SURVIVAL OF *CAMPYLOBACTER JEJUNI* WITHIN MIXED POPULATION BIOFILMS OF POULTRY ISOLATES ON STAINLESS STEEL

by

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(Under the direction of Joseph F. Frank)

ABSTRACT

A method was developed to determine the attachment and survivability of *Campylobacter jejuni* in mixed biofilms on stainless steel. Confocal scanning laser microscopy (CSLM) and epifluorescence microscopy were used for visualization of *C. jejuni* transformed with a $P_c$gfp plasmid (*C. jejuni*1221gfp). The data in this study indicated that *C. jejuni* 1221gfp was able to form a biofilm on stainless steel when incubated with and without other bacteria at 37 °C up to 7 days. The numbers of *C. jejuni*1221gfp were enhanced on the stainless steel when incubated with mixed poultry isolates to form biofilms. *C. jejuni*1221gfp was visualized being attached to 16 hr biofilms formed on stainless steel at temperatures of 13, 20, 37 and 42 °C. The frequency of attachment at the four temperatures was statistically the same even though biofilm surface coverage was less at 37 and 42 °C when compared to 13 and 20 °C. Culturable *C. jejuni*1221gfp was recovered only from biofilms formed at 13 and 20 °C, however, according to epifluorescence microscopy cells were seen at 37 and 42 °C, attached to biofilm and stainless steel but they may be in the viable-but-nonculturable phase or nonviable. *C. jejuni*1221gfp was able to attach to stainless steel with preexisting biofilms and survive in nutrient limited environments. In diluted tryptic soy broth (1:10 and 1:50 TSB), biofilms of mixed culture were

allowed to form and *C. jejuni*1221gfp was allowed to attach at 20 and 37 °C. Biofilm surface area coverage was low (approximately 2%) in both broths at both temperatures. Attached *C. jejuni*1221gfp differed in numbers between 1:10 TSB (20 °C) and 1:50 TSB (37 °C) with numbers from 1:10 TSB being the highest between the two samples. Counts of *C. jejuni*1221gfp were recovered only from 20 °C. Epifluorescent microscopy displayed *C. jejuni*1221gfp directly attaching to the coupon and not areas of biofilm in both broth and temperature treatment combinations in a 48 hr period.

INDEX WORDS: *Campylobacter jejuni*, biofilm, confocal scanning laser microscopy, CSLM, epifluorescent microscopy, stainless steel, green fluorescent protein, gfp, temperature, nutrient limitation

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DEDICATION

This dissertation is dedicated to my late grandmother Ethel Sanders, my mother Cynthia Sanders, and my aunt Valine Grant.

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CHAPTER 1

INTRODUCTION

In both the United States and United Kingdom, *Campylobacter jejuni* is the leading cause in foodborne illnesses. Approximately, 2 million cases of campylobacteriosis annually occur. Reported symptoms of campylobacteriosis include diarrhea, fever, abdominal cramping and vomiting. Deaths are rare in healthy adults, however infants, the elderly and immune suppressed individuals are susceptible. Guillain-Barré syndrome is the most common sequela disease of *Campylobacter* infection. This disease is a demyelating disorder that leads to acute neuromuscular paralysis (Altekruse *et al*, 1999).

Since *C. jejuni* can form a viable-but-nonculturable (VBNC) state when stressed, it can be present in the environment but undetectable by traditional culture methods. Pathogens such as *C. jejuni* could be surviving in poultry processing plants by attaching to stainless steel equipment and recontaminating broiler carcasses as they move though the processing line. *C. jejuni* is very sensitive to ambient atmospheric conditions, therefore, it must be surviving in a favorable environment or/and transforming into another physiological state (VBNC) during poultry processing since it has been found on retail chicken. *C. jejuni* has already been shown to survive in biofilms on polyvinyl chloride plastics (Trachoo *et al*, 2002) and water microcosms (Buswell *et al*, 1998) but none have investigated the use of stainless steel as a substratum for *C. jejuni* biofilm formation. The objective of this study is to determine if *C. jejuni* is able to attach to biofilms or form biofilms and survive on stainless steel equipment in poultry processing plants. Confocal scanning laser microscopy and epifluorescent microscopy were used to

visualize *C. jejuni*, transformed with a $P_c$gfp plasmid (*C. jejuni*1221gfp), attached to biofilms and stainless steel. There are three research chapters in this dissertation. The first chapter objective is to determine if *C. jejuni* is able to attach to and survive on stainless steel coupons without existing biofilms. The second chapter objective is to determine if temperature has any effect on the attachment and survivability of *C. jejuni* in mixed biofilms. The objective of the last chapter is to detect if nutrient deprived environments have any effect on the attachment and survivability of *C. jejuni* in mixed biofilms and on stainless steel.

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CHAPTER 2

LITERATURE REVIEW

Campylobacter jejuni

Origin of *Campylobacter jejuni*

Campylobacter jejuni belongs to the genus *Campylobacter* and the family *Campylobacteraceae* (*82, 119*). This organism originally was classified as a *Vibrio* species (*Vibrio jejuni*) but due to its low DNA base composition, microaerophilic growth requirements, and non-fermentative metabolism, it was later renamed genus *Campylobacter* (*119*). In the 1980s, the genus *Campylobacter* had only eight species and subspecies. Since then, it has grown to have approximately 18 species and subspecies (*82*). *C. jejuni* currently consists of two subspecies which are *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doyeli*. Other genera such as *Arcobacter*, *Sulfurospirullum,* and *Bacteroides* are also included in the family *Campylobacteraceae* (*91, 119*).

Characteristics

C. jejuni are gram-negative, microaerophilic, non-spore-forming, slender, spirally curved rods that are 0.2 to 0.8 μm wide and 0.5 to 5 μm long in size (*82, 119*). Old cultures or cells overexposed to air may form a coccoid shape which is considered to be a degenerative form of *Campylobacter* (*23, 44, 47, 82, 115*). *C. jejuni* cells are motile and possess a singular polar flagellum at one or both ends and move in a cork-screw type motion (*67, 82, 119*). In terms of biochemical reactions, *C. jejuni* is catalase(+), oxidase(+), urease(-), alpha-hemolysis(+) and

hippurate hydrolysis(+) (*67, 119*). The growth temperature range for *C. jejuni* is approximately 34 to 44°C and its optimal growth temperature is 42 °C, which could reflect its adaptation to the intestines of warm-blooded birds (*67*). Atmospheric growth conditions for *C. jejuni* consist of an oxygen, carbon dioxide, and nitrogen mixed environment (*4*). *Campylobacter* has also been reported to possess antibiotic resistance. Ge *et al (41)* reported *C. jejuni* from raw retail chicken and turkey meat as showing resistance against ciprofloxacin, doxycycline, erythomycin, nalidixic acid, and tetracycline antimicrobial agents. The *C.jejuni* isolates from the turkey meat showed greater resistance than those from the chicken meat. In another study, Bea *et al (11)* isolated *C. jejuni* from cattle where resistance was shown against doxycycline.

Importance of *C. jejuni*

C. jejuni is one of the leading causes of bacterial gastroenteritis in the United States and worldwide. It is estimated that 2.1 to 2.4 million cases of *Campylobacter* enteritis occur each year in the United States (*4, 82*). In developing countries, *Campylobacter* is one of the most frequently isolated microorganisms from the stool of infants with diarrhea (*30*). However, there are few national surveillance programs for campylobacteriosis even though it is prevalent in these countries. Campylobacteriosis is due largely to food and water contamination (30). Many cases in developing countries are non-pathogenic, and *Campylobacter* infections are mainly a pediatric disease. Most of the time *Campylobacter* is involved in polymicrobial infections with other organisms such as *Salmonella*, *Escherichia coli*, *Shigella,* and *Giardia lamblia* (*30*).

Disease and incidence

Infection by *C. jejuni* usually causes enteritis in which the symptoms result in fever, abdominal cramping, and diarrhea (with or without blood) (*30, 82*). *Campylobacter* enteritis usually occurs after 1-7 days and the disease usually lasts for 5-8 days. The symptoms are self-limiting in most healthy people, however, infants, elderly persons, and the immuno-compromised have the highest rate of complications and even death due to *Campylobacter* enteritis (*104*). *C. jejuni* can also cause extraintestinal infections such as bacteremia, bursitis, urinary tract infection, meningitis, endocarditis, peritonitis, erythema nodosum, pancreatic, abortion, neonatal sepsis, reactive arthitis, and Guillain-Barré syndrome (*82, 104*). Guillain-Barré syndrome (GBS) is one the diseases most affiliated with *C. jejuni* infection. GBS is an autoimmune disorder of the peripheral nervous system that causes symmetrical weakness which extends over a period of several days (*30, 45*). Symptoms of GBS include rapid development of weakness in limbs, respiratory muscles, and areflexia (loss of reflexes). GBS is a self-limiting disease where muscles reach a baseline of strength within 2 to 3 weeks (*45*). The bottoming of strength is followed by partial or complete recovery that can range from a time period of weeks to months. GBS can leave severe neurologic deterioration (*83*). Mortality rates are much higher in developing countries than developed countries (*30, 45*). In the U.S., the most common pathologic pattern of GBS is known as acute inflammatory demyelinating polyneuropathy (AIDP) (*45*). This pattern's mechanisms are immune-mediated attacks on myelin and involve lymphocytic infiltration. During severe cases, axonal degeneration occurs (*82, 83*). Other patterns such as acute motor-sensory axonal neuropathy are commonly found in China, Japan, and Mexico (*45*). In developed countries, the median annual incidence of GBS is 1.3 per 100,000 and this disease has been found to be remotely more common in males than females

(*45*). Serotypes such as *C. jejuni* O:19, have been associated with having the highest risk of development of GBS after infection (*30, 45, 82*). As for molecular mimicry and GBS, several studies have supported how *Campylobacter* structural characteristics evoke auto-immune mediated attack against nerve tissue. Lipopolysaccharides (LPS) of some *C. jejuni* have ganglioside-like structures to which GBS patients possess antiganglioside antibodies. Nerve fibers have also been reported as having ganglioside-like parts located on them as well (*45*).

