown to significantly enhance both the growth and the virulence of a variety of pathogenic bacteria [16, 17]. The two main stress catecholamines in the intestinal lumen are norepinephrine (NE) and dopamine (DA), both of which are also important neurotransmitters in the ENS. Although the effects of NE and DA have been extensively studied in food-borne bacteria such as pathogenic *Escherichia coli*, *Vibrio* spp., and *Salmonella* spp., the effects of DA on *C. jejuni* have not been previously examined.

Previous work has demonstrated that the inclusion of NE and epinephrine (EPI) into iron-restricted medium significantly enhanced the growth and virulence of *C. jejuni* strain NCTC 11168 [18, 19]. This project extended current findings on the effect of stress catecholamines on *C. jejuni* by adding DA into examination together with NE. By examining the growth response of three *C. jejuni* strains (NCTC 11168, 81-176, and ML2126) to NE and DA, this project revealed multiple key findings. In general, the inclusion of NE or DA into iron-restricted medium significantly enhanced the growth of *C. jejuni*. However, the response was strain specific, with *C. jejuni* strain 81-176 being less sensitive to both NE and DA than strain NCTC 11168 and ML2126. Furthermore, we identified a key factor, pyruvate, that is responsible for the DA-mediated but not the NE-mediated growth stimulation in *C. jejuni*, suggesting that different mechanisms are involved in the response of *C. jejuni* to NE and DA. Since this is the first time pyruvate, a bacterial glycolysis product generated in large amount in the intestine [20, 21], has been identified to modulate to response of enteropathogenic bacteria to catecholamines, we examined the role pyruvate plays in the process. These findings expanded our understanding on the catecholamine-stimulated growth in *C. jejuni*, which may contribute to the development of effective therapies or preventive measures against bacterial gastroenteritis through modulating intestinal stress catecholamines.

1.2 Thesis Organization

This thesis consists of three chapters. The first chapter is a literature review including a general overview of *C. jejuni* growth requirements; *C. jejuni* infections; *in vivo* and *in vitro* findings on the effect of stress on enteropathogenic bacteria; and the interaction between stress catecholamines and enteropathogenic bacteria. The second chapter describes a study on the effect of stress catecholamines, norepinephrine and dopamine on *C. jejuni* growth. The third chapter is the general conclusion.

1.3 Literature Review

1.3.1 Microbiology and Growth Requirements

C. jejuni is a gram-negative bacterium with a curve or spiral shape, belonging to the class Epsilonproteobacteria. Another well-known member within same group is *Helicobacter pylori*, which is the principal cause for some severe gastric and duodenal disorders in human such as chronic gastritis, gastric and duodenal ulceration, and gastric cancer [22]. The optimum growth of *C. jejuni* is maintained in lab under microaerophilic condition (85% N_2, 10% CO_2, and 5% O_2) and incubation temperature between 37°C and 42°C [23]. *C. jejuni* grow well in complex media such as Muller Hinton (MH), Brucella broth, and Brain heart infusion (BHI), as well as tissue-based culture media such as DMEM. A defined minimal medium, MCLMAN (Medium Cysteine Leucine Methionine Aspartic acid Niacinamide) is also developed for *C. jejuni* [24].

C. jejuni grows best under microaerophilic condition with oxygen tension (pO_2) of 2-10%. At high cell densities (usually more than 10^6 initial colony forming unit (CFU)/ml depends on the strain), the bacterium can also grow under fully aerobic conditions (pO_2 around 21%) [25]. However, at low cell densities, the optimum microaerophilic condition is required for *C. jejuni* to initiate growth [25]. The bacterium cannot grow anaerobically regardless of initial cell densities [25]. The microaerophilic nature of *C. jejuni* is believed to be due to the presence of iron-sulfur

essential enzymes in the bacterium that are sensitive to oxygen. For example, the citric acid cycle (CAC) in *C. jejuni* is especially sensitive to molecular oxygen and reactive oxygen species (ROS) due to the presence of two iron-sulfur enzymes pyruvate:ferredoxin oxidoreductase (POR) and 2-oxoglutarate oxidoreductase (OOR). POR carries out the entry of pyruvate into CAC, while OOR is responsible for decarboxylating 2-oxoglutarate into succinyl-CoA in CAC. The oxygen-sensitive POR and OOR are usually found in many obligate anaerobes but not aerobes, who utilize the oxygen-stable counterparts of these enzymes instead [26, 27]. Another important enzyme, serine dehydratase (SdaA), which is essential for the bacterium to colonize chicken intestine, is also an oxygen-sensitive iron-sulfur enzyme. Although *C. jejuni* can produce an oxygen-binding protein, hemerythrins to protect the two oxygen-sensitive oxidoreductase from aerobic exposure damage, there is a limit to the protection [28]. Once oxygen-stressed, the poor iron-sulphur cluster repairing mechanisms of *C. jejuni* resulted in prolonged recovering time for damaged Por and Oor, further contributing to the sensitivity of cells to oxygen exposure [28].

C. jejuni has a narrow growth temperature range from 30°C to 47°C, with optimal growth observed at 42°C. Although temperature change near the maximum and minimum growth temperatures significantly reduces the growth rate of the bacterium, less effect is observed when altering temperature within the range of 37°C to 42°C [29], indicating the bacterium is well suited for colonizing a variety of warm-blooded animals whose body temperatures are within this range. Furthermore, transient temperature change from 37°C to 42°C only alters *C. jejuni* global gene expression for less than 50 minutes, after which the gene expression patterns are essentially the same between the two growth temperatures [30]. These findings suggest that *C. jejuni* can rapidly adapt to altered temperatures, which contributes to the transmission of this bacterium among different reservoirs.

C. jejuni has a complete citric acid cycle (CAC) that it heavily relies on for energy [31]. Although being unable to use many common carbohydrates such as glucose and galactose, *C. jejuni*

can make use of amino acids and organic acids, the catabolic products of which are fed directly into the CAC. Amino acids are considered the most important carbon source for *C. jejuni* [32], who readily uses the amino acids aspartate, glutamate, serine, and proline, all of which are also found in high concentrations in chicken feces [33]. In complex amino acid-rich media, only these 4 amino acids are significantly depleted. L-serine is especially favored by *C. jejuni*, since the amino acid can be converted to a central metabolite, pyruvate, directly through serine dehydratase (SdaA). Although L-serine is not necessary for *C. jejuni* growth *in vitro*, the inactivation of SdaA rendered the bacteria unable to colonize chicken intestine [34].

Apart from amino acids, *C. jejuni* can also utilize CAC cycle intermediates such as pyruvate, succinate, and α-ketoglutaric acid (also known as 2-oxoglutarate or 2-OG), as well as other organic acids such as formic acid and lactic acid [35]. Formic acid (the salt form of formate), fumaric acid, α-ketoglutaric acid, succinate, and pyruvate are preferably used by *C. jejuni* compared with other organic acids such as citrate, propionate and acetate [36]. In a recent study, formate has been shown to modulate respiration and enhance important colonization phenotypes in *C. jejuni* [36]. Specifically, formate is not only a strong chemoattractant for *C. jejuni*, supplementation of Muller Hinton broth with physiologically relevant levels of formate, around 3 to 8 mM in mice ileum [37], was also shown to significantly enhance the growth, biofilm formation, and respiratory activity of the bacterium, with some of the effects being unique to formate [36]. The findings on formate suggest that metabolic byproducts generated by gut microbiota, other than serving as nutrient sources for *C. jejuni*, may also be important stimulus for its colonization.

Although having a rather fastidious growth requirement, *C. jejuni* survives well in a variety of environments where it is unable to grow. *C. jejuni* can survive for 3-5 days in fresh broiler feces [38], 1-8 days in water [39], and 56 days on retail chicken skin when stored at -20°C [40]. Furthermore, the bacterium also persists on retail chicken meat throughout usual storage time at 4°C (7 days), with little reduction in viable cell count [40, 41]. One intriguing factor that

contributes to the survival of *C. jejuni* on chicken meat is the presence of other bacteria species, for example *Pseudomonas* spp., a bacterium commonly found on chicken meat. It has been shown that the presence of *Pseudomonas* spp. significantly enhanced the aerotolerance of *C. jejuni* on chicken meat [42]. This finding is not entirely surprising, since *C. jejuni* is known for its ability to utilize and respond to metabolites produced by the gut microbiota such as short chain fatty acids and organic acids [43]. In general, lower temperature, higher humidity, microaeroplilic atmosphere, and the presence of other bacteria usually enhance the survival of *C. jejuni*. Once the survived *C. jejuni* gets access to the host intestine through consumption of contaminated food or water sources, they can start proliferating again to be transferred to other reservoirs.

1.3.2 The Iron Regulation in *C. jejuni*

Iron has limited availability to the gut microbiota. The major source of iron in the intestine is dietary. However, 5% to 15% of the dietary iron is taken up by the small intestine enterocytes. The rest of the iron is also subject to chelation by host iron-binding protein such as lactoferrin, which is secreted to the intestinal mucosa to further reduce the amount of iron accessible to the gut microbiota [44].

Apart from a few bacteria, such as *Lactobacillus* spp. and two obligate intracellular parasites, *Borrelia burgdorferi* and *Treponema pallidum*, that can grow without iron sources in most of the tested culture media [45, 46, 47, 48], the vast majority bacteria require iron as an essential nutrient. Iron is important for *C. jejuni* as it is required for the formation of iron-sulfur complexes that are present in the active site of many essential enzymes including those involved in amino acid and short-chain fatty acid utilization pathways and electron transport chain, all of which are crucial for the growth of *C. jejuni* [31].

C. jejuni can acquire iron in various ways including direct import of Fe^{2+} through a divalent metal transporter FeoB [49] or direct utilization of host iron-containing complexes such as heme

[50] and ferric-lactoferrin/transferrin (Lf/Tf-iron) [51]. It is noteworthy that the receptor (CtuA) mediated Lf/Tf-iron utilization alone in *C. jejuni* may not be sufficient for acquiring iron from these high-affinity iron binding proteins as shown by the fact that the bacterium is unable utilize the Lf/Tf-iron in agar culture [52]. Another way used by many bacteria to acquire Tf/Lf-iron is thorough the production and secretion of siderophores, a group of low molecular weight compounds that liberate iron from host proteins due to their higher affinity for ferric iron [44]. Siderophores can be categorized into three types according to the functional groups responsible for iron removal. They are catecholate, carboxylate, and hydroxamate siderophores. Among the three types of siderophores, catecholate siderophores such as enterobactin and salmochelin that are produced by bacteria of the family Enterobacteriaceae exhibit the highest affinity for iron under physiological pH, contributing to the growth of the bacteria in Lf/Tf-rich environment *in vivo* [44].

Most *C. jejuni* strains lack the ability to synthesize their own siderophores, including the two commonly used strains NCTC 11168 and 81-176. However, *C. jejuni* can utilize siderophores produced by other microorganisms such as enterobactin and ferrichrome [53, 54]. The mechanisms through which *C. jejuni* acquire enterobactin, the siderophore with the highest known affinity to ferric iron, have been well characterized. The acquisition of ferric enterobactin (FeEnt) starts with this complex binding to the outer-membrane receptors (CfrA, CfrB) of the bacterium, after which a functional TonB-ExbB-ExbD energy transduction system energizes the transportation of FeEnt across the outer-membrane [55, 56, 57]. Once inside the periplasm, the imported FeEnt can be either transported by an inner-membrane ABC transporter system, CeuBCDE, or being hydrolyzed by a periplasmic enzyme, Cee, both facilitate the entry of the iron source into cytosol [58].

In *C. jejuni*, impaired iron uptake system is usually associated with colonization defects. It has been demonstrated that *C. jejuni* mutants with non-functioning transporters of Fe^{2+} (FeoB), ferric-lactoferrin/transferrin (CtuA), or ferric-enterobactin (CfrA/CfrB) have significantly compromised ability to colonize live animals [49, 51, 56, 57]. However, mutants with impaired heme uptake

system failed to show colonization defects in chicken [50]. Therefore, iron sources such as Fe^{2+}, ferric-lactoferrin/transferrin, and ferric-enterobactin, but not heme may be limiting factors determining the proliferation of *C. jejuni* inside host intestine.

Although iron is an essential nutrient for most bacteria, uncontrolled intracellular iron level can lead to generation of the highly damaging hydroxyl radical through Haber-Weiss reaction where iron serves as a catalyst [46]. Therefore, in *C. jejuni* and other gram-negative bacteria, intracellular iron level is strictly regulated by the ferric-uptake regulator protein (Fur). Specifically, when iron is in excess, the binding of Fe^{2+} to Fur protein inhibits the expression of genes associated with iron acquisition, such as those involved in ferric-enterobactin, lactoferrin, and heme uptake [59, 60].

1.3.3 Pathogenesis and Clinical Manifestation

Although *Campylobacter* colonization in many food-producing animals are predominately asymptomatic, in human, the infection gives rise to acute, self-limiting gastroenteritis characterized by fever, abdominal cramps, and watery-mucous diarrhea, usually with the presence of leukocyte (75% of the cases) and/or blood (60% of the cases) as the result of intestinal inflammation [61, 62]. On rare occasions, campylobacteriosis can lead to its most serious complication: Guillain-Barré syndrome—a rare neurological disorder occurring 1 per 1000 infections [63]. *C. jejuni* is the principal cause of human *Campylobacter* infection contributing to more than 80% of the cases [64]. The minimal infection dose of *C. jejuni* in human ranges from several hundreds to several thousands cells [65], which is considered high compared with other food-borne bacteria that can be readily transmitted from person to person. Because of this, campylobacteriosis cases are seldom associated with outbreaks and are mainly sporadic.

Symptoms of campylobacteriosis in human is likely due to the invasion of colonic epithelial cells by *C. jejuni*, which, in chicken, is a less frequent event [66, 43]. Later studies further elucidated the pathogenisis of *C. jejuni* by showing that the bacterium can adhere to and translocate across

intestinal epithelial cell layer [67, 68], which consequently induce pro-inflammatory cytokines secretion [69, 70]. These cytokines can recruit neutrophils and macrophages which although contribute to the efficient clearing of the infection, can also result in cellular damage that gives rise to the characteristic inflammatory diarrhea during a *C. jejuni* infection [71].

1.3.4 Epidemiology

The thermofilic and mircoaerophilic nature of *C. jejuni* makes it unlikely to grow in places other than the intestine of warm-blooded animals. As a result, the major reservoirs of campylobacteriosis are the gastrointestinal tracts of poultry, cattle, sheep, and swine, where *Campylobacter* can present in high concentrations. It is noteworthy that *Campylobacter coli* is the main *Campylobacter* spp. isolated from swine, with *C. coli* isolated from more than 90% of the *Campylobacter* positive samples compared with *C. jejuni* which only present in 1% to 2% of the samples [72, 73].

Campylobacteriosis cases are usually sporadic in humans, caused by consuming or handling of under-cooked meat, unpasteurized milk, or untreated water that have been contaminated with animal manure [74]. It is widely accepted that poultry meat, especially chicken meat, is the most important source of campylobacteriosis, contributing to 38% to 77% of the infections [75]. Second in place is cattle, which contributed to 16% to 54% of the cases [75]. The importance of chicken meat to the disease is explained by the fact that *Campylobacter* is not only most frequently isolated from retail chicken meat [74, 76], but the bacterium also present at higher levels on chicken carcasses compared with other types of meat [74, 77]. For cattle and swine, although *Campylobacter* spp. are commonly isolated on farm, incidence of *Campylobacter* positive samples at retail are low (2.7% in beef and 2% in pork) compared with that of chicken (57.4%) [74]. Such difference is likely caused by the slower slaughtering process for swine and cattle which has more steps than poultry slaughtering process [74, 77]. Additional steps in red meat slaughtering process such as skin removal, flaming, chilling, and desiccation that can significantly reduce *Campylobacter* contamination level [77].

In poultry production cycle, a flock is usually *Campylobacter* negative at the beginning due to rarity of vertical transmission from parent to egg [76]. However, once the bacteria are introduced into the flock, they spread rapidly via contaminated fecal shedding, where they reach a high concentration of up to 10^8 CFU/g of cecal contents [76, 78]. This high contamination level usually persist throughout the production cycle and eventually be carried to slaughter facilities [76].

1.3.5 Modulation of Stress-Induced Bacterial Infections by Microbiota-Gut-Brain Axis

Evidence suggests the bidirectional connection between the gut and the brain. Development wise, the enteric nervous system (ENS) arises from precursor cells from neural crest, which, during embryo development, migrate along the vagus to the gut, forming enteric neurons and glial cells [79]. Signaling wise, the ENS can not only function independently utilizing its own intrinsic signaling pathways, it can also exchange signals with central nervous system (CNS) through the parasympathetic and sympathetic arms of the autonomic nervous system (ANS) [80].

The nerve connections between the CNS and the ENS form the physiological basis for bidirectional communication the brain and gut. Intestinal neurotransmitters released from the ENS nerve endings have been shown to modulated the growth and virulence of gut microbiota [81], meanwhile, metabolites produced by gut microbiota can also modulate the ENS [81]. Microbial endocrinology is an emerging field of research that studies such bidirectional communication between the host and the microbiota through neurotransmitters [81]. This interdisciplinary research area forms an experimental framework to study the microbiota-gut-brain axis, which provides a novel perspective to examine the pathogenesis of infectious diseases [82].

One branch of microbial endocrinology specifically studies the effect of stress on enteric bacteria, since stress is known to predispose animals to bacterial infections. Stress is defined as the effect of a stimuli that compromise homeostasis. The nature of the stimuli can be physiological or

psychological, and depends on the duration of the stress, it can be defined as acute or chronic. The stimuli that initiates stress is termed as the "stressor", and the response of an organism to the stressor is described as the "stress response". Brain is the central component of stress response pathway, which takes into account of the homeostatic information throughout the body and evaluates whether the animal is under stress [83]. Feedback from the brain is then passed down to the gastrointestinal tract, which may alter the living environment of gut microbiota [82, 84, 85, 86]. Considerable amount of progress has been made on understanding the effects of stress on gut microbiota, which can be divided into two main categories: *in vivo* and *in vitro* evidence.

1.3.5.1 *In vivo* Evidence on the Effects of Stress on Gut Microbiota

Numerous studies done in human, laboratory animals, and farm animals have shown that both physiological and psychological stress can alter gut microbiota, which usually result in increased susceptibility to pathogenic bacterial infection. In human, stress is a well-known trigger of inflammatory bowel disease (IBD) [6], the patients of which had altered microbiome composition including enriched *Escherichia coli*, *Clostridium clostridioforme*, and *Clostridium symbiosum* [7]. Similarly, maternal prenatal stress in human was associated with higher abundances of bacteria in Proteobacteria phylum in mothers, which includes many common enteric pathogenic bacteria such as *Escherichia coli*, *Salmonella* spp., and *Yersinia* spp. [8].

In mice, restrained stress was shown to result in overgrowth of both gram-negative and gram-negative aerobic bacteria, as well as reduced microbial richness [9]. Furthermore, after orally challenged with *Citrobacter rodentium*, a murine enteric pathogen, a nearly 10,000-fold increase in terms of *C. rodentium* colonization level was also observed in the stressed mice compared with the nonstressed controls [9]. According to Dréau et al. [10], mice subjected to social conflict stress had 2.26 $\log_{10}$ (lg) higher *E. coli* O157:H7 count in the bacterium inoculated semipermeable chamber implanted in the peritoneal cavity compared with the controls. Hendrickson et al. [11] found that stress induced by surgery (hepatectomy) and starvation resulted in a 7500-fold increase in the num-

ber of *E. coli* adhering to the cecum. Similarly, in rats, maternal separation stress was also shown to alter the fecal microbiota composition [87]

Most of the findings on stress-induced gut microbiota alternation are on human and laboratory animals, compared with which the effects of stress on the gut microbiota of food-producing animals are not adequately studied. In chicken, heat stress was shown to alter the gut microbiota composition [12]. Similar result was also observed in dairy cows underwent heat stress, where the stress reduced the fecal microbial diversity [13].

1.3.5.2 *In vitro* Evidence on the Effects of Stress on Intestinal Microbiota

Although *in vivo* evidence is accumulating in terms of the relationship between stress and increased susceptibility of animals and humans to bacterial infections, the exact mechanism of how stress induces such effect is not clear. One of the ways for stress to contributes to infections is through the enhancement of bacterial growth and the virulence mediated by neurotransmitters, especially those of the catecholamine family, which are released into the gastrointestinal tract during stress [88]. Past studies have demonstrated that stress catecholamines can enhance both the growth and the virulence of a wide range of bacteria, many of which are bacteria associated with food-borne illness such as *E. coli*, *Salmonella* spp., and *Vibrio* spp. [16, 17]. Norepinephrine (NE) and dopamine (DA) are the two major catecholamine neurotransmitters that can be found in high concentrations in the intestine, whose secretion is modulated by stress [88]. Because of this, *in vitro* examination of the effects of NE and DA on enteric pathogenic bacteria is a physiologically relevant way to study the effects of stress on the pathogenesis of these bacteria.