Food and animal reservoirs for *C.jejuni*

C. jejuni can be found in many foods. Raw animal products such as chicken, beef, pork, lamb, turkey, offal, and shellfish have been reported to have *C. jejuni* present on them (*13, 30, 56, 82, 107*). Along with raw foods, raw milk, and contaminated water supplies frequently harbor *C. jejuni* which is mainly due to fecal shedding (*12, 125*). Salad vegetables or other uncooked vegetables are also at risk for having *C. jejuni* present on them, due to contaminated water supplies (*39*). Food handlers can be a pertinent source in spreading *C. jejuni* during improper food preparation. Unhygienic food handling and undercooking are usually the cause of infection (*30*).

***C. jejuni* association with poultry**

C. jejuni is a zoonotic organism and some of its animal reservoirs include of rabbits, rodents, wild birds, sheep, horses, cows, pigs, and domestic pets (82). However, poultry is where this organism is the most prevalent (*100, 132*). Shortly after chicks are hatched, *C.jejuni* can be detected on them. While newly hatched chicks are free of *Campylobacter*, they can become colonized with *C. jejuni* by 8 to 10 days of age (*31*). It only takes approximately 2 to 3

weeks for most flocks to become infected after chicks are placed into broiler houses (*42, 87*). Experimentally, birds can be infected with very low doses of *Campylobacter*. A low dosage such as 40 CFU of *C. jejuni* (*31*) is able to colonize chicks and chickens. Colonization also depends on the bacterial strain and chicken strain (*12*). Once *C. jejuni* has colonized a broiler chicken, it persists for the lifespan of the bird. However, colonization levels decrease with age (*31, 42*). *C. jejuni* tends to colonize the lower gastrointestinal tract of chickens (*12, 31, 77, 85, 92*). Principal sites of localization have shown to be in the ceca, large intestine, cloaca, and in the intestinal mucas layer in the crypts of intestinal epithelial where *C. jejuni* is usually found (*12*). *C. jejuni* is generally detected in caecal contents at levels of 10^4 to 10^8 CFU/g (*31*). Even though, chickens are colonized with *C. jejuni*, there are usually no observable clinical symptoms of infection. Extraintestinal sites such as the liver and spleen may also be colonized by *C. jejuni* (*85*).

Sources of contamination

Infection of chickens can be traced to several environmental sources. *C. jejuni* can be present in non-chlorinated drinking water from reservoirs and water lines (*94*). Feces from wildlife (rodents, rabbits, deer, foxes, birds, etc.) and domestic animals (cats and dogs) can harbor *C. jejuni* or it can be tracked into the chicken house on boots of workers (*125*). In Denmark, a study by Hald *et al* (*47*) showed that flies caught outside a broiler house had the potential to transmit *C. jejuni* to chickens. Therefore, insects may play an important role in *Campylobacter* infection to chickens.

Even though, vertical transmission of *C. jejuni* (breeder hen via egg) remains unclear and controversial, some studies have shown possibilities of this being a source of infection. Results

by Cox *et al* (*34*) demonstrated that some *Campylobacter* isolates from commercial broiler breeder flocks and from their respective broiler progeny may be of clonal origin and that breeder hens could be a source of contamination. Evidence of this was provided by DNA sequence analysis of the short variable region (SVR) of the *fla*A gene (*fla*A SVR). Other sources of *C. jejuni*, can be from aerosols in humid atmospheres and equipment taken into the broiler houses (*85, 86, 105*).

Poultry processing (A summarization from Sams *(101)*)

The slaughter process of poultry consists of unloading, stunning, killing, scalding, feather removal, evisceration and chilling. When birds first arrive at the processing plant, they are either dumped onto a conveyor belt from and/or manually removed from the coops. This procedure is traditionally performed in a dark room, lit with black lights or dim red lights (the dark room is used as a calming effect for the birds). After chickens are removed from the coops, they are hung on shackles by their feet. Next, the birds are stunned to render them unconscious for 60 to 90 seconds, by lowering their heads to come in contact with approximately 1% NaCl solution that is charged so that electrical current flows though their bodies. Stunning is considered to be a humane form of slaughter for the birds. Some benefits from proper stunning include immobilization for improved killing machine efficiency, more complete blood loss, and better feather removal during picking. Another method of stunning is by gas (carbon dioxide or argon/nitrogen mixture) which is used in Europe. After stunning, the birds are moved to a killing machine within seconds. While moving on the shackle conveyor, a rotating circular blade cuts the jugular veins and carotid arteries on one or both sides of the neck of the bird. Most killing machines cut both sets of the blood vessels. After the neck cutting, a time frame of 2 to 3

minutes is allowed for bleeding. During bleeding, 30 to 50% of blood loss takes place, leading to brain failure and death. Next, the chicken carcasses are submersed into a scald tank of water with a temperature of approximately 53° C (128° F) for 120 seconds. This is called "soft scalding" which is the normal procedure used by industry (*130*). Hard scalding is a procedure in which the carcasses are scalded at 62 to 64°C (145 to 148° F) for 45 seconds (*31*). Both loosen feathers for easier removal, however hard scalding causes loss of the waxy cuticle which may increase attachment of bacteria (including pathogens) to skin during processing. Feather removal is the next step after the carcasses have been scalded. Carcasses move though a picking machine which consists of rows of rotating clusters of flexible, ribbed, rubber fingers. In order to remove feathers, these rubber fingers rotate rapidly to rub against the carcass to produce enough abrasion to pull out loosened feathers. After feathers are picked, carcasses go though another process called singeing. During singeing, carcasses are quickly passed though a flame to burn off hair-like structures on the skin. This gives the carcass an aesthetically pleasing look for consumers. Right before the carcasses exit the picking area, the heads, feet, and inedible viscera (together referred to as offal) are removed and sent to other locations in the plant to be sorted for sale or discarded. Lastly, before evisceration, the birds are transferred from the kill shackle to the evisceration shackle line. This is done manually or by machine.

Evisceration is the removal of edible and inedible viscera from a carcass. The three basic objectives of evisceration are: 1) Opening of the body cavity by making a cut from the posterior tip of the breastbone to the cloaca; 2) Removal of internal organs by a scooping motion of the viscera (the gastrointestinal tract and organs, reproductive tract, heart and lungs); 3) Giblets separation where edible viscera (heart, liver, gizzard) are removed from the extracted viscera, washed and inspected. After viscera removal, the carcass is inspected for bruising, fecal

contamination or other defects. If inspection fails, the bird is reworked if the problem can be corrected. If inspection passes, the carcass is passed though an inside/outside (I/O) bird washer which directly sprays the interior and exterior of the bird before it enters the chill tank .

The purpose of the chilling process is to reduce microbial growth to a level that will maximize food safety (*31*). After evisceration, the broiler carcass temperature of 4°C or less is usually achieved. In the U.S., regulations require this temperature to be reached within 4 hours of death of the broiler. Water or air can be used for chilling. Water chilling is mainly used in the U. S. while Europeans commonly use air chilling (*31*). The first step in water chilling is removing the carcasses from the shackles and placing them in the tank where they are slowly pushed though the water by a paddle-like device. Once in the chill tank, the carcasses will go though two phases of chilling. The first phase is the prechiller phase. In this stage, the water is 7 to 12°C and the carcass goes though this chill process for 10 to 15 minutes. The primary objective of this step is to allow water absorption, however some washing and chilling of the carcasses do take place. At the entrance of the prechiller tank, the carcass temperature is approximately 38°C and after is 30 to 35°C. Next is the main chiller step, where the tank is larger and the water temperature is 4° C at entrance and approximately 1° C at the exit. This process usually lasts 45 to 60 minutes. After the main chiller phase, the carcass temperature should be down to 4 °C.

C. jejuni presence in the poultry processing plant

Before the scald process, *C. jejuni* numbers are usually at its highest on broiler carcasses (*14, 125*). During scalding, *C. jejuni* counts drop significantly, however counts increase again after defeathering (*13, 14*). Counts are sufficiently high during this phase that an immediate

second scald of the broilers would not make a significant reduction of this organism (*15*). This could be due to the mechanical picker fingers cross-contaminating the carcasses. Picker fingers may cause such a force on the carcass abdomen to release fecal matter from the gut, where *C. jejuni* is heavily found (*13*). As the birds are processed, *C. jejuni* generally decreases (evisceration to chill tank). After the chill process, the counts of *C. jejuni* are lowest in detection (*14*). The presence of *C. jejuni* on retail chicken parts and whole carcasses is very common. Studies have shown that 98% of retail chicken is positive for *C. jejuni* (*4*). Levels of *C. jejuni* are not significantly different between broiler parts with and without skin (*16*).

C. jejuni metabolism

C. jejuni is a fastidious, microaerophilic organism with an aerobic metabolism (59, *61, 66, 74, 76, 118*). This bacterium has a highly branched respiratory chain and the ability to operate a complete oxidative citric acid cycle (*67*). Its respiratory chain is considered to be branched due to oxidation being mediated by more than one oxidase because it contains high concentrations of *c*- and *b*- type cytochomes in its electron transport chains. It possesses homologues of key enzymes such as α and β subunits of succinyl-CoA synthetase and nicotinamide adenine dinucleotide-linked malate dehydrogenase to operate such as system (*67*). *C. jejuni* is unable to metabolize external carbohydrates due to its lack of the key glycolytic enzyme phosphofructokinase. For aerotolerance and oxidative stress resistance, *C. jejuni* uses alkylhydroperoxide reductase (AphC) (*9*). Proteins such as superoxide dismutase catalase and ferritin help *C. jejuni* defend against oxidative stress, even though it is oxygen sensitive (*47, 67*).