Norepinephrine (NE) is the major neurotransmitter released by postganglionic sympathetic nerves that innervate different regions of the intestinal wall including the circular muscle of sphincters, the mucosa, and the Peyer's patches [89]. A large portion of sympathetic outflow in human is directed to mesenteric organs (37%) such as gastrointestinal tract, pancreas, and spleen, contribut-

ing to the NE released to these organs during sympathetic discharges [90]. Furthermore, according to Yang et al. [91], removal of the majority of intestinal tract from rats prevented the increasing plasma NE level induced by sepsis and surgery, further suggesting that the gut is a major source of NE. NE released from the adrenergic nervous endings into the gut lumen can then be accessed by enteric bacteria, leading to enhanced growth [92].

Alternatively, dopamine (DA) is released from the dopaminergic nerve of the ENS [93]. Apart from that, several types of dopamine receptors, namely dopaminergic receptors are also found in the mucosal layer and the nerve ending layer of the intestinal wall [94, 95, 96]. Dopaminergic receptors are different from adrenergic receptors which is also present in the intestinal tract in that they recognize DA, while adrenergic receptors recognize NE and EPI [97, 98]. A communication pathway between the CNS and the enteric dopaminergic nerves may exist. In Crohn's disease (CD) patients, reduced sympathetic innervation was observed in the inflamed colon tissue accompanied with reduced levels of NE, DA, and 5-HT [99, 100]. The findings suggest that enteric sympathetic innervation, originated from the autonomic nervous system (ANS) that directly communicates with the brain, may be able to alter the dopamine level in the colon mucosa through activation of dopaminergic nerves in ENS [101]. Furthermore, according to Zhang et al. [102], rats experienced cold-restraint stress had increased DA levels in their colon smooth muscle, suggesting the modulation enteric DA release by stress.

It is widely accepted that the growth and virulence enhancement effects of stress-related catecholamines on bacteria is mediated by their ability to release iron restricted in host high-affinity iron-binding proteins such as lactoferrin (Lf) and transferrin (Tf), through which the iron is made more accessible to bacteria [103, 104]. Otherwise, it is hard for bacteria to acquire Lf/Tf-restricted iron, especially when they lack the ability to synthesis siderophores or to produce sufficient levels of siderophores. The catechol moiety in stress catecholamines is responsible for removing iron restricted in Lf and Tf [103]. A similar mechanism is also used by catecholate siderophores to re-

move Lf/Tf-restricted iron. Upon complexation with iron, catecholate siderophores forms a similar structure compared with iron-catecholamine complexes [103]. Furthermore, other compounds with the catechol moiety were also shown to stimulate bacterial growth under iron-restricted conditions. Many of them are of plant origin such as caffeic acid and tannic acid, while others are either the precursors or the metabolites of stress catecholamines [105, 106].

Considering the similar structures of cateholate-siderophore and stress-catecholamine iron complexes, it is not surprising that the response of some bacteria to stress catecholamines in iron-restricted media is associated with their ability to produce or to utilize catecholate siderophores (e.g. enterobactin and salmochelin) [106]. This is also true for *C. jejuni*. Lacking the ability to synthesis siderophores, *C. jejuni* takes advantage of the siderophore synthesized by other members of the enteric microbiota. It has been demonstrated that the inclusion of NE and EPI into iron-restricted media significantly enhanced the growth of *C. jejuni* [18, 19]. However, *C. jejuni* mutants with non-functional ferric-enterobactin receptor (CfrA) had reduced, although not fully abolished catecholamine-stimulated growth [57], suggesting the involvement of siderophore receptors in the mechanism of catecholamine-induced growth stimulation in *C. jejuni*.

Both physiologically relevant culture media and inoculum are important when examining stress-catecholamine mediated growth stimulation in bacteria. For food-borne bacteria, the culture media should reflect the iron-restricted environment in the gastrointestinal tract. Apart from that, the bacterial inoculum should be small, since food-borne bacterial infections are usually caused by consumption of a small amount of bacteria. The bacteria, most of the time, are also weakened, due to exposure to environments that are not suitable for their growth or even survival. Commercial culture medium such as BHI and MH are usually iron-replete. However, in the gastrointestinal tract, the availability of iron is restricted due to the active uptake by small intestine enterocytes [107, 108], as well as the chelation by host-derived high-affinity iron-binding proteins such lactoferrin (Lf) and transferrin (Tf). Specifically, apo-form Lf is secreted from exocrine epithelial cells into

gastric fluid, bile, and pancreatic juice to protect the gastrointestinal mucosal surfaces [109, 110]. Tf, on the other hand, is mainly found in the serum and functions by delivering iron to host cells through receptor-mediated endocytosis [111]. Together, Lf and Tf are essential elements of host innate immunity system against bacterial infections.

One of the ways to simulate the iron-restricted environment bacteria encounters in the gastrointestinal tract is through using serum-containing media, since serum contains the iron-binding protein Tf which has essentially the identical iron-binding site as Lf found in the intestine [112]. Serum-containing media is frequently used to examine stress catecholamine mediated growth stimulation in bacteria, with the most commonly used one being SAPI+serum medium developed by Lyte and Ernst [113]. The serum component of the medium contrains Tf which restricts the amount of iron in the medium that is easily accessible to the bacteria. Furthermore, Tf has essentially the same iron binding site compared with Lf which is the main iron-binding protein found in the intestine [114]. Utilizing iron-restricted medium is crucial for examining the catecholamine-stimulated growth in bacteria. According to Freestone et al. [105], *Escherichia coli* O157:H7 and *Salmonella enterica* SV Enteriditis failed to respond to various catechols in commercial culture media Tryptone Soy Broth (TSB) or Luria broth (LB) [105]. However, when the same concentrations of these catechols were included into SAPI+serum medium, several lg fold increase of bacterial growth was observed, which is in consistent with the growth stimulation effect of stress-catecholamines.

Apart from using iron-replete culture media, using a small bacterial inoculum is also crucial for studying stress-catecholamine mediated growth stimulation in bacteria. According to O'Donnell et al. [115], using a small inoculum with bacterial concentration of less than 100 CFU/ml is essential for *Enterobacter sp.*, *Shigella sonnei*, and *Staphylococcus aureus* to show the NE-mediated growth stimulation in SAPI+serum medium, which was either absent or less obvious when a higher inoculum was used instead [116, 117]. Similarly, according to Burton et al. [106], SAPI+serum medium was bacterial static when *E. coli* was inoculated to achieve an initial concentration of

less than 10^3 CFU/ml, and the growth inhibitory effect of the medium was reversed by either NE inclusion or using a higher bacterial inoculum. Higher bacterial inoculum may enhance the ability of bacteria to cope with iron-restricted environment, which, as a result, allows the bacteria to grow well without the help of stress-catecholamines. For example, *E. coli* can produce enterobactin (Ent) to access the iron restricted by Tf in serum-containing media [118]. With higher bacterial inoculum, the resulted higher level of Ent can assist the bacteria to acquire sufficient amount of Tf-restricted iron for growth in serum-containing media [118].

1.3.5.3 The Importance of Studying the Effects of Stress-Catecholamines on *C. jejuni*

Food-producing animals raised in modern agricultural facilities are unavoidably exposed to stressors such as extreme weathers, high stocking density, long-term confinement, frequent human handling, and lack of environmental enrichment [119]. The adverse effects of stress on animal production include reduced feed efficiency, disrupted reproduction, and increased disease susceptibility [120]. Furthermore, with the trend of removing antibiotics from animal production, it becomes increasingly important to develop new ways to reduce disease susceptibility and to increase feed efficiency [121]. In chickens, although removing antibiotics were shown to reduce the prevalence of *C. jejuni* resistant to ciprofloxacin, the opposite trend holds for the prevalence of multidrug resistant *C. jejuni* and *C. jejuni* resistant to non-quinolone antibiotics [122]. Reducing the level of *Campylobacter* colonization in food-producing animals is important for controlling campylobacteriosis. It is estimated that reducing the *Campylobacter* numbers on chicken carcasses by a factor of 100 could reduce the incidences of campylobacterisis associated with consumption of chicken 30 times [123]. Numerous attempts have been made to intervene *C. jejuni* colonization in food-producing animals such as through reduction of environmental exposure (biosecurity, sanitation/hygiene, barn management) [78], vaccination [124], antibody supplementation [125], phage therapy [126], and probiotics [127]. However, all of these interventions had limited success. Studying the stress-catecholamine mediated growth stimulation in *C. jejuni* using microbial endocrinology approach

may provide novel understandings on the pathogenesis of the bacterium thus contributes to the development of effective measures against it.

1.3.6 The Growth Inhibitory Effect of Catecholamines

Apart from enhancing bacterial growth in iron restricted media, catecholamines such as dopamine (DA), epinephrine (EPI), and norepinephrine (NE) may also be toxic to bacteria. According to Guita et al. [128], the inclusion of DA into both Mueller-Hinton broth and agar is growth inhibitory to *Staphylococcus aureus*. The inhibition effect is inoculum dependent, with lower bacterial inoculum being more susceptible DA. Similar trend was also observed with EPI, the cytotoxic effect of which appeared to be more potent than DA [129]. Similarly, according to Burton et al. [106], NE added at a concentration of more than 10^{-4} M into SAPI medium inhibited the growth of *E. coli*, the effect of which was absent at lower NE concentrations. Furthermore, the susceptibility of bacteria to catecholamines varies depending on bacterial strains and species. *Staphylococcus* spp. was shown to be more susceptible to catecholamines compared with *E. coli*, *Pseudomonas aeruginosa*, and *Enterococcus* spp. [128].

Other compounds with the catechol moiety may also have similar growth inhibitory effect as catecholamines. Taguri et al. [130] screened 10 different plant polyphenols for their antibacterial effect against multiple strains of *Staphylococcus aureus*, *Escherichia coli*, *Vibrio* spp., and *Salmonella* spp. All of the 10 polyphenols were growth inhibitory to the tested bacteria, albeit with varied potencies. Overall, *Staphylococcus aureus* and *Vibrio* spp. were more sensitive to the polyphenols compared with *Escherichia coli*, and *Salmonella* spp. [130]. This is in consistent with the previous findings, where *Staphylococcus aureus* was also shown to be more sensitive to catecholamines than *E. coli* [129, 128]. The toxicity of catecholamines and other compounds with the catechol moiety on bacteria is likely due to the reactive oxygen species (ROS) generated when these compounds are oxidized by O_2 [131]. This hypothesis is supported by findings by Guita et al. [128], where the inclusion of antioxidants such as metabisulfite and cysteine, as well as oxygen-protective enzymes

such as catalase (CAT) and superoxide dismutase (SOD) were able to abolish the growth-inhibition effect of DA on *Staphylococcus aureus*.

Since catecholamine supplementation can be both growth stimulative by providing iron to bacteria and growth inhibitive due to the generation of ROS, to avoid complicating results, antioxidants may need to be supplemented into the culture medium when studying catecholamine-stimulated growth on bacterial species that are sensitive to ROS. In a study by Roberts et al. [132], such result complication might have happened due to the lack of antioxidant component in their culture medium, since their study demonstrated that the supplementation of NE and EPI into iron-restricted medium enhanced the growth of some bacteria species such as *Eikenella corrodens* and *Campylobacter gracilis* while inhibited the growth of other bacteria species such as *Porphyromonas gingivalis* and *Bacteroides forsythus*. Furthermore, their data showed that the optical density of medium controls treated with NE and EPI increased over time, which is likely due to the color change as a result of catecholamine oxidation. The growth inhibitory effect of NE and EPI observed in their study for some of the bacteria may be caused by ROS generated during the oxidation of these catecholamines, and the varied response of different bacteria species to catecholamines may be explained by their varied susceptibility to ROS. Including antioxidants into iron-restrict medium allows researchers to control for the ROS and only examine the catecholamine-stimulated growth. Lastly, it is noteworthy that *C. jejuni* is very sensitive to ROS, since the bacterium possess less than half the antioxidant enzymes compared with *E. coli* and *Bacillus subtilis* [133]. It has been shown that *C. jejuni* is 50 times more sensitive to peroxide compared with *E. coli* [134]. Because of the sensitivity of *C. jejuni* to ROS, there is a need to add antioxidants into culture medium when examining the catecholamine-stimulated growth of the bacterium.

1.3.7 Variation of the Effects of Different Catecholamines

As discussed previously, the mechanism of catecholamine-stimulated growth involves the removal of Lf- and Tf-restricted iron by the catechol moiety in catecholamines, after which the resulted catecholamine-iron complexes can be accessed by bacteria using receptors for catecholamine siderophores (e.g. enterobactin) uptake. This mechanisms may not be the complete story however, since different catecholamines were shown to affect bacteria differently, which cannot be explained the mechanism. According to Freestone et al. [135], in gram-negative bacteria such as *Escherichia coli*, *Salmonella enterica*, and *Yersinia enterocolitica*, the growth enhancement effect of catecholamines can be blocked by adrenergic or dopaminergic receptor antagonists. Specifically, the growth enhancement effect of EPI and NE are blocked by the adrenergic receptor antagonists, while the effect of DA is blocked by the dopaminergic receptor antagonists but not the other way around. This finding suggests that catecholamine-stimulated growth in bacteria may not be merely explained by the catechol moiety shared by all catecholamines. The slight structural differences between NE, EPI, and DA are reflected by the different receptor binding sites they occupy and used to access different catecholamine-iron complexes. Furthermore, in SAPI+serum medium, NE was shown to have the strongest growth stimulatory effect on pathogenic *E. coli*, followed by EPI, then DA, which has the weakest effect among the three [106]. The varied growth stimulation effect mediated by different catecholamines further suggests that the effect of different catecholamines on bacteria may vary.

The response of *C. jejuni* to catecholamines is different from the three gram-negative bacteria mentioned above. For *C. jejuni*, neither α– nor β– adrenergic receptor antagonists can block the growth stimulation effect of NE, suggesting that the acquisition of NE-iron complex in *C. jejuni* is different from the three gram-negative bacteria [18]. Furthermore, *C. jejuni* lacks the ability to internalized NE [18], whereas *Escherichia coli*, *Salmonella enterica*, and *Yersinia enterocolitica* can all internalize NE. Since the NE-internalization process for the three gram-negative can be inhibited by α– adrenergic receptor antagonists [135], the results suggest that *C. jejuni* may have

different receptor(s) for NE-iron complex uptake that can not be blocked by α– adrenergic receptor antagonists. Although Cogan et al., [18] failed to demonstrate the different effects of NE, EPI, and DA on *C. jejuni* from a receptor standpoint, Xu et al. [19] successfully demonstrated the different effect of NE and EPI from a transcriptome standpoint by showing that more than one third of the differentially expressed genes were unique to either NE or EPI.

1.4 Experiment Justification

Microbial endocrinology is an interdisciplinary field of study that provides novel insight into the mechanisms which contribute to bacterial pathogenesis by studying the interactions between host neurohormones and the infectious bacterium. Of particular significance is the examination of the role of stress-related neurochemicals on the pathogenesis of infectious disease due to the well-recognized ability of stress to facilitate infections. The two major stress neurotransmitters in the intestinal tract are NE and DA, both of which are catecholamines produced within enteric nervous system and can be released into the gut lumen during period of stress.

C. jejuni is a leading food-borne bacterium that causes gastroenteritis in human. While the ability of stress-related neurochemicals to directly influence the growth and pathogenesis of food-borne pathogenic bacteria such as *E. coli*, *Salmonella* spp., and *Vibrio* spp. have been investigated by numerous groups worldwide, *C. jejuni* has not received the same level of investigation [17, 16]. Although NE and EPI has been previously shown to significantly enhance the growth and the virulence of *C. jejuni* [18, 19], both of the studies were done using a single *C. jejuni* stain, NCTC 11168, and neither study examined DA. Furthermore, although the growth stimulation effect of stress catecholamines on *C. jejuni* has been demonstrated, the mechanisms behind such effect was not fully understood. The purpose of the study, therefore, was to understand the effect of two major stress catecholamines, NE and DA, on the growth of *C. jejuni*. This study examined multiple strains of *C. jejuni*, and for the first time examined the effect of DA on *C. jejuni* growth. The findings in

this study provide further understanding on the mechanisms of catecholamine-stimulated growth in *C. jejuni*.

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CHAPTER 2. DOES CATECHOLAMINE-STIMULATED GROWTH OF DIFFERENT *CAMPYLOBACTER JEJUNI* STRAINS REQUIRE PYRUVATE?

Modified from a paper submitted to *PeerJ*

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2.1 Abstract

Given both human and food-producing animals are constantly affected by stress, the effect(s) of stress-related catecholamines on bacteria are of high interest. It has been well established that as the consequence of stress, the release of catecholamines, specifically norepinephrine (NE) and dopamine (DA) from nerve terminals enhances both the growth and the virulence of pathogenic bacteria, contributing to the possible development of gastrointestinal infections. Food-borne bacteria such as *Escherichia coli*, *Salmonella* spp. and *Vibrio* spp. have been extensively studied in terms of their response to catecholamines. In contrast, less is known about the effect of catecholamines on *Campylobacter jejuni*, the leading cause of bacterial food-borne infections in humans. The present study focuses on the effect(s) of stress catecholamines DA, NE, and epinephrine in iron-restricted medium, exploring their impact on the growth of the *C. jejuni* strains NCTC 11168, 81-176, and ML2126. Results demonstrated that DA- and NE-enhanced growth of *C. jejuni* in iron restricted medium may involve different mechanisms that can not be explained by the current understanding on catecholamine-mediated iron delivery, indicated by the DA-mediated growth

enhancement requiring pyruvate. We find significant and strain-specific dependence of the *C. jejuni* growth on various catecholamines. This study provides novel insights into the effect of stress catecholamines on the growth of *C. jejuni* in iron-restricted environments, including the intestinal tract, indicating the existence of possible mechanisms by which stress influences the intestinal microbiota and subsequent disease susceptibility.

2.2 Introduction

Campylobacter spp. are the leading cause of food-borne bacterial disease worldwide, of which *Campylobacter jejuni* is the most frequently isolated species from patients with *Campylobacter*-associated infections (campylobacteriosis) [1]. Although *C. jejuni* is an intestinal commensal in a variety of mammals and birds, human campylobacteriosis is characterized by varying clinical symptoms including fever, abdominal cramps, vomiting, mild to severe diarrhea or the immune mediated neuropathy Guillain-Barre syndrome [2, 3, 4]. Food-producing animals such as poultry, cattle and sheep are the main reservoirs for human campylobacteriosis. The disease is mainly transmitted through consumption and handling of contaminated meat, milk, or water. Numerous attempts made to curb the level *C. jejuni* colonization of food-producing animals without considering the stress factor resulted in limited success [5, 6, 7, 8, 9]. Therefore, it might be instrumental to consider the effect of stress on *C. jejuni* growth in a simulated environment that is physiologically relevant.

Recognized as a factor contributing to the development of enteric infection, in vivo stress may also be crucial to the successful colonization of *C. jejuni* in food-producing animals. Both psychological and physiological stress have been shown to alter the gut microbiome composition, resulting in the overgrowth of pathogenic bacteria in a wide variety of hosts, including human [10, 11, 12], mice [13, 14, 15], chicken [16], and dairy cows [17]. Food-producing animals are exposed to a variety of unavoidable stressors such as extreme weathers, long confinement, high population density, and regular human handling [18]. We expected that understanding of the mechanism(s) on how stress contributes to growth of *C. jejuni* may provide novel intervention strategies.

One possible reason for stress to contribute to the susceptibility of infectious diseases is its influence over the release of neurotransmitters into the intestinal lumen, especially those of the catecholamine family released from enteric nervous system (ENS) [19]. Norepinephrine (NE) and dopamine (DA) are the two main stress-related catecholamines in ENS that can be found in high concentrations in the gut lumen [20, 21]. During acute stress, NE can be released from the axons of sympathetic neurons into the gut lumen, where intestinal microbes are exposed [22, 23]. Dopamine can be alternatively released from the dopaminergic nerves that constitute part of the ENS [24]. Furthermore, dopamine receptors are located in the mucosal layer, as well as the nerve ending layer of the intestinal wall [25]. When rats underwent cold-restrained stress, increased DA concentration in colon tissue was shown [26], suggesting the existence of neural pathways that may contribute to the release of DA into the intestinal lumen during periods of stress, leading to the possible interaction with *C. jejuni*. Both NE and DA containing nerve fibers are found in the Peyer's patches in porcine jejunum [27]. Moreover, contraluminal NE administration enhanced the enteropathogenic bacteria uptake by Peyer's patches, a process that may contribute to the production of antibodies against these bacteria [27]. The finding suggests that catecholamine-containing nerve fibers modulate the mucosal immune response and infection outcome of enteric infections caused by pathogenic bacteria. Findings referred to above suggest that stress could alter availability of catecholamines, specifically NE and DA, in the intestinal lumen, where their interactions with gut microbes takes place.