C. jejuni pathogenesis

Due to lack of fit animal models, much information on *C. jejuni* pathogenic mechanisms is predicted rather than known for certain. As stated earlier, *C. jejuni* is a motile organism which moves in a corkscrew form. Its motility is a necessary characteristic for colonization of the intestine (*82*). Since intestinal cells are covered with a mucus barrier, it is pertinent that *C. jejuni* be able to penetrate this layer in order to infect the cell (*52*). Motility of *C. jejuni* is made possible by a single, polar, unsheathed flagellum located on one or both sides of the bacterium. The flagellar motility makes invasion of eukaryotic cells possible (52, *84*). Evidence supporting flagellar motility has been provided by research demonstrating how flagellar mutants with truncated or absent flagellum were partially motile and inhibited *C. jejuni* from colonizing cells (*120*). Chemotaxis is another feature about *C. jejuni* which is essential for colonization. By using chemotaxis, *C. jejuni* has the ability to detect chemical gradients and move up or down them. Non-chemotactic mutants of *Campylobacter* are unable to colonize intestinal cells (120). *C. jejuni* is chemically attracted to substances such as mucins, L-fucose, and L-serine (*53*).

It has been proposed that invasion by *C. jejuni* begins with the uptake of the bacterium by host cells. *C. jejuni* binds with the cell surface by using adhesions such as CadF and PEB1 proteins (*5, 82*). The CadF protein is a fibronectin-binding protein while PEB1 is linked with adherence of HeLa cells (*52*). After binding, a series of host cell signaling mechanisms begin. Next, *C. jejuni* is internalized after it produces at least 14 new proteins which also contribute to host cell cytoskeletal component rearrangement. Host cell signaling is obscured, and inflammatory cytokines (e.g. interleukin-8) are released. Following cytokine release, lymphocytes and phagocytes are attracted to the infection area (*52, 82*).

Other virulence factors of *C. jejuni* are its surface polysaccharide structures, oxidative stress defense, and toxins. Lipo-oligosaccharide (LOS) and lipopolysaccharide (LPS) outer membrane structures can aid in serum resistance, endotoxicity, and adhesion (*5*). LOS has always been expressed in *C. jejuni* because the bacterium contains an operon consisting of genes that produce LOS/LPS biosynthesis (*5, 95*). Since *C. jejuni* is microaerophillic, it must have a defense mechanism against oxygen toxicity. *C. jejuni* uses superoxide and peroxide defense systems. The superoxide defense system can manipulate the superoxide dismutase protein (SOD) to convert superoxides into hydrogen peroxide. This protein is encoded by the *sodB* gene and contains iron (*67*). The peroxide defense system uses two main proteins, catalase (KatA), and alkyl hydroperoxide reductase (AhpC) (*9, 67*). The catalase breaks down hydrogen peroxide to water and oxygen which removes hydrogen peroxide produced by SOD or other metabolic mechanisms. AhpC reduces alkyl hydroperoxides to alcohols and is important for aerobic survival (*9*).

C. jejuni infections are associated with production of cytotoxins and enterotoxins. Even though, many researchers disagree because of lack of genetic evidence of *Campylobacter* toxicity, some studies link the cytolethal distend toxin (CDT) with *Campylobacter* toxin (*40*). CDT is produced in 40% of over 700 strains of *C. jejuni*, however its role in pathogenesis is unknown (*96*). This could be due to CDT titre disparity. Many bacterial pathogens have genes that are regulated in response to iron-like virulence factors such as toxin and heamolysin genes (*82*). The Fur protein is usually responsible for this type of iron-responsive regulation. However, *C. jejuni* has two genes that encode Fur like homologues, *fur* and *perk* (*120*). This *C. jejuni* Fur protein has the main iron-responsive regulator and the PerK protein is the second. Other regulatory systems of *C. jejuni* are the RacR and CheY proteins (*52, 82*). The RacR

protein is involved with colonization and thermoregulation. The CheY protein affects the flagellar motor switching rate.

C. jejuni and temperature

C. jejuni survives temperatures ranging from -70 to 50°C. When attached to chicken skin and then stored at -20 and -70 °C, *C. jejuni* remains viable and is able to proliferate at 4 °C and at room temperature according to Lee *et al* (*75*). Yang *et al* (*130*) observed *C. jejuni* surviving 50 °C scalding water for five minutes, however, at 55 °C the organism died during this time frame (*130*). Cold stressed *C. jejuni* have survived storage at 4 °C and -20 °C if incorporated into food such as ground chicken and on chicken skin (*17*). Rollins and Colwell (*98*) showed that *C. jejuni* can survive in the VBNC for four months at 4 °C and that DNA maintenance can persist as well as cell viability. Investigations by Lazaro *et al* (*73*) observed *C. jejuni* sustained intact DNA, cellular integrity and adequate respiration when kept at 4 °C for 116 days. However, Chan *et al* (*24*) reported that *C. jejuni* cell morphology is not strongly related with survival at 4 °C or with viability after freeze-thawing, and that clinical isolates of *C. jejuni* were more robust in cold tolerance than poultry-derived strains. Culturability can also be retained at low temperatures. Hazeleger *et al* (*49*) observed that *C. jejuni* cells in nutrient-poor and nutrient-rich media sustained culturability the longer at 4 °C than at 12 or 25 °C before transforming into the nonculturable coccal cells (*49*). They also observed that the intracellular levels of ATP were the highest in cells during this temperature storage and coccoid cell protein profiles and membrane fatty acid are more stable at 4 °C than at 25 °C. Therefore, cocci could be able to contribute to pathogenesis as well as spiral forms. Cold shock proteins have not been found yet in *Campylobacter*, however, other functions such as chemotaxis and aerotaxis are observed at 4 °C,

which contribute to this pathogen's ability to move to favorable places in the environment (*50*). In planktonic forms and in biofilms, *C. jejuni* can also survive longer at 4°C than at 22 or 37 °C (*20*).

C. jejuni does not usually grow below 30°C and not above 47 °C (*65, 88*). Heat injury occurs at 46 °C, and thermal inactivation happens at 48 °C. The incubation temperatures of 37 and 42 °C is optimal for growth (*62, 118, 119, 129*) for this organism. Solow *et al* (*106*) observed when *C. jejuni* was inoculated onto raw, irradiated chicken and pork skin under microaerobic conditions, viable counts declined at 25 °C. Around 22°C, *C. jejuni* tended to be more susceptible to NaCl inactivation and acidic conditions than at 4 °C (*63*). *C. jejuni* DNA is barely detectable after cells are incubated at 20 °C (*73*).

C. jejuni morphology

C. jejuni possesses two morphological forms (spiral and coccal). In liquid culture, *C. jejuni* has shown over time extreme morphological changes (*116*). During exponential phase, cells are in the typical short spiral shape. Mid-stationary phase, cells become twice the length of the forms from the previous phase. In late-stationary and early decline phase, *C. jejuni* cells can be seen in both coccal and spiral morphology and the spiral cells appear to be 3 to 4 times longer than the cells in the exponential phase. Ultimately, all the cells turn into coccal forms (*44*).

Coccal forms of *C. jejuni* are considered viable but non-culturable (VBNC) cells. VBNC cell morphology is also found in other human pathogens such as *E. coli*, *Salmonella enteritidis*, *Vibrio cholera,* and *Legionella pneumophila* (*115*). Even though, VBNC cells are potentially pathogenic, they can not be recovered by standard culture methods (*115*). The viability of these cells is often determined by optical microscopic methods (3) such as direct viable counting

(DVC). The DVC method usually employs salts such as 5-cyano-2,3,ditolyl tetrazolium chloride (CTC) which can determine viable, respiring cells (*47, 73, 115*). This method consists of counting membrane filtered cells or fields of cells on a slide that have been stained with CTC. CTC is monotetrazolium redox dye which produces a fluorescent formazan when it is biologically or chemically reduced. Therefore, respiring, viable cells produce a red fluourescence when stained with CTC. Some scientists consider VBNC to be a degenerate state while others say it is a dormant state. Studies by Harvey *et al* support the VBNC degenerative theory by showing how *C. jejuni* cells converted to the VBNC state after applying oxidative stress and how the outer and inner membranes became considerably separated to yield a collapsed appearance in the cell (*47*). Under starved conditions, *C. jejuni* converts to the VBNC phase and can even build up resistance to some environmental stresses, such as temperature, due to protein synthesis at the onset of starvation (23). *C. jejuni* can survive in natural aquatic environments (98). This survival could be largely due to the VBNC transformation because of low nutrient availability and other environmental stresses. VBNC cells may be able to revert back to culturable, spiral morphology if passed though the favorable medium. A study by Chaveerach *et al* (26) reported some VBNC cells of *C. jejuni* being resuscitated via amniotic and yolk sac injection into specific-free-pathogen fertilized eggs. Studies by Capplier *et al* (22) have produced similar results that support the VBNC forms being resuscitated back to spiral forms.

Biofilms

A biofilm is an accumulation of microorganisms colonizing a surface and forming a single layer or multilayers of cells (*7*). Biofilm formations tend to occur on solid surfaces that come in contact with water and can form on both inanimate and living surfaces (*20, 21, 48, 127*).

Biofilms cover surfaces entirely or in patches of growth. The construction of a biofilm basically consists of microbial cells, water, and extracellular polymeric substances (EPS) (*27*). EPS can consist of polysaccharides, other polymers, debris, exoenzymes, etc. (*32, 70*). EPS created by bacteria differ greatly in chemical and physical composition. Most EPS tend to be polyanionic because of the presence of uronic acids such as D-glucuronic acid or ketal-linked pyruvate. Inorganic residues (phosphate and sulphate) can also cause polyanionic properties in EPS. Polysaccharides of EPS matrices are long, thin molecular chains having a molecular mass of 0.5-2.0 x 10^6 Da (*112*). The formation of biofilms can be a result of transport interfacial transfer and transformation (growth) processes (*127*).