The experimental framework of microbial endocrinology studies the effects of biogenic amines, notably catecholamines, on enteric pathogens [19]. Microbial endocrinology represents the union of the fields of microbiology and neurobiology and, as such, views the microbiota as neurochemical responsive, and neurochemical producing, microorganisms [28, 23]. Microbial endocrinology-based research has shown that the stress-related catecholamines NE and DA can significantly enhance the growth and virulence of enteric pathogens such as *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* [28, 23]. Similarly, adding NE and epinephrine (EPI) into

iron-restricted media also resulted in increased growth and enhanced virulence of *C. jejuni* NCTC 11168 [29, 30]. NE and DA may benefit *C. jejuni* by mediating iron delivery under iron-restricted environment such as the intestine, since iron is crucial for the proliferation of the bacterium [31]. The catechol moiety found in neurotransmitters such as NE, EPI, and DA, is able to release lactoferrin- and transferrin-bound iron (Lf/Tf-iron) thus making it accessible to these bacteria [31]. Although a common mechanism of releasing Lf/Tf-iron is shared by these neurotransmitters, individual catecholamines may not act in a uniform manner solely based on iron binding, but may in fact differentially influence bacterial physiology. Transcriptomic analysis of *C. jejuni* cultures have shown that a majority of genes were similarly impacted by NE or EPI, but more than one-third of the deferentially expressed genes were either unique to NE or EPI treatments [30].

Although DA is an abundant catecholamine in the gastrointestinal tract [32, 33, 21], the effect of DA on *C. jejuni* growth is unknown. Thus, the present study tested the effect of DA on *C. jejuni* growth in a similar iron-restricted medium system that had been previously used to examine the effect of NE and EPI on *C. jejuni* [29, 30]. By comparing the effect of DA and NE on growth of 3 *C. jejuni* strains, we demonstrated that the enhanced growth response is dependent on both the type of catecholamine as well as the bacterial strain. Moreover, we identified a new mechanism involving pyruvate, which was shown to modify the response of *C. jejuni* to DA but not NE. It is noteworthy that pyruvate is a bacterial metabolite produced in high amount in the intestine, where it stimulates intestinal immune response [34]. Therefore, our data suggest that the NE- and DA-mediated growth enhancement mechanisms in *C. jejuni* may be different in a way that the later requires a physiologically relevant factor, pyruvate, to be present.

2.3 Materials and Methods

2.3.1 Bacterial Strains

Three *C. jejuni* strains were used in the study: NCTC 11168, 81-176, and ML2126. Both NCTC 11168 and 81-176 are well characterized *C. jejuni* human isolates that are commonly used to infect animals with [35, 36]. ML2126 was provided by Dr. Qijing Zhang (Iowa State University). This strain was isolated from the cecal contents of a healthy chicken in November 2018, and was identified with MALDI-TOF (Bruker, Billerica, MA, USA). All strains were stored at -80°C in Muller Hinton (MH) broth (Difco, Sparks, MD, US) supplemented with 20% (v/v) glycerol.

2.3.2 Chemicals and Media

Dopamine hydrochloride (DA) was obtained from AlfaAesar (Tewksbury, MA, USA). L-Norepinephrine bitartrate (NE) was obtained from TCI (Portland, OR, USA). L-Epinephrine bitartrate (EPI), adult bovine serum, sodium pyruvate, sodium metabisulfite, L-glutathione reduced (GSH), and ferrous sulfate heptahydrate were obtained from Sigma (St. Louis, MO, USA). Adult bovine serum was stored at -20°C. Prior to inclusion into culture medium , the serum was thawed at 4°C overnight and sterilized by passing through a 0.2 µm syringe filter.

The *C. jejuni* minimal medium, MCLMAN (Medium Cysteine Leucine Methionine Aspartic acid Niacinamide), was prepared as described [37]. Muller Hinton broth (MH; Difco, Sparks, MD, USA) and peptone water (Difco, Sparks, MD, USA) were prepared according to manufacturer's instructions. Two iron-restricted media were prepared as described in previous publications, with the modification that adult bovine serum was used in substitution of fetal bovine serum [29, 30]. Specifically, 10% (v/v) adult bovine serum was added into MH and MCLMAN medium, which yielded serum-supplemented MH (MHs) and serum-supplemented MCLMAN (MCLMANs) respectively. Blood agar plates (TSA supplemented with 10% sheep blood) were employed for recovery and plating *C. jejuni* strains from frozen stocks and for quantitative CFU counts (Remel, San Diego, CA, USA)

2.3.3 Growth Assay

C. jejuni strains from frozen stocks were cultured on blood agar plates overnight at 41°C in a microaerophilic jar (Anoxomat Mark II, Advanced Instruments, Norwood, MA, USA) containing 6% O_2, 7.1% H_2, 7.1% CO_2, and 79.7% N_2. A working solution was prepared by harvesting bacterial colonies using a sterile cotton swab then suspending into 4 ml sterile peptone water to yield an absorbance of $OD_{600} = 0.2$. Immediately prior to inoculation of experimental cultures, the 0.2 OD_{600} bacterial suspension was further diluted 1000-fold into sterile peptone water. In order to achieve an initial inoculum of 10^4 colony forming unit (CFU)/ml, 40 µl of the diluted *C. jejuni* suspension was inoculated into 2 ml of iron-restricted medium. Cultures were grown statically in sterile 5 ml round-bottom polystyrene tubes (Falcon) containing 2 ml of cultures/tube for 24h in microaerophilic jars. Following incubation, the CFU/ml in each culture tube was determined using the drop plate method [38], with minor modifications by plating 25 µl for each dilution instead of 10 µl.

Growth assays were performed with the following objectives: (1) To examine the effect of iron supplementation on *C. jejuni* growth in iron-restricted medium: The above growth assay was conducted using *C. jejuni* strain ML2126 in MHs supplemented with different concentrations of ferrous sulfate heptahydrate (0, 5, 10, 20 and 40 µM). (2) To test the effect of stress catecholamines on *C. jejuni* growth: NE or DA were included at physiologically relevant 100 µM in iron-restricted media [39, 40]. Both types of the iron-restricted media, MHs and MCLMANs, were supplemented with 1 mM sodium pyruvate, giving rise to pMHs and pMCLMANs respectively. The growth of *C. jejuni* strains 81-176, NCTC 11168, and ML2126 in culture medium with and without NE or DA was examined using the growth assay. (3) To test the effect of catecholamine concentration on *C. jejuni* growth: Growth assay was carried out for *C. jejuni* strains NCTC 11168 and 81-176 in pMHs and pMCLMANs supplemented with different concentrations of NE or DA: 0 µM (no supplementation), 10, 50, 100, 200 and 500 µM. (4) To test the effect of pyruvate supplementation on the response of *C. jejuni* to catecholamines: Growth assay was conducted on *C. jejuni* strain

NCTC 11168 in MHs and MCLMANs with and without 1 mM sodium pyruvate supplementation. (5) To examine the possibility of pyruvate functioning as an antioxidant: The growth assay was carried out on *C. jejuni* strain NCTC 11168 in NE or DA supplemented MCLMANs with and without sodium pyruvate and two other antioxidants, sodium metabisulfite or GSH, each of which were supplemented to achieve a concentration of 2 mM in the iron-restricted medium.

2.3.4 Growth Curve Protocol

Preparation of the growth curve inocula were performed by statically culturing NCTC 11168 or 81-176 at 42°C in the broth phase of biphasic MH broth and agar (2% w/v) in a microaerophilic environment (containing 5% O_2, 10% CO_2 and 85% N_2 gas), as described previously [41]. The motility of different *C. jejuni* used in these studies were assessed each time growth curves were performed. Growth curves were performed with 10 replicates using microplates in a Bioscreen C plate reader (Growth Curves USA, Piscataway, NJ, USA), measuring OD_{600} every 1h for 48h. To limit aggregation, which may impact optical density values, microplates were shaken for 30 seconds prior to each OD_{600} reading. Inocula were diluted 1:2500 in 400 µl of MH broth with or without serum, pyruvate, DA, NE or EPI supplementation at 42°C in a microaerophilic environment. Uninoculated media served as a control to subtract OD_{600} background.

2.3.5 Statistical Analysis

For growth assay data with triplicate samples, two-tailed t-tests were performed using Prism v8.1.2 (Graph Pad Software Inc., San Diego, CA, USA) to analyze for significant differences between groups. Growth curve data were analyzed using the R package growthcurver [42], which measured the logistical area under the curve, growth rate and generation time.

2.4 Results

2.4.1 Effect of Iron and Catecholamine Supplementation on *C. jejuni* Growth

Addition of iron, in a dose-dependent manner, to iron-restricted MHs broth reversed the inhibitory growth effect on *C. jejuni* (Fig. 2.1). The addition of NE or DA (100 µM) to pyruvate treated iron-restricted medium pMHs enhanced growth of *C. jejuni* strains ML2126 and NCTC 11168 (Fig. 2.2). Without DA or NE, these strains failed to grow in pMHs (Fig. 2.2). *C. jejuni* strain 81-176 was unique since DA, but not NE, increased its growth to levels comparable with strain ML2126 and NCTC 11168 grown in pMHs (Fig. 2.2). Growth curves of strains NCTC 11168 and 81-176 were performed in pMHs supplemented with DA, NE or EPI (Figs. 2.3A and 2.3B). As a control, both strains grew in MH broth alone (Figs. 2.3A and 2.3B). In pMHs, DA significantly ($p < 0.001$) enhanced growth of strain 81-176. Area under the curve (AUC) analysis demonstrated DA had a greater ($p < 0.00001$) growth enhancement effect on strain 81-176 compared with NE or EPI. Analysis of AUC for strain NCTC 11168 demonstrated that both DA and NE significantly ($p < 0.003$ and $p < 0.04$, respectively) enhanced its growth compared to EPI. The growth enhancement effect of NE on 81-176 growth was much less compared with the other two strains.

Consistent with the result observed in pMHs, a *Campylobacter* minimal medium supplemented with pyruvate and serum (pMCLMANs) with or without 100 µM DA or NE also enhanced growth of NCTC 11168 and ML2126 in iron-restricted conditions (Fig. 2.4). *C. jejuni* strain 81-176 failed grow in MCLMAN-based iron-restricted media, regardless of catecholamine supplementation. As for the effect of catecholamine concentration on *C. jejuni* growth, regardless of catecholamine used, no growth was observed at the end of the 24-hour incubation in pMHs when the concentration was less than 10 µM (Fig. 2.5). Addition of NE (> 100 µM) or DA (> 50 µM) enhanced growth of strain 81-176 whereas strain NCTC 11168 was enhanced by addition of DA or NE at ≥ 50 (Fig. 2.5).