Biofilms were reported to be observed first by van Leeuwenhoek during the 17^{th} century (*122*). They were in the form of dental plaque on teeth. Today, biofilms are known to play many roles in the environment. Biofilms provide beneficial processes such as in water treatment (*10*) (sewage removing organic and inorganic pollutants) and growing on root cells to provide nutrients for plants in the fermentation industry for quick-vinegar processes (*97*). Some negative aspects of biofilms include biofouling, corrosion, dental disease, and causing infections from build-up on medical devices (*7, 27, 32, 70, 127*).

Bacterial attachment and biofilm formation process

In the attachment process, a bacterial cell binds to a surface using polymers that initially produce reversible adhesion but later becomes irreversible (*27*). After adhesion, cell division and proliferation take place that produce cell colonies bound within a glycocalyx matrix. As microcolonies continue to develop within the biofilm, planktonic bacterial cells attach from the surrounding fluid. Therefore, biofilms ultimately end up consisting of single cells and

microcolonies embedded in bacterial exopolymers and trapped extraneous macromolecules (*117, 132*). The attachment rate of bacteria depends on the surface properties, environmental conditions, bacterial species, and cell density (*8, 57, 70*). Bacterial pili, flagella, and fimbriae also help to contribute to anchorage of cells to biofilms. Likewise, bacterial cells can possess complementary molecules that interact with other cells or with molecules of the biofilm to form attachment (*95, 98*). Physio-chemical binding interactions such as ionic, electrostatic, bipolar, hydrophobic, and hydrogen bonding are also forms of bacterial attachment (*7, 127*).

Biofilm detachment (Summarized from Rittmann *et al (97)*)

Biofilm detachment is the loss of cells from the biofilm. Detachment can occur by four different mechanisms: grazing, erosion, abrasion, and sloughing. Grazing detachment results from protozoa feeding on the outer surface of the biofilm. Erosion is caused by shear stress from continuous water flowing which removes small particles from the surface of the biofilm. Abrasion is caused by the impingement of particles which are covered with biofilm. Therefore, biofilm is removed from the particles when they are rubbed together. Finally, sloughing occurs when large chunks of biofilm break off and float away. This detachment could be caused by the accumulation of daughter cells from the biofilm separating into the liquid or a result of nutrient exhaustion in which the biofilm microbes separate in search of a more favorable environment. The physical conditions of the surface substratum can contribute to detachment as well as attachment. If a surface has crevices, biofilms embedded in these areas can be protected from shear stress and abrasion as opposed to them being attached on smooth surfaces.

Quorum sensing

Within a biofilm community, bacteria communicate with one another (*126*). This intercellular signaling is called quorum sensing. Quorum sensing is a cell density dependent regulation and it allows bacteria to coordinate behavior which allows the biofilm community to develop functional structure (*69*). Quorum sensing may play an important role in the accessory gene regulator (agr) system in *Staphylococcus aureus*, which contributes to virulence in model biofilm-associated infection (*131*). The signaling molecule homoserine lactone (HSL) which controls expression of various traits is found in numerous Gram-negative bacteria (*38*). Some of the expression traits controlled by HSL are bioluminescence, antibiotic production, and virulence factor production. Contrarily, Gram-positive bacterial quorum sensing is mediated by peptide signaling molecules (*38*). Another quorum sensing system is the signaling molecule autoinducer-2 (AI-2) system. The AI-2 system is found in both gram negative and gram positive bacteria and is highly conserved in both (*29*). A study by Elvers and Park (*38*) demonstrated how *C. jejuni* can produce functional A1-2 activity though the ability of cell-free extracts specifically inducing bioluminescence in a quorum sensing reporter strain *Vibrio harvey*. Here, the *C. jejuni* luxS gene product was used as regulation in the production of this signaling compound.

Bacteria embedded in biofilms are more resistant to antimicrobial agents than their planktonic counterparts (*70*). Numerous studies have shown how difficult it is for sanitizers and biocides to remove and inactivate organisms in biofilms when used at safe concentrations (*70, 99, 122*). Therefore, the matrix of the biofilm must be serve as some type of protection for organisms intertwined deep inside it. Biofilm formation also changes bacterial cell morphology. Some strains of *Listeria monocytogenes* undergo morphotypic conversion during biofilm

formation (*80)*. Contrarily, normal cell morphology can also be maintained during biofilm formation. For instance, Lazarevic *et al (72)* observed enzyme α-phosphoglucomutase contributing to retention of normal cell morphology of *Bacillus subtilis* during biofilm development.

Biofilm formation in the processing plant

Stainless steel is one of the most prevalent material found in poultry processing plants. The majority of the processing equipment is made from of it. Many wet chicken carcasses contact the equipment during processing, leading to bacterial attachment and ultimately to biofilm formation. Within six hours of poultry processing, biofilms can be formed on equipment (*7,8*). Heavy bacterial biofilm build up can develop and sloughing of pieces of biofilm can occur due to high pressure water spraying of the equipment during processing and clean up. This biofilm formation can increase chances of cross-contamination of carcasses and spread of pathogens though out the plant. Pathogens such as *Campylobacter* are able to adhere to chicken muscle and membrane collagens (*25 ,71 ,95*) associated with skin, providing a means for subsequent broiler carcasses to be cross-contaminated with bacteria from the equipment.

Nutrient Limitation

Nutrients for bacterial cells can come in the forms of micro- and macromolecules. However, nutrient limitation can decrease or increase survivability (*1, 36, 51*). Nutrient limitation can cause changes in growth rate, metabolism, culturability, and morphology (*2, 55, 88, 90*). Gene regulation, regulation of molecular functions, and development of resistance to stress are other cellular functions that are induced by nutrient limitation (*43, 58, 113, 121, 128*). Nutrient

limitation affects transcriptional control for major outer-membrane porins in *E. coli* (*78*) and induce survival in *Staphylococcus aureus* (*28*). Anderl *et al* (*6*) have shown that nutrient-deprived *Klebsiella pneumoniae* was less susceptible to antimicrobials such as ampicillin and ciprofloxacin. In biofilms, Mueller *(81)* showed that cells in a solid-water interface tend to detach in low nutrient environments, while Hunt *et al (54)* observed detachment of *Pseudomonas aeruginosa* biofilms when exposed to nutrient starvation. Nutrient limitation can also effect biofilm formation. Kim *et al (68)* reported that a reduction in levels of phosphate decreased biofilm development of *Listeria monocytogenes* on stainless steel surfaces.

Green fluorescent protein (GFP)

The green fluorescent protein (GFP) is an intrinsic fluorescing protein derived from the jellyfish *Aequorea victoria* (*18, 111*). This protein is responsible for a green bioluminescence in *Aequorea victoria* as well as other marine coelenterates. In 1971, GFP was first discovered by scientists Morin and Hastings, however most of the early characterization of this protein was done by research teams of Frank Johnson, Osamu Shimomura, and John Blinks between 1975 and 1978 (*111*). The green light of GFP is acquired though aequorin that is a calcium-ion activated photoprotein. Aequorin does not require exogenous cofactors to emit light. Its luminescence is blue, however, though energy transfer to the GFP, this blue light is emitted as green. The chomophore (a chemical group that absorbs light at a specific frequency and imparts color to a molecule) of this protein is derived from posttranslational cyclization (formation of one or more rings in a chemical compound) of a serine-tyrosine-glycine tripeptide followed by dehydration of the tyrosine and oxidation (*111*).

Wild-type GFP is made up of 238 amino acids, folded into a sequence of six alpha helices, and eleven beta strands. These strands are connected by loops that form a classical beta barrel (cylindrical beta sheet with anti-parallel strands). The beta sheet strands produce a regular pattern of hydrogen bonding. The GFP cylinder structure has dimensions made of a 24 Å diameter and length of 42 Å that is tightly packed. The stability of GFP fluorescence is due to the chomophore being buried deep within the beta barrel which distorts the helix positioned in the center. In this formation, the chomophore is protected from beta strand solvent interactions (*111*).

Wild-type GFP has two absorption maxima, 395nm and 475nm. The non-ionized form of the chomophore peaks at 395 nm, while the ionized form peaks at 475 nm. Ultraviolet light induces ionization and over time the chomophore neutral state returns (*33, 103, 111*). Interactions with GFP residues induce transitions within neutral ionized forms of the chomophore. Photobleaching is the photoinduced destruction of a chomophore molecule. The photobleaching of GFP is considerably less than fluorescein under common conditions. Resistance of photobleaching by GFP could be a result of the protection of the chomophore in the tightly packed beta-can structure (*111*). Since wild-type GFP chomophore structure is in an intramolecular rotation, it connects the two absorption peaks (395 nm and 475 nm) to each other. The structure is in an isomerization form that can be induced by irradiation at either peak which can allow both photoisomerization and photobleaching to occur if irradiated at 488 nm. Initially, photoisomerization causes high emission intensity then intensity decreases due to photobleaching. GFP brightness is also affected by pH. Wild-type GFP displays even brightness at a pH range from 5 to 10 (*111*).

GFP is usually visualized by using a fluorescence microscope. The components of a fluorescence microscope are an excitation light source, objective source, objective lens, filter cube, and detection. A mercury lamp or argon-ion laser (*25, 33, 103*) used to excite GFP. Quantitative imaging of GFP requires two significant components: the emission filter and detector. A filter cube used in qualitative imaging combines an excitation filter, dichoic mirror, and emission filter. However, visualization of GFP occurs only if the expression level of GFP is high enough (*111*).

GFP has been used as marker in both prokaryotic and eukaryotic cells. For bacteria, GFP is inserted into a plasmid then transduced into the cell (*18, 25, 33, 103*). For eukaryotes, GFP chimeras, fusion proteins (centromeric plasmids), and gene insertion are used for targeting (*111*). There are many bacterial studies that use GFP markers for various applications. A study by Skillman *et al (103)* used GFP to observe the individual interactions of GFP- labeled *Enterobacter agglomerans* and *E. coli* with non-fluorescent bacterial species in biofilm development. Bloemberg *et al (18)* used GFP as a marker for *Pseudomonas* strains to visualize cells in a mixed bacterial biofilm and analyze its association with tomato seedling roots. To study environmental regulation of bacterial major outer membrane protein (MOMP) porin expression, Dedieu *et al* (*35*) constructed a *momp*-gfp fusion from a *C. jejuni* MOMP and inserted into *E. coli*. In a direct microscopic observation analysis, Chantarapanont *et al* (*25*) used GFP-labeled *C.jejuni* to observe its attachment to various sites on chicken skin.

Lectins

Lectins are defined as carbohydrate-binding proteins other than enzymes or antibodies (*79*). In 1953, Boyd and Shipleigh first recognized these cell-agglutinating and sugar-binding

proteins. The word lectin originated from the latin word *legere* which means to pick out or choose. Lectins are oligomeric proteins with several sugar binding sites, commonly one site per subunit. Many lectins are glycoproteins with carbohydrate matter of 50% or more (*37*). Lectin molecular weights can range from 11,000 to 335,000 Da (*79*). Over 100 lectins have already been characterized and purified. Lectins are derived from many forms of life (Table 1).

Table 1: Sources of Lectins in Nature and the Environment (*37*)

Source	Location
Bacteria	Cell wall, cytoplasm, cytoplasmic membrane, fimbriae (pili), outermembrane, periplasm
Viruses	Bacteriophages, spikes from animal viruses
Yeasts, protozoa	Surface structures
Plants	Flowers, fruit, leaves, roots, saps, seeds, stems
Mammals	Eggs, lymphocytes, serum, sperm, tissues
Avian	Eggs, serums, tissues
Invertebrates	Crustaceans, insects, slugs, snails

Over 1000 plant species have been reported to have lectins and lectin-like activities. Most lectins are multivalent and have the ability to combine reversibly and noncovalently with mono- or oligosaccharides that are simple or complex, free in solution, or bound to a cell surface (*79*). Lectin receptors (cell-surface sugars) are located where binding involves forces such as hydrophobic and hydrogen bonds (most are hydrophobic) and rarely electrostatic forces. Lectin specificity depends on the monosaccharide or simple oligosaccharide that mostly prevents the lectin-induced cell agglutination or precipitation reaction. Effective concentrations of these specific inhibitors are in the millimolar range and lower (*79*). It is possible for lectins to possess

similar specificity toward monosaccharides, however, they may vary in their affinity to disaccharides, oligosaccharides and glycoproteins (*37, 79*). Even though lectins are similar to antibodies, they are not products of the immune system. They are diverse in structure, and they are only specific to carbohydrates. The specificity of lectins can be determined though hapten-inhibition experiments once the lectin has demonstrated binding to a particular cell (ex. bacteria, fungi, red cells, etc) (*37, 79*). Once specificity is determined, affinity purification methods for isolation of the glycoconjugate-binding proteins can be performed.

Specificities of lectins

Numerous lectins can bind to Gal or GalNac residues and most Gal or GalNac binding lectins complex with alpha- or beta-linked saccharides (*26, 79*). A small numbers of lectins are specific for anomeric linkages. Albeit a lectin binds to a certain saccharide, it may not bind to those same residues on a microbial surface (37). Quite often, hydrophobic residues enhance saccharide-lectin interactions (*79*). Lectins may also bind to metal ions and hydrophobic ligands (other proteins, glycolipids, lipoteichoic acids, etc). There are various microbial substrata to which lectins can bind (Table 2) (*37*).

Table 2: Lectin-Reactive Sites on Microorganisms (*37*)

Organism	Site(s)
Bacteria	Capsules, glycolipids, glycoproteins, group-specific polysaccharides, levans, lipomannas, lipooligosaccharides, lipopolysaccharides, lipoteichoic acids, peptidoglycans, surface array layers, teichoic acids, teichuronic acids
Viruses	Envelope glycoproteins
Protozoa	Galactomannans, glycolipids, glycoproteins, lipophosphoglycans, phosphoglycans

Lectins in microbiology

Lectin applications are used for various types of research in microbiology. Some of the applications are the following: affinity sorbents for microbial polymers and products; detection of microorganisms *in situ*; reagents for diagnostic microbiology; studies on adhesion mechanisms of microorganisms; and use in identification of antigens (*26, 37*). Studies have shown that lectins have no effect on cell viability, however they can prevent uptake of DNA by bacteria and prevent the expression of new genetic markers. Lectins can also induce physiological responses in an organism. For example, growth rates and culture yields of *Bacillus cereus* are stimulated by lectin ConA. Con A was the first lectin to have its specificity studied in detail. This lectin binds to unsubstituted nonreducing α-D-glucose (Glc) or α-D-mannose (Man) residues (*37*).

In microbiology, lectin derivatives (a reporter or sensitive tag bound to the lectin) are sometimes used instead of lectins. Derivatives can be used to monitor glycoconjugates in

solution or on surfaces of microbes. Some of the following are used in microbiological applications as lectin derivatives (*37*).

1) **Fluorescein isothiocyanate (FITC) derivatives -** Used to detect microorganisms and spores, and to analyze wall polymer distribution.
2) **Indirect agglutination**- Lectins bound directly to latex spheres resulting in passively sensitized particles to be used for aggregation reactions and establishing lectin specificities.
3) **Enzyme-linked lectinsorbent assays (ELLA)** - Lectins coupled with enzymes and used for detection of low concentrations of bacteria and bacterial spores.
4) **Salt-enhanced ELLA assays (SELLA)** - Detect extremely low concentrations of microbial glycoconjugates. It uses the process of ammonium sulfate to promote binding of a lectin or protein antigen to polystyrene.
5) **Fluorescent ELLA assays (FELLA)** - Used to detect glycoconjugates and microbes.
6) **GELLA assays** - Represent lectin-collodial gold mixtures that detect low densities of microbes or low concentrations of glycoconjugates.

Other assays are WELLA assays (Western blot modified to allow lectins to bind a macromolecule in a gel), BELLA assays (biotin-conjugated lectin that can be detected by an avidin-enzyme conjugate) and RELLA assays (lectins label with radioactive ^{3}H, ^{14}C, ^{131}I, or ^{125}I.

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CHAPTER 3

CULTURE AND DETECTION OF *CAMPYLOBACTER JEJUNI* WITHIN MIXED MICROBIAL POPULATIONS OF BIOFILMS ON STAINLESS STEEL[1]

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[1]Sheriase Q. Sanders, Dorothy H. Boothe, Joseph F. Frank, and Judy W. Arnold. To be submitted to *Journal of Food Protection*.

ABSTRACT

The objective of this paper was to elucidate the formation and composition of biofilms that contain *Campylobacter jejuni*. Biofilms containing natural populations of bacteria from the poultry processing environment and the target pathogen, *C. jejuni* 1221 gfp, were produced. *C. jejuni* growth was assessed with four media, two temperatures, and two atmospheric conditions to develop culture methods for liquid media that would allow growth within the biofilms. Growth kinetics were followed at four cell densities to determine temporal compatibility with biofilm mixtures. Thus, a protocol was developed for growing *C. jejuni* within biofilms on stainless steel coupons. Analysis of the biofilms by confocal laser scanning microscopy showed that *C. jejuni* 1221 gfp formed a biofilm on stainless steel when incubated without other bacteria. The average surface area of steel covered by *C. jejuni* 1221 gfp increased from 24 hours to approximately equivalent levels at 48 and 96 hours, respectively. *C. jejuni* 1221 gfp and natural bacterial populations formed biofilms on stainless steel. This mixture was characterized by an initial increase and subsequent decrease of the surface area coverage of stainless steel by *C. jejuni* 1221 gfp for each time period. Data on the surface area of stainless steel associated with *C. jejuni* 1221 gfp when incubated with either of two different initial inoculum densities of other bacteria suggested that the presence of natural populations of bacteria enhanced the numbers of *C. jejuni* on stainless steel. This work provides the basis to study interactions of *Campylobacter jejuni* with other bacteria.

The reduction and elimination of microbial pathogens in food products is the most pressing food safety problem today. To establish scientifically based regulatory guidelines, information is needed on growth and survival of specific pathogens *in situ,* such as *Campylobacter jejuni*. Although *C. jejuni* is a commensal organism in poultry *(14)*, it is also a major food pathogen associated with poultry products. In humans, *C. jejuni* is the etiologic agent of acute inflammatory enteritis which may be associated with Guillain-Barré syndrome, a disease of the peripheral nervous system *(9)*. Consumption of undercooked or improperly handled poultry products is considered to be a major route of infection *(14)*. Reduction of the incidence of this bacterium on raw poultry products will lead to a decrease in related food-borne illness.

One source of *Campylobacter* in poultry processing facilities may be equipment surfaces which have come in contact with contaminated broiler carcasses *(10, 11)*. When bacterial cells attach to a surface, their extracellular fibrils form a complex matrix with other microbes and debris *(1)*. The ultimate composite is a biofilm. Release of bacteria, such as *C. jejuni*, from biofilms may subsequently contaminate product passing over the surface of processing equipment.

Little is known about the presence of *C. jejuni* in biofilms on surfaces in poultry processing facilities. However, recent work by Trachoo *(18)* demonstrated that *C. jejuni* survives in biofilm formed on polyvinyl chloride used in poultry house water systems. Research on the attachment and viability on chicken skin of *C. jejuni* constitutively expressing green fluorescent protein (gfp) indicated that the bacterium can attach to skin, survive, and multiply during product storage at refrigeration or room temperature *(7)*. Use of *C. jejuni* gfp allowed the non-destructive detection of the pathogen on surfaces by confocal laser scanning microscopy

(CLSM). The green fluorescent protein of *Aequorea victoria*, encoded by the reporter gene *gfp,* is particularly useful for this type of study because it has been shown to be stable, resistant to photobleaching, and it does not require an exogenous substrate *(6)*.

The specific objective of this paper is to elucidate the formation and composition of biofilms that contain *C. jejuni*. Methods were developed for producing mixed biofilms on stainless steel. The biofilms included bacteria isolated from the poultry processing environment and the target pathogen, *C. jejuni*. The pathogen, constitutively expressing *gfp*, was observed by confocal laser scanning microscopy and epifluorescence microscopy. We investigated the incidence, viability, and fluorescence of *C. jejuni* on stainless steel with and without concomitant exposure to biofilms of other microbial populations during incubation periods of up to 7 days.