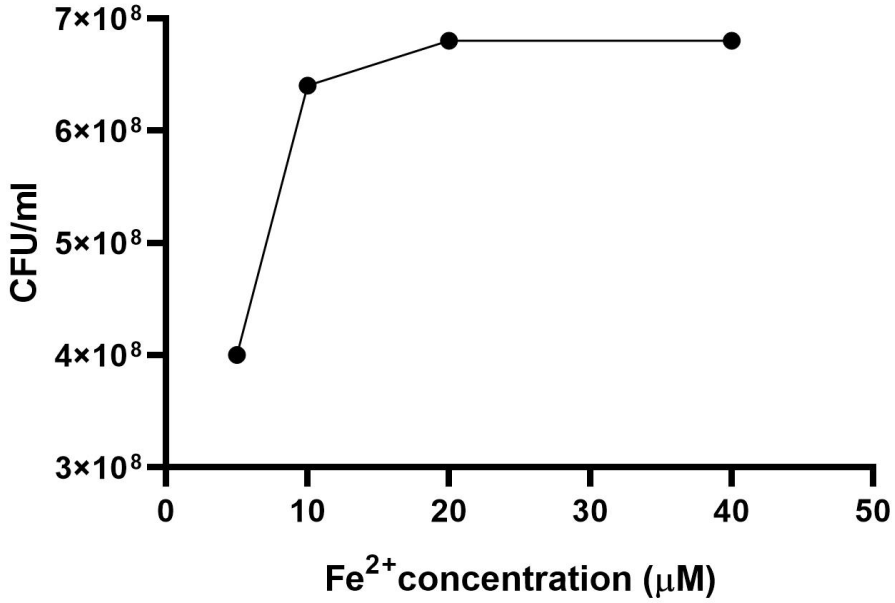

Figure 2.1 The growth of *C. jejuni* ML2126 in MHs supplemented with different concentrations of iron for 24h. Data represent the mean of duplicate samples.

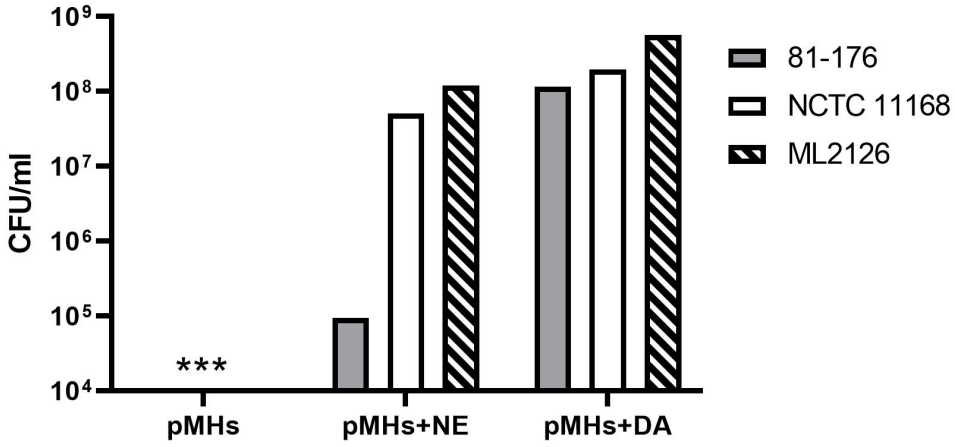

Figure 2.2 The growth of 3 *C. jejuni* strains in pyruvate-supplemented MH broth with serum (pMHs) with and without the supplementation of 100 µM NE or DA for 24h. An asterisk (∗) indicates no detectable growth at the end of incubation. Each bar represents the mean of duplicate samples. These data are representative of two independent experiments.

2.4.2 Effect of Pyruvate on the Response of *C. jejuni* to Catecholamines

Addition of catecholamines may induce oxidant stress in *C. jejuni* strain NCTC 11168 [30]. Pyruvate may protect bacteria from oxidative stress [43]. To define a mechanism by which pyruvate affects DA or NE-enhancement of growth, iron-deficient rich (MHs) or minimal (MCLMANs) iron-deficient media were used. *C. jejuni* NCTC 11168 failed to grow in both iron-restricted medium whether or not pyruvate was present (Fig. 2.6). Addition of DA required pyruvate to enhance growth of strain NCTC 11168 in rich or minimal iron-deficient media (Fig. 2.6). In the absence of pyruvate, addition of NE only significantly ($p < 0.1$) affected growth of strain NCTC 11168 in rich media, and the presence of pyruvate rescued growth in rich or minimal iron-restricted media ($p < 0.05$) (Fig. 2.6). The addition of antioxidants pyruvate, GSH or sodium metabisulfite enhanced growth of *C. jejuni* strain NCTC 11168 in NE supplemented iron-restricted pMCLMANs ($p < 0.05$) (Fig. 2.7). However, addition of GSH or sodium metabisulfite failed to enhance growth

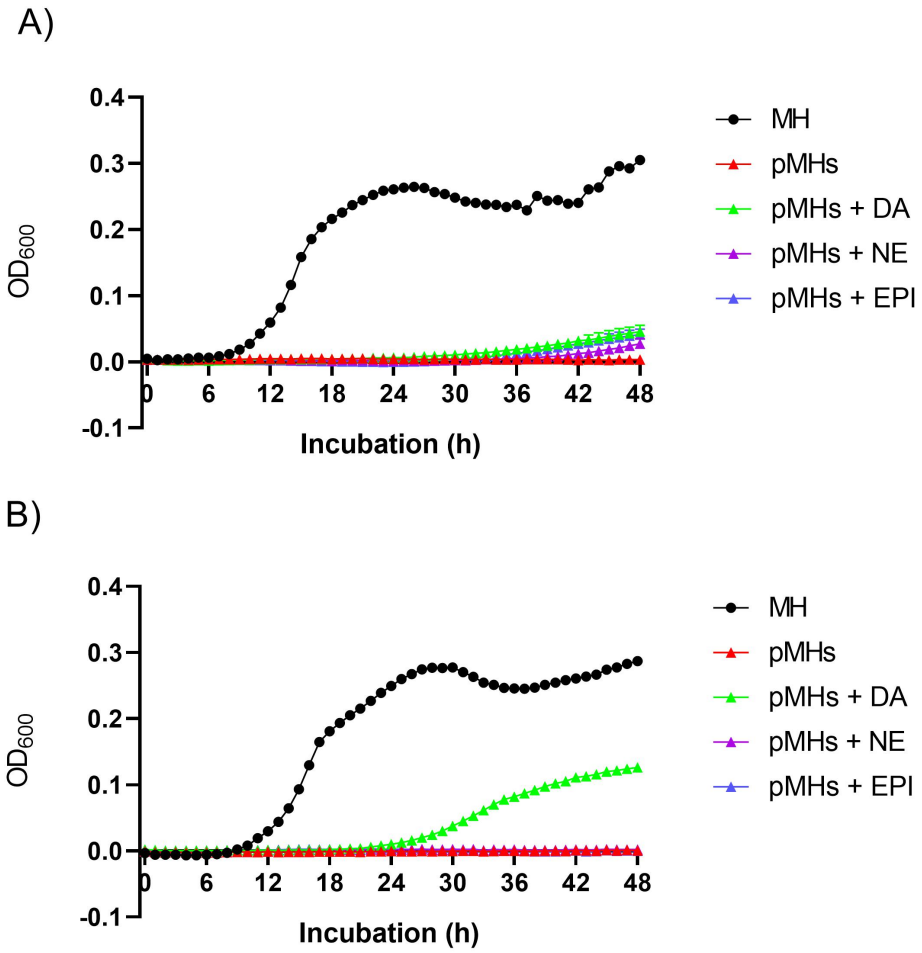

Figure 2.3 Growth curves of *C. jejuni* strains NCTC 11168 (A) and 81-176 (B) in MH broth, MH broth with serum and pyruvate (pMHs), pMHs and 100 µM dopamine (pMHs + DA), pMHs and 100 µM norepinephrine (pMHs + NE) or pMHs and 100 µM epinerpherine (pMHs + EPI). Growth curves were performed for 48h at 42°C in a microaerophilic environment (5% O_2, 10% CO_2 and 85% N_2). Data represent the mean ± SEM OD_{600} of 10 cultures measured every hour for a total of 48h with the background value of unincolated media subtracted. Data are representative of 2 independent experiments.

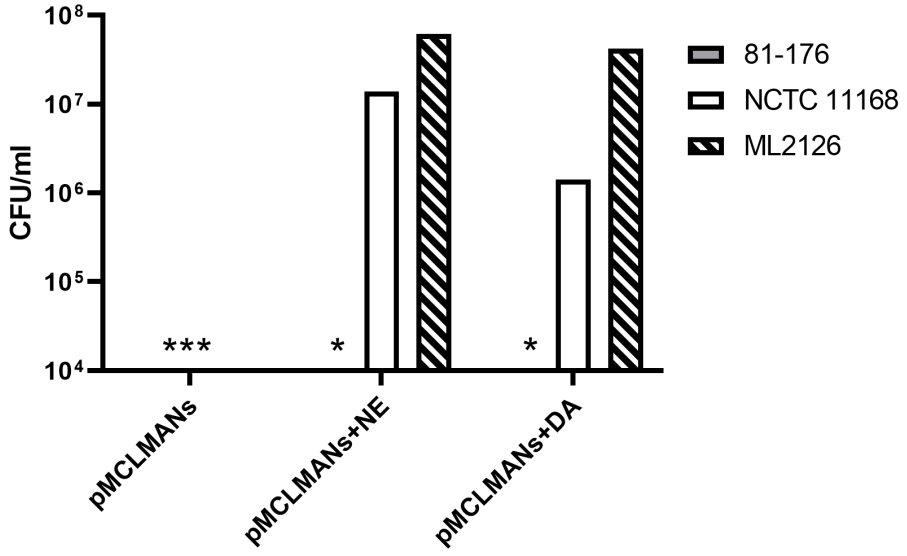

Figure 2.4 The growth of 3 *C. jejuni* strains in pyruvate-supplemented MCLMAN medium with serum (pMCLMANs) with and without the supplementation of ND or NE (100 µM). An asterisk (∗) indicates no detectable growth at the end of incubation. Each bar represents the mean of duplicate samples. The graph is representative of two independent experiments.

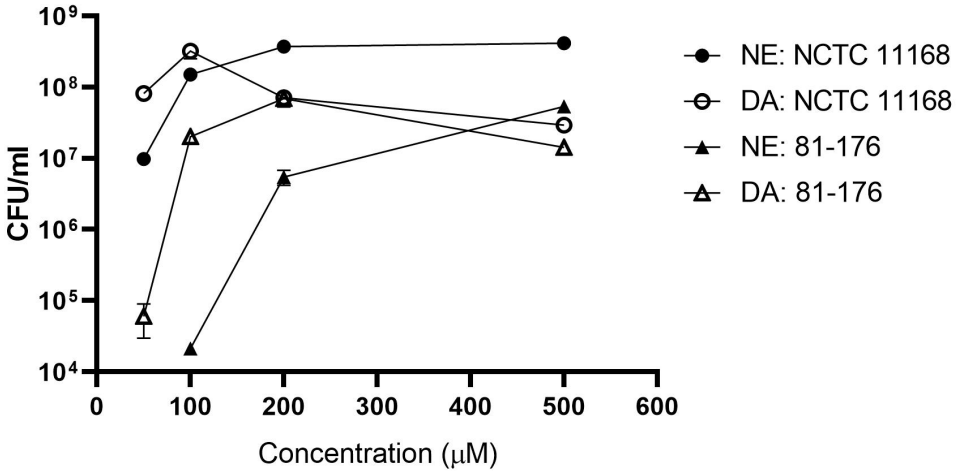

Figure 2.5 The growth of *C. jejuni* NCTC 11168 and 81-176 in pMHs supplemented with different concentrations DA or NE for 24h. Each point represents the mean ± standard deviation of triplicate samples.

of strain NCTC 11168 in DA supplemented iron-restricted pMCLMANs, but pyruvate enhanced its growth ($p < 0.01$) (Fig. 2.7).

2.5 Discussion

Microbial endocrinology is an emerging field of research that studies the bidirectional communication between the host and the bacteria through neurotransmitters [44]. As an interdisciplinary research area, the theoretical framework can be employed to examine mechanisms that may be involved in the pathogenesis of infectious disease to the microbiota-gut-brain axis and the ability of nutrition to influence host physiology and dietary preferences [44, 45]. For the present study, we employed a microbial endocrinology-based approach to examine the role of stress-related neurochemicals to influence the growth of *C. jejuni*. Studies examining the effects of stress on bacterial physiology through direct, non-immune mediated, interactions between host neurophysiology and bacteria are critical to our understanding of infectious disease pathogenesis since both human and food-producing animals unavoidably experience stress. Such studies have shown that stress expo-

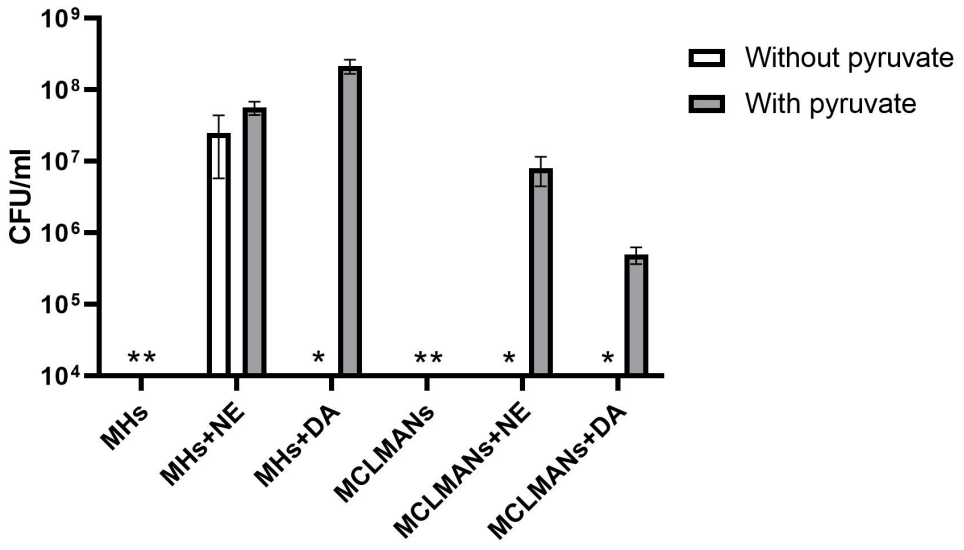

Figure 2.6 Growth (24h) of *C. jejuni* strain NCTC 11168 to DA or NE (100 µM in iron-restricted media MHs or MCLMANs with and without 1 mM sodium pyruvate supplementation. An asterisk (∗) indicates no detectable growth at the end of incubation. Each bar represents the mean ± standard deviation of triplicate samples.

sure increased susceptibility to bacterial infections [10, 11, 12, 16, 17]. The present study used a microbial-endocrinology approach to examine the effect of two major intestinal stress neurotransmitters, NE and DA, on the growth of the leading food-borne bacteria, *C. jejuni*. The results shown in the present study extend our understanding of the role of host neurotransmitters in bacterial pathogenesis.

To survive and successfully replicate in the intestinal tract, *C. jejuni* needs to adapt to restricted access to essential nutrients, most notably iron [46, 47, 48]. Within the gut lumen, *C. jejuni* is also exposed to a variety of molecules that are either secreted by the host or by other organisms. One group of these molecules is the catecholamine neurotransmitters, especially NE and DA, which are secreted into the intestinal lumen from the ENS nerve terminals during periods of stress [22, 23, 24, 26]. These catecholamines may also be provided by other members of the

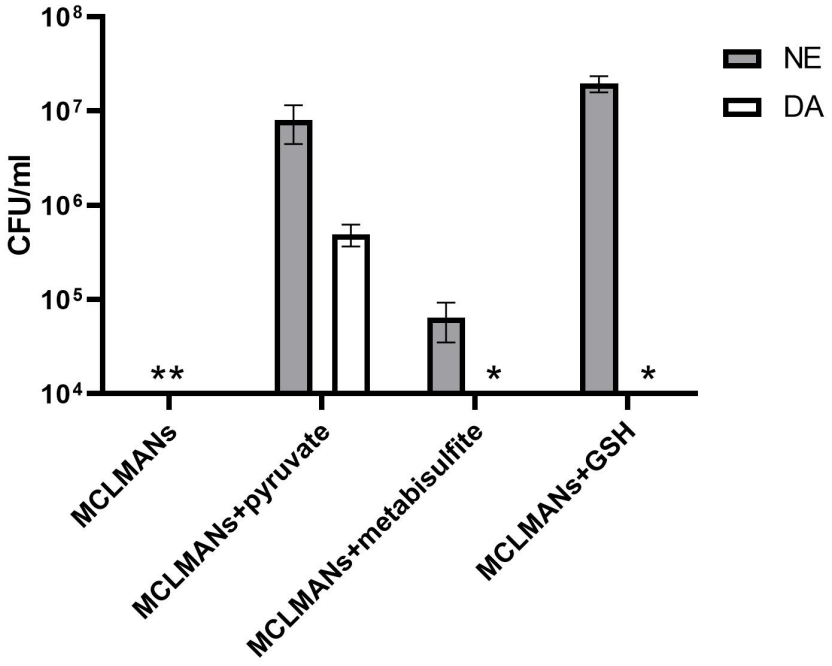

Figure 2.7 Growth (24h) of *C. jejuni* NCTC 11168 in NE or DA supplemented MCLMANs with and without different antioxidants included at a concentration of 2 mM. An asterisk (∗) indicates no detectable growth at the end of incubation. Each bar represents the mean ± the standard deviation of triplicate samples.

gut microbiota such as *Enterococcus faecium*, *E. coli*, or *Clostridium* spp. whether generated from catecholamine precursors or glucuronide-conjugated catecholamines [32, 49]. In the intestine, iron is one of the key limiting nutrients making the environment restrictive for *C. jejuni* growth. Apart from active iron uptake by small intestine enterocytes [46, 47], the host can also produce high affinity iron-sequestering proteins such as lactoferrin and transferrin, which serves as a primary innate mechanism to limit some bacterial infections. Lactoferrin is secreted from exocrine glands located at the gateway of the digestive system [48] and can be found in mucosal secretions such as saliva, bile, pancreatic juice, and gastric fluids [48, 50]. Transferrin, on the other hand, is mainly found in serum and is responsible for delivering iron to host cells through receptor-mediated endocytosis [51]. To simulate the iron-restricted environment bacteria encountered in the gastrointestinal

tract, serum-supplemented medium system was developed to examine the effect of catecholamines on enteric pathogenic bacteria [39, 52]. In the present study, a chemically defined and a complex serum-containing media were developed based on a medium used to examine the response of *C. jejuni* strain NCTC 11168 to NE and EPI [29, 30]. Because supplementing the serum-containing medium with iron rescued the growth of *C. jejuni* (Fig. 2.1), these data suggest that iron-restriction is, in part, responsible for growth inhibition. These data are consistent with previous studies where iron rescued growth of enteric pathogenic bacteria such as enteroaggregative *E. coli* [53], *Salmonella enterica* [54], and *Vibrio cholerae* [55] grown in serum-containing medium.

Stress-related catecholamines such as NE, EPI, and DA have been shown to enhance both the growth and the virulence of a variety of bacteria in iron-restricted media [28, 23]. It is widely accepted that catecholamines help remove the iron restricted in host iron-binding proteins such as lactoferrin and transferrin, through which the iron becomes more accessible to bacteria [52]. However, the catecholamine-mediated effects on bacteria may also involve other unknown mechanisms. In the present study, the inclusion of NE into iron-restricted MH or MCLMAN medium significantly enhanced the growth of *C. jejuni* strains NCTC 11168 and 81-176 (Figs. 2.2 and 2.4), which is in consistent with the findings in previous *C. jejuni*-based studies [29, 30]. To test whether the NE-mediated growth stimulation effect is present in a more physiologically relevant culture medium, the growth assay was also conducted in an iron-restricted simulated small intestinal medium. The simulated small intestinal medium (sSIM) was prepared as described [49], followed by adding 10% (v/v) adult bovine serum into the medium to restrict iron. Similar to the results obtain in iron-restricted MH and MCLMAN media, in the iron-restricted sSIM, the addition of 100 µM NE also significantly enhanced the growth of *C. jejuni* (result not shown). Dopamine is another stress catecholamine found in high concentrations in the intestine [32, 33, 21], however, its effect on *C. jejuni* is unknown. Our results suggest that DA enhanced *C. jejuni* growth in iron-restricted medium similar to the effect of NE (Figs. 2.2 and 2.4). The mechanism of the DA-enhanced growth may not be the same as the NE-enhanced growth, which is supported by

the observation that pyruvate is a requisite for the DA-enhanced but not the NE-enhanced growth (Figs. 2.6 and 2.7). The varied response to different catecholamines has been previously shown in *C. jejuni* [30] and *E. coli* [56], demonstrated by the large proportion of genes (more than 30% of total differentially expressed genes) that were uniquely expressed with either NE or EPI supplementation.