MATERIALS AND METHODS

Test surfaces. Stainless steel used for the coupons in this study was 11 gauge (3.04 mm thick) 304 American Iron and Steel Institute SS601-477-25M-GP stainless steel plate with a 2B mill finish. Coupons (1 x 4 cm) cut from the plate were obtained from Stork Gamco, Inc., Gainesville, GA, U.S.A. Prior to use in experiments, stainless steel pieces were soaked at room temperature in a 2% detergent solution (Micro®, Cat # 6732, International Products, Burlington, NJ, U.S.A.) for 5 min, followed by rinsing for 5 min under tap water to remove residual detergent. Coupons were placed in sterile test tubes containing at least 5 ml of distilled water, covered, and treated by sonication for 30 min to remove extraneous metal and other material from the surface**.** The steel pieces were air dried and placed in sterile uncovered Petri plates under ultraviolet light (100 microwatt seconds per cm/2) for 48 h on each side, to eliminate bacterial contamination prior to use.

Cultures. *Campylobacter jejuni* expressing green fluorescent protein (*C. jejuni* 1221 gfp) and its parent strain, *C. jejuni* RM1221 (isolated from a 1 *M* NaCl wash of a retail chicken carcass), were obtained from Robert Mandrell (Food Safety and Health Research Unit, ARS-USDA, Albany, CA, U.S.A.) via Robert Phillips (USDA-FSIS, Athens, GA, U.S.A.). Characterization of these strains is provided by Miller *et al. (12)*. *Campylobacter jejuni* subsp. *jejuni* #49943 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, U.S.A.) for use as a positive control. Identification of parent, fluorescent, and ATCC strains of *C. jejuni* was confirmed in this lab by a combination of latex slide agglutination and polymerase chain reaction (PCR) assays. Cultures were initially identified as *C. jejuni*, *C. coli*, or *C. laridis* using the INDX®-Campy (jcl)™ (Panbio Inc., Columbia, MD, U.S.A.) polyclonal antibody test. Subsequently, cultures were confirmed as *C. jejuni* by the presence of a specific 735-base pair product in agarose gels following electrophoresis of PCR products *(8)* from DNA extracts of these cultures.

Growth conditions. The maintenance and growth characteristics of *C. jejuni* 1221gfp were assessed prior to use in biofilm studies. *C. jejuni* 1221gfp was grown in Brucella broth (Hardy Diagnostics, Santa Maria, CA, U.S.A.) at 42^{o}C in a microaerobic environment (5% O_2, 10% CO_2, and 85% N_2) to an optical density (O.D.) of 0.8 at 410 nm. Aliquots (0.5 ml) of the culture were subsequently dispensed into 2 ml cryovials containing 40% glycerol (0.5 ml) and frozen at -40 ^{o}C. Growth characteristics of cultures of *C. jejuni* were determined by spectrophotometry (Beckman Instruments, Inc., Fullerton, CA, U.S.A.) in duplicate trials for each of three types of broth: Bolton's (Oxoid Ltd., Ogdensburg, NY, U.S.A.) without blood, Brucella and trypticase soy broth (TSB) (Becton, Dickinson and Company, Sparks, MD, U.S.A.).

A natural bacterial population for biofilm formation was obtained, as previously described by Arnold and Silvers *(3)*, from a saline rinse of defeathered, whole broiler carcasses collected in a commercial poultry processing facility. The whole carcass rinse was incubated at 37 ^{o}C for 18 h in TSB. Equal volumes (0.5 ml) of the rinse culture (WCR) were mixed with 20% glycerol in cryovials and stored at -40 ^{o}C. To resuscitate, 200µl of WCR was added to 9 ml Bolton's broth and incubated for 18 h at 37 ^{o}C. To determine bacterial concentration, 1 ml aliquots of culture were serially diluted into TSB, and dilutions from 10^{-1} to 10^{-10} were plated (0.1 ml aliquot) in triplicate on plate count agar. Plates were incubated 24 h at 37^{o}C, and the number of bacterial colony forming units (cfu) per ml in the original cultures was determined.

Biofilm formation. The following protocol was used to inoculate coupons with cultures of *C. jejuni* 1221gfp, WCR, or a combination of *C. jejuni* 1221gfp and WCR prior to CLSM analysis. *C. jejuni* 1221gfp in cryovials was thawed, and 100-µl aliquots were spread on Campy-Cefex agar plates which were incubated in a microaerobic environment at 37^{o}C for 48 h. Subsequently, *C. jejuni* 1221gfp was removed from agar plates and suspended in Bolton's broth without blood to an O.D. value of 0.70 to 0.80 (approximately 10^{9} cells/ml). *C. jejuni* 1221gfp was diluted five-fold to an O.D. value of 0.10 to 0.20 (approximately 2 x 10^{8} cells/ml) in a final volume of 10 ml Bolton's broth without blood. Thawed WCR cultures that were grown by adding 200 µl to 9 ml Bolton's broth and incubating for 18 h at 37 ^{o}C were diluted (from an O.D. of 1.5-1.6) either ten-or fifty-fold in the same manner as *C. jejuni* 1221gfp. The final O.D. in each test tube of WCR culture ranged from 0.1 to 0.5 for cultures diluted fifty- or ten-fold, respectively. Irradiated stainless steel coupons were added to duplicate or triplicate test tubes containing one of the following in 10 ml Bolton's broth without blood: *C. jejuni* 1221gfp (1:5 dilution), WCR (1:10 or

1:50 dilution), or *C. jejuni* 1221gfp (1:5 dilution) and WCR (1:10 or 1:50 dilution). Table 3.1 summarizes bacterial O.D. values of each of the treatments with stainless steel coupons after designated incubation periods. Test tubes with coupons in sterile Bolton's broth without blood were used as negative controls. After incubation of tubes at 37^{o}C for 24, 48, 96 or 168 h, coupons were aseptically removed for observation by confocal microscopy.

Counts. Numbers of C. *jejuni* 1221 gfp in cultures used in experiments were determined by ten-fold serial dilution of a culture in Bolton's broth without blood and subsequent spread plating of individual dilutions (0.1 ml) on Campy-Cefex agar plates prepared using a modified protocol of Englen *et al*. *(8)*. Modifications included replacing 0.2 g sodium cycloheximide with 0.05 g nystatin (1 ml of 0.5g nystatin in 10 ml sterile water) and 0.01 g rifampicin (2 ml of 0.1 g rifampicin in 10 ml 100% methanol and 10 ml sterile water) per liter of media. All antibiotics were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Numbers of colony-forming units (cfus) on plates were determined after plate incubation in a microaerobic environment at 42^{o}C for 48 h, and the number of bacterial cells per ml of original culture was calculated.

Fluorescence microscopy. CLSM was performed with a Leica TCS NT SP2 confocal microscope (Leica Microsystems, Heidelberg, Gmbh) equipped with an argon laser (excitation wavelength = 488 nm) to excite green fluorescent protein in cells of *C. jejuni* 1221gfp. Emitted light was collected though a triple dichoic mirror (TD) 488/568/633. A 63X water immersion objective lens was used for microscopic observation of treated and control stainless steel coupons secured on glass slides under 22 x 40 mm coverslips (#12-543-A, Fisher Scientific). TCS NT software (version 1.6.551; Leica Microsystems) was used to obtain and process 3 types

of images collected at a scan rate of 400 Hz (lines/sec). First, images assigned to the green channel were those with emitted light (499-539 nm) associated with gfp fluorescence. Second, images assigned to the red channel were those with emitted light (552-591 nm) associated with autofluorescence of the stainless steel. Third, images assigned to the gray channel were those with reflected light (476-496 nm). Two- or three-color images were obtained by overlaying images from individual channels using the TCS NT software. The surface area (μm^2) covered by *C. jejuni* 1221gfp cells was assessed using the histogram statistical function of the software. Regions of interest (ROIs) in micrographs identified by size, shape, and intensity of fluorescence represented cells (single or in clumps) of *C. jejuni* 1221 gfp. Cultures of *C. jejuni* 1221gfp grown on Campy-Cefex plates at 37^{o}C for 24, 48, 96, or 168 h were used as positive controls for confocal microscopy. Wet mounts on glass slides were prepared by suspending cells from the plates in Bolton's broth without blood.

The duration of fluorescence of *C. jejuni* 1221gfp grown in either broth or on solid media was tested. For both tests, *C. jejuni* 1221gfp (100-µl aliquots of a thawed culture) was initially spread onto Cefex plates, as described previously, and incubated in a microaerobic environment at 37^{o}C for 48 h. For testing the fluorescence of *C. jejuni* 1221gfp grown on solid media, cells were removed from plates every 48 h during a two-week period and suspended in 2 ml of Bolton's broth without blood. A wet mount of the suspension was prepared on a glass slide with 22 x 40 mm coverslips (#12-543-A, Fisher Scientific) and low fluorescence immersion oil (Cargille Type DF, R.P. Cargille Laboratories, Inc., Cedar Grove, NJ, U.S.A.) for observation by epifluorescence microscopy. For testing the fluorescence of *C. jejuni* 1221gfp grown in broth, cells were first removed from plates after 48 h of incubation and suspended in Bolton's broth without blood (30 ml) to yield a suspension with an O.D. value of 0.7-0.8. Subsequently, 2 ml of

the *C. jejuni* 1221 gfp broth suspension was diluted five-fold in each of six test tubes with Bolton's broth without blood (final volume of 10 ml). The diluted cultures (O.D. value of 0.1-0.2) were incubated at 37^{o}C for up to 168 h. After 72, 120, and 168 h of incubation, subsamples of the culture were removed from duplicate test tubes to prepare wet mounts, as previously described. Wet mounts of *C. jejuni* 1221gfp grown on Cefex plates or in Bolton's broth without blood were observed by epifluorescence microscopy (Nikon Eclipse E600, Southern Micro Instruments, Marietta, GA, U.S.A.) using a fluorescein optical filter (495-nm excitation and 518-nm emission) and a 100X oil immersion objective lens. Images were acquired using an Optronics Magnafire camera (Optronics, Goleta, CA, U.S.A.) and Lucius TM software (version 4.1, Image Content Technology LLC, New Britain, CT, U.S.A.).

The viability of *C. jejuni* 1221gfp was determined using the CTC (5-cyano-2,3-ditolyl tetrazolium chloride) staining protocol of Polyscience, Inc. (Warrington, PA, U.S.A.). A 4.0 mM solution of the CTC stain was prepared by adding 5.75 ml of distilled water to 0.5 ml of 50 mM CTC. *C. jejuni* 1221gfp (100-µl aliquots of a thawed culture) was initially spread onto Campy-Cefex plates, as described previously, and incubated in a microaerobic environment at 37^{o}C for up to 168 h. After 48, 96, and 168 h of plate incubation, *C. jejuni* 1221gfp cells were removed from plates and suspended in 2 ml of R2A broth (Difco Laboratories, Detroit, MI, U.S.A.). Cells were stained by adding 200 µl of the 4 mM CTC working solution to the suspension and incubating for 1 h at 37^{o}C. Stained cells were captured by microfiltration though 25 mm black polycarbonate membrane filters, 0.2 µm pore size (Osmonic Inc., Minnetonka, MN, U.S.A.). Filters were air-dried prior to mounting and viewing by epifluorescence microscopy using a 100x oil immersion lens with a fluorescein-Texas red double optical filter (470-nm excitation and 615-nm emission).

RESULTS AND DISCUSSION

A protocol was developed for growing *C. jejuni* 1221gfp alone or within biofilms containing bacteria from the poultry processing environment on stainless steel coupons. Twenty-two bacterial isolates from the WCR cultures have been identified previously *(5)*. Optimum growth characteristics of *C. jejuni* for compatibility within biofilms containing multiple species were determined in duplicate trials for each of three types of broth: Bolton's broth without blood, Brucella broth, and TSB. An incubation temperature of 37^{o}C was used because it was the approximate temperature in early processing after defeathering where the broiler carcasses were collected, and it is a favorable temperature for pathogen survival. Bolton's broth without blood was selected for growth of cultures used in biofilm studies because the O.D. values for *C. jejuni* 1221 gfp grown in Bolton's broth without blood exceeded those observed in Brucella broth or TSB under the conditions tested. Additional studies showed that the number of *C. jejuni* 1221 gfp cells grown in Bolton's broth without blood at 37^{o}C and 42^{o}C differed by less than one log unit (data not shown).

Biofilm formation of pure *C. jejuni* culture. CLSM micrographs showed that *C. jejuni* 1221 gfp formed a biofilm on stainless steel when incubated without WCR cultures (Figure 3.1a). The size, shape, and fluorescence intensity of single cells and microcolonies on steel were observed. The surface area (μm^2) of ROIs (regions of interest, corresponding to single cells and clumps of *C. jejuni*) was assessed using the histogram statistical function of the TCS NT software. The average area (in a 5.66 x 10^4 μm^2 field of view) covered by *C. jejuni* 1221 gfp increased from 24 h (40 μm^2) to approximately equivalent levels (65 and 66 μm^2) at 48 and 96 h, respectively (Table 3.2). Similar levels of C. *jejuni* 1221 gfp were also detected on steel exposed to this bacterium with a 1:10 dilution of WCR culture after incubation periods of 48 or 96 h.

Subsequently, the average surface area covered by *C. jejuni* 1221 gfp decreased (to 28 μm^2 after 168 h of incubation). In parallel to the increase in surface area of coupons associated with *C. jejuni* 1221 gfp over time, the number of discrete cells or cell clumps increased during 24 h to approximately equal levels at 48 and 96 h and decreased after 168 h.

***C. jejuni* and WCR biofilm formation.** CLSM micrographs also showed that mixtures of *C. jejuni* 1221 gfp and WCR formed biofilms on stainless steel (Figure 3.1b). These biofilms were characterized by an initial increase and a subsequent decrease of the surface area associated with *C. jejuni* 1221 gfp in the presence of WCR cultures (Table 3.2). When grown together, a 1:5 dilution of a *C. jejuni* 1221gfp culture and a 1:10 dilution of WCR culture, the average surface area (230 μm^2) initially covered by *C. jejuni* 1221gfp after 24 h of incubation exceeded that of *C. jejuni* 1221 gfp alone. Similar to results with a pure culture of *C. jejuni* 1221gfp, the average area covered by *C. jejuni* 1221gfp decreased to equivalent amounts (71 and 69μm^2) after 48 and 96 h of incubation when mixed with WCR cultures. The area covered by *C. jejuni* 1221gfp after 168 h of incubation of coupons with these diluted bacterial cultures was the least observed during the time course. For biofilms containing a 1:5 dilution of a *C. jejuni* 1221gfp culture and a 1:50 dilution of WCR culture, the average area (164 μm^2) covered at 24 h was greater than that observed with only *C. jejuni* 1221 gfp. However, less coverage was observed when mixed with a 1:10 dilution of WCR culture. A decrease in coverage of stainless steel with time was also observed with the mixture of a 1:5 dilution of a culture of *C. jejuni* 1221 gfp and a 1:10 dilution of WCR culture.

Data presented in Table 3.2 suggest that the presence of natural populations of bacteria enhanced the numbers of *C. jejuni* on stainless steel. CLSM micrographs (Figure 3.1b) showed

greater coverage of stainless steel by *C. jejuni* 1221 gfp after incubation with WCR. Sashahara and Zottola *(16)* demonstrated a similar enhancement of attachment by *Listeria monocytogenes* to stainless steel in flowing systems after initial colonization by *Pseudomonas fragi*. These authors suggested that the production of exopolymeric material by the initially colonizing pseudomonad enhanced the attachment to stainless steel of *L. monocytogenes*, which formed a sparse biofilm when grown in pure culture.

Stainless steel was selected as the substrate for biofilm formation in these studies because stainless steel is the most common food contact material found in the poultry processing plants *(2)*. Bacterial attachment to stainless steel typifies the attachment process for most other processing materials *(3)*. However, CLSM images of stainless steel coupons exhibited a yellowish-green autofluorescence. Light associated with this autofluorescence was subsequently defined as that with a wavelength between 552 and 591 nm and was displayed in a separate channel, designated as the red channel. By overlaying images from the gray, green, and red channels, areas of a coupon associated with *C. jejuni* 1221gfp (evidenced by only a green color) could be differentiated from those associated with autofluorescence (evidenced by red and green colors overlaying the same area). This autofluorescence diminished with increasing incubation periods of the coupons with WCR cultures, possibly due to coverage of the steel with non-fluorescent bacteria. Problems in distinguishing gfp from endogenous autofluorescence have been well documented, and a review of techniques for differentiating the two phenomena is available *(4)*. However, the major sources of autofluorescence discussed in the review by Billinton and Knight *(4)* are cellular or media in origin. In our studies, the autofluorescence originated with the stainless steel and not an organic component of the sample, as indicated by the presence of autofluorescence from stainless steel observed by CLSM with water as a negative

control (Figure 3.1c). This autofluorescence appeared to be related to the surface structure of the steel and was not uniform across the surface of an individual coupon.

Autofluorescence from the stainless steel coupons was confirmed (data not shown) using a commercially available gfp meter (Opti-Sciences Inc., Tyngsboro, MA, U.S.A.), which utilizes fiber optics to detect green fluorescent protein. This portable instrument was previously used to quantify fluorescence from gfp in intact plant organs *(13)*. Fluorescence counts were obtained from coupons incubated using the same protocol for observation by CLSM. Counts from coupons with sterile Bolton's broth (negative control) and from those with either *C. jejuni* 1221gfp or a mixture of *C. jejuni* 1221gfp and WCR cultures were similar, primarily due to the high background fluorescence counts obtained from the negative control. Significantly lower fluorescence counts of *C. jejuni* 1221gfp on glass slides, in either Bolton's broth or water, further indicated that much of the background fluorescence was due to autofluorescence of the stainless steel and not that of the Bolton's broth or an interaction of the broth with the stainless steel. The magnitude of the autofluorescence should be taken into account when assessing the presence of gfp expressed by bacteria or other organisms associated with stainless steel having a similar surface finish.

Viability and fluorescence duration. It is feasible that both live and dead cells of *C. jejuni* 1221 gfp were fluorescing during biofilm formation. A study by Skillman et al. *(17)* showed that dead *E. coli* cells with plasmid encoded gfp fluoresced as long as gfp was synthesized before death. Nonetheless, no studies have shown the continuation of fluorescence from gfp synthesis in cells after death which leads us to monitor fluorescence and check viability when observing the duration of fluorescence. In this study, cell viability was determined by CTC-staining. After

staining with CTC at a concentration of 4.0 mM, bright red fluorescence was detected in *C. jejuni* 1221 gfp cells when observed by epifluorescence microscopy at 48, 96, and 168 h. Red fluorescence from the formazan remained up to 168 h (Figure 3.2). This indicates that the cells remained viable up to seven days, since reduction of CTC to red, formazan requires actively respiring cells *(15).* In order to verify whether fluorescence from *C. jejuni* 1221 gfp diminished during this study, fluorescence was assessed by epifluorescence microscopy. *C. jejuni* 1221 gfp grown in Bolton's broth without blood and on solid media were observed for fluorescence. *C. jejuni* 1221 gfp from broth was observed after 72, 120, and 168 h of incubation under the conditions described above for biofilm formation. Fluorescence persisted for 168 h. *C. jejuni* 1221 gfp grown on solid media was monitored in 48 h intervals for two weeks. Fluorescence was strongly displayed though day 14 (Figure 3.3). Whether the cells were grown on solid media or in broth, fluorescence was similar.

In conclusion, biofilms containing natural populations of bacteria and *C. jejuni* were produced on stainless steel. The surface area covered by *C. jejuni* increased during the seven days when grown in Bolton's broth without blood at 37 ^{o}C. Viability of *C. jejuni* within the biofilms was maintained for seven days and fluorescence of gfp was maintained for 14 days, . Although WCR cultures are not necessary for attachment of C. *jejuni* 1221 gfp to stainless steel, the presence of WCR was associated with increased coverage of C. *jejuni.* Additional studies are needed to determine why this occurs. In addition, it is unclear whether nutrient limitation affected attachment because media was not replenished during the incubation. Future work with continuous cultures and *in situ* cultures during processing will be used to clarify the role of *C. jejuni* 1221 gfp and WCR bacteria in biofilm interactions. Identifying the role that pathogens play in bacterial attachment and biofilm formation will help to determine the relative importance

of pathogens found in the poultry processing plant to food safety. Our ultimate goal is to reduce the risk of food-borne disease by determining how the pathogen interacts with other bacteria in biofilms in food processing environments and to use this information to develop effective intervention strategies that minimize contamination of products.

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Table 3.1 Optical density of gfp[a] and WCR[b] bacterial cultures in Bolton's broth without blood after designated incubation periods with stainless steel coupons at 37 °C.

Bacterial cultures		Incubation period			
	Trial #	24 h	48 h	96 h	168 h
1:5 dilution gfp	1	ND[c]	0.171	0.160	0.276
		ND	------	0.186	0.300
	2	0.205	0.218	0.269	0.222
		------	0.223	0.243	0.231
1:5 dilution gfp + 1:10 dilution WCR	1	ND	1.459	1.339	1.478
		ND	1.320	1.358	1.359
	2	1.269	1.543	1.451	1.505
		1.308	1.434	1.466	1.389
1:10 dilution WCR	1	ND	1.408	1.422	1.400
	2	1.342	1.378	1.466	1.505
1:5 dilution gfp + 1:50 dilution WCR	1	ND	1.501	1.358	1.369
		ND	1.408	1.339	1.389
	2	1.334	1.422	1.498	1.402
		1.317	1.459	1.571	1.473
1:50 dilution WCR	1	ND	1.459	1.389	1.389
	2	1.361	1.485	1.322	1.429

[a] C. jejuni1221gfp (cell concentration before dilution = $1x\ 10^9$)

[b] Whole carcass rinse bacterial cultures (cell concentration before dilution $1x\ 10^9$)

[c] Not done

Table 3.2 Average area (μm^2), number of adherent cells and microcolonies of gfp[a] on stainless steel coupons over incubation periods with designated bacterial cultures in Bolton's broth without blood at 37^{o}C.

	1:5 dilution gfp			1:5 dilution gfp + 1:10 dilution WCR[b]			1:5 dilution gfp + 1:50 dilution WCR		
Time	Area[c]	# of ROIs[d]	N[e]	Area	# of ROIs	N	Area	# of ROIs	N
24h	40(14)	6	6	230 (41)	33	9	164 (35)	25	9
48h	65 (8)	11	9	71 (15)	14	12	88 (22)	15	12
96h	66 (6)	13	6	69 (13)	11	9	30 (9)	5	6
168h	28 (7)	5	6	17 (2)	4	10	12 (3)	3	9

[a] *C. jejuni* 1221gfp (cell concentration before dilution=1x10^9)

[b] Whole carcass rinse bacterial cultures (cell concentration before dilution 1x 10^9)

[c] Area=mean (standard error).

[d] ROI=region of interest, representing areas of gfp associated with stainless steel

[e] N=number of samples

Fig. 3.1 Confocal laser scanning microscopic images of stainless steel coupons incubated for 24 h at 37^{o}C with either (A) 1:5 initial dilution of *C. jejuni* RM1221 gfp or (B) 1:5 initial dilution of *C. jejuni* RM1221 gfp + 1:10 initial dilution whole carcass rinse cultures. (C) Negative control sample=coupon with water. Images were obtained using a 63X water immersion objective. Field of view is 238 µm x 238 µm.

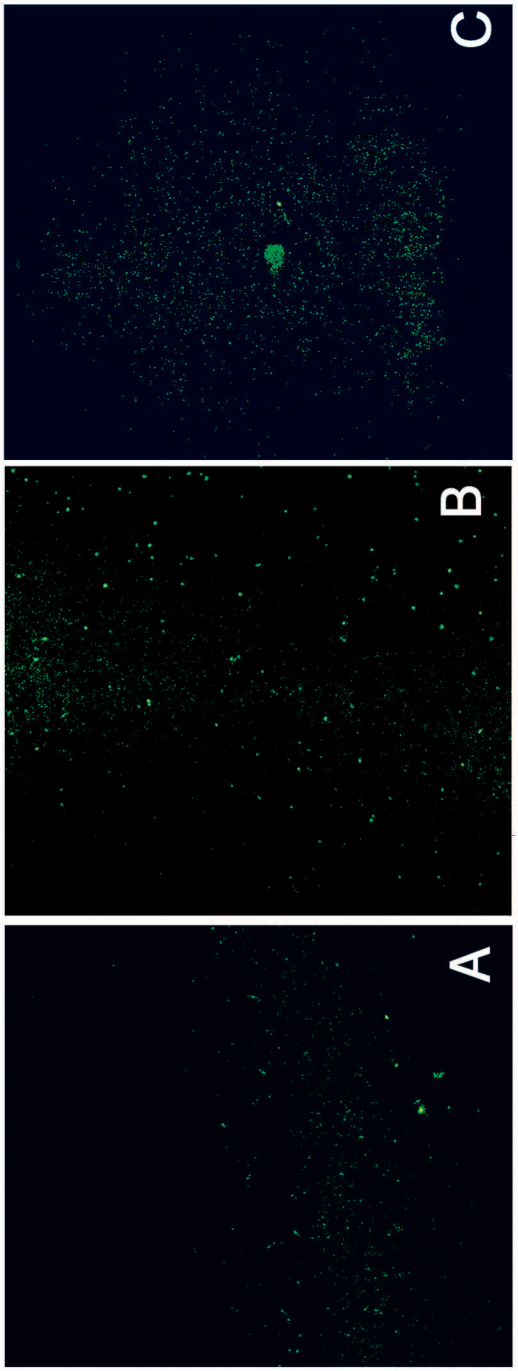

C
B
A

Fig. 3.2 Epifluorescence image at 168 h of *C. jejuni* RM1221 gfp stained with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) stain and captured on black polycarbonate membrane filter. Cells were grown at 37^{o}C and observed after 48, 96 and 168 h of incubation (100x oil immersion).

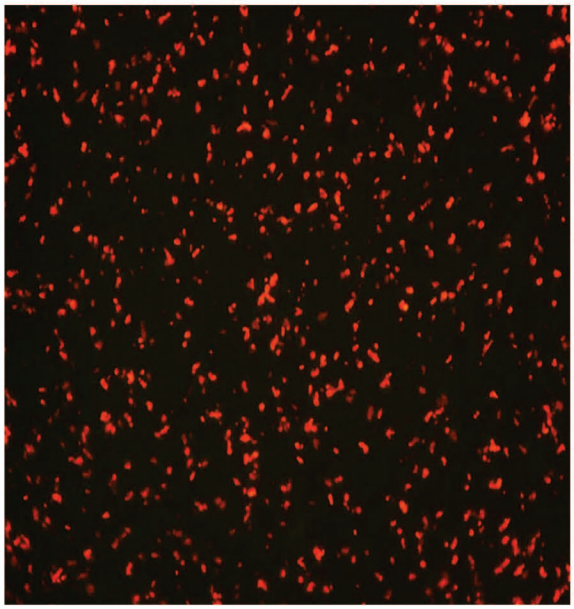
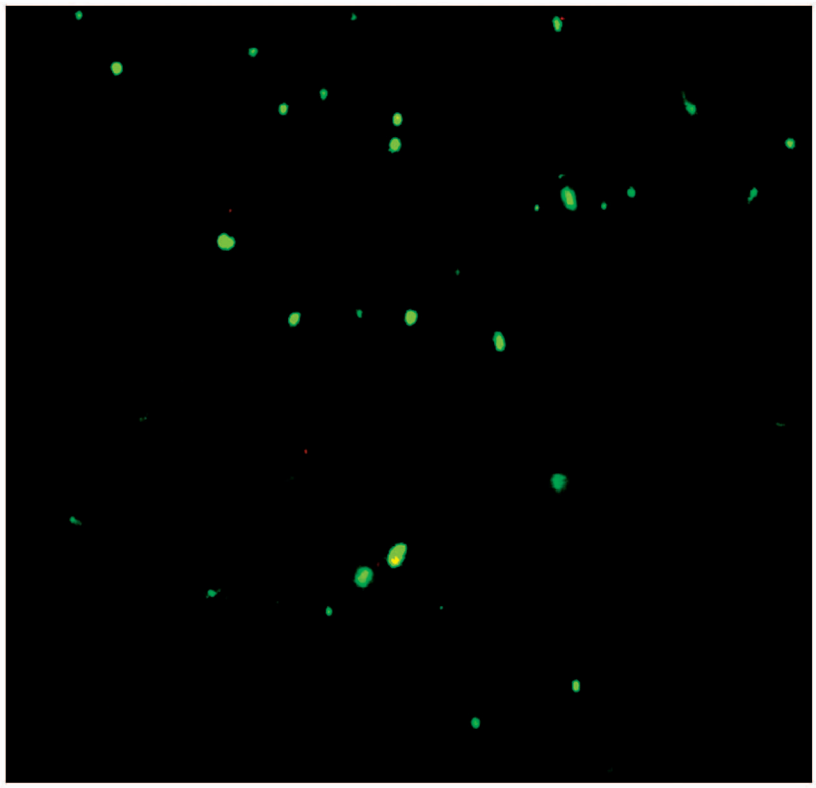


Fig. 3.3 Epifluorescence