Results from this study suggest a different mechanism by which *C. jejuni* responds to DA and pyruvate. To our knowledge, this mechanism has not been described previously. Our initial experiments using serum supplemented MCLMAN (MCLMANs) completely failed to demonstrate the NE-stimulated growth in *C. jejuni*, which is in contrast to a previous study where a tissue-based culture medium, αMEM, was used instead [30]. Comparing the formulation of MCLMAN and αMEM, we suspected that sodium pyruvate, which is present in αMEM but not in MCLMAN medium, may be responsible for the different result. Supplementing MCLMANs medium with the same level of sodium pyruvate found in αMEM medium successfully reproduced the NE-mediated growth stimulatory effect observed in the previous study, suggesting that pyruvate is an important factor in the catecholamine-mediated growth stimulation among *C. jejuni* strains. Catecholamine neurotransmitters such as NE, DA, and EPI are known to generate ROS that are harmful to bacteria [57, 58, 59, 39]. Since pyruvate has been previously shown to protect bacteria against the lethal effect of reactive oxygen species (ROS) [43], we examined the possibility of pyruvate acting as an antioxidant. To test this hypothesis, we did a preliminary experiment in MCLMAN and MH without serum supplementation to which NE or DA was added at a final concentration of 100 µM to the medium. Addition of either catecholamine at 100 µM concentration prevented growth of *C. jejuni* in MCLMAN medium, suggesting that *C. jejuni* is sensitive to ROS. In MH, a significantly lower CFU/ml was also observed in catecholamine supplemented cultures. Critically, when antioxidants such as sodium pyruvate, sodium metabisulfite and GSH were included into MCLMAN or MH, the growth inhibitory effect of catecholamines were reversed (results not shown). These data are consistent with previous studies with *Staphylococcus aureus*, where the toxicity of catecholamines was also blocked by addition of antioxidants [58, 59]. Following testing

in growth media without serum supplementation, the above antioxidants were further examined in the iron-restricted medium, pMCLMANs. Interestingly, although the inclusion all 3 antioxidants enabled NCTC 11168 to positively respond to NE supplementation as reflected by increased growth, a similar result was not observed with DA supplementation, where only pyruvate supplementation was effective (Fig. 2.7). Since, pyruvate, a TCA cycle intermediate, is also a recognized energy source, we further tested some other energy sources that are known to be utilized by NCTC 11168, including L-serine, L-glutamic acid potassium salt, monosodium succinate [60]. None of the other tested energy sources were able to replace pyruvate to induce the DA-mediated growth stimulation (results not shown). In aggregate, our findings therefore suggest that an antioxidant, such as pyruvate, metabisulfite, or GSH are required in culture medium supplemented with NE or DA to protect *C. jejuni* against ROS generated by the catecholamines. However, in iron-restricted medium, the DA-mediated growth stimulation can only be seen with pyruvate supplementation but not with the addition of other antioxidants or energy sources. Thus, DA-mediated growth stimulation may involve a different mechanism compared with the NE-mediated growth stimulation in *C. jejuni* where pyruvate plays an important role.

What is also evident in this study is the strain specific response to catecholamines. Although *C. jejuni* strains NCTC 11168 and ML2126 had similar response to NE and DA in both types of iron-restricted medium, the response of strain 81-176 to the catecholamines is significantly different from the previous two strains (Figs. 2.2 and 2.4). The strain specific response to NE and DA is better illustrated in the concentration curves on *C. jejuni* NCTC 11168 and 81-176 (Fig. 2.5), where NCTC 11168 was shown to be much more sensitive to NE and DA compared with 81-176, being consistent with the results shown in Figs. 2.2 and 2.4. The difference is more prominent with their response to NE, where NCTC 11168 reached a population density of around 1×10^7 CFU/ml when NE was supplemented at 50 µM, whereas the same NE concentration failed to initiate growth for 81-176. Another trend revealed by the concentration curves is that both *C. jejuni* strains were more responsive to low concentrations (less than 100 µM) of DA than NE in pMHs,

with higher CFU/ml achieved in DA supplemented media than in NE supplemented media at the end of the incubation. The results further emphasize that different mechanisms between NE- and DA-mediated growth stimulation may be operative in different strains of *C. jejuni*.

The strain specific response may be explained by the differences in the iron uptake system between *C. jejuni* strains NCTC 11168 and 81-176. Considerable progress has been made on understanding the iron acquisition mechanism in *C. jejuni*. NCTC 11168 was able to utilize transferrin- and lactoferrin-bound iron (Tf/Lf-iron) in liquid culture through a proposed receptor CtuA [61]. However, the ability of *C. jejuni* to utilize Tf/Lf-iron may not be adequate in vivo, because all *C. jejuni* strains tested in the present study were unable to show growth in adult bovine serum (10% v/v) supplemented media. Another mechanism how bacteria access Tf/Lf-iron is through the production of siderosphores, a group of high affinity iron chelators with low molecular weight. For example, members of *Enterobacteriaceae* are known for their ability to produce the siderophore enterobactin [62, 63]. Two of the *C. jejuni* strains used in this study, NCTC 11168 and 81-176, are known for their ability to utilize siderophores, such as enterobactin and ferrichrome produced by other intestinal microorganisms [64, 65, 66]. Previous studies have associated NE-mediated growth stimulation to the enterobactin uptake system, which may be explained by the structural similarity between Fe-Ent and Fe-NE complexes [31]. The NE-mediated growth stimulation in *C. jejuni* is associated with a ferric enterobactin (FeEnt) receptor, CfrA [65]. NCTC 11168 CfrA mutant showed significantly impaired the NE-mediated growth stimulation compared with the wild-type strain, although the mutation did not abolish the NE-mediated growth [65]. Similar finding was also seen in *E. coli*, where strains with mutations on FeEnt uptake failed to response to NE [53]. The uptake of FeEnt may only be partially responsible for the catecholamine-mediated growth stimulation, since, a *C. jejuni* mutant that is fully incapable of utilizing FeEnt has been shown to have similar NE-mediated growth stimulation compared with the wild-type strain [66]. The result suggests that apart from FeEnt utilization pathway, other unknown mechanisms may also contribute to the catecholamine-mediated growth stimulation. Another important component in iron uptake

system associated with catecholamine-mediated growth stimulation is the TonB-ExbB-ExbD energy transduction system (TonB complex). Studies on *E. coli* and *Bordetella bronchiseptica* have demonstrated that a functional TonB complex is required for the NE-mediated growth enhancement in iron-restricted media containing Tf/Lf-iron [67, 68]. In gram-negative bacteria, TonB complexes are known to provide energy for iron uptake mediated by other membrane receptors. For example, in *C. jejuni*, the uptake of FeEnt through outer membrane receptor CfrA and CfrB both require an intact TonB-ExbB-ExbD energy transduction system [69]. Unlike *C. jejuni* strain NCTC 11168 which has 3 TonB complexes, strain 81-176 only has a single TonB2/ExbB2/ExbD2 complex [35], which may explain its less sensitivity to NE and DA observed in the present study.

2.6 Conclusions

By employing a microbial endocrinology-based approach, the present study has identified novel aspects regarding the mechanism(s) by which stress-related neurochemicals, especially members of the catecholamine family, have on the growth of *C. jejuni* in physiologically relevant iron-restricted media. Our results demonstrated that the intestinal stress-related catecholamines, NE and DA, enhanced the growth of *C. jejuni* in a strain-dependent manner. Furthermore, the DA-enhanced growth in *C. jejuni*, unlike the NE-enhanced growth, requires pyruvate as a key factor. The results shown in the present study extend our understanding of the mechanisms by which stress may contribute to the pathogenesis of food-borne bacterial infections.

2.7 Acknowledgments

The authors would like to acknowledge the technical support of Matt H. Inbody for growth curve assays. We are also grateful to Dr. Qijing Zhang (Iowa State University) for providing the chicken *C. jejuni* isolate (ML2126) used in this study. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture (USDA). USDA is an equal opportunity provider and employer.

2.8 References

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CHAPTER 3. GENERAL CONCLUSION

In this study, we examined three *C. jejuni* strains, NCTC 11168, 81-176, and ML2126 for their response to stress catecholamines NE and DA. Our result showed that NE and DA addition into iron-restricted medium can significantly enhance the growth of *C. jejuni*. The growth stimulation is strain-specific, with *C. jejuni* strains NCTC 11168 and ML2126 being more sensitive to NE than strain 81-176. Since iron uptake system plays an important role in the response of bacteria to catecholamines, such difference may be explained by their different iron uptake systems which *C. jejuni* strain 81-176 are known to have a weaker version than strain NCTC 11168. Furthermore, both *C. jejuni* strains NCTC 11168 and 81-176 are more sensitive to DA than NE, as shown by the finding that both strains exhibit similar level of growth enhancement at a lower DA than NE concentration.

Another major finding in this study is that pyruvate is an essential factor in DA- but not NE-mediated growth stimulation. In one of the iron-restricted media used in this study, MCLMANs, all *C. jejuni* strains failed to show enhanced growth with NE or DA addition, and pyruvate, which is lacking in MCLMANs is responsible for the lack of response of *C. jejuni* to catecholamines in the medium. For NE-mediated growth stimulation in *C. jejuni*, other antioxidants such as metabisulfite and reduced glutathione can substitute pyruvate in MCLMANs. However, only pruvate addition into MCLMANs enabled the bacterium to show DA-mediated growth stimulation. Thus, the findings suggest that different mechanisms are involved in NE- and DA-mediated growth stimulation in *C. jejuni*, where the DA-mediated growth stimulation requires pyruvate as a key factor.

In conclusion, this study provided some novel findings on the response of *C. jejuni* to host intestinal neurotransmitters, NE and DA, which are released during stress. Findings in this study demonstrated that the stress catecholamines are important for the pathogenesis of *C. jejuni*. Thus modulating the level of these stress catecholamines as well as other factors involved in mechanisms of the catecholamine-mediated growth stimulation, possibly through modulating gut microbiota, is a promising direction of future research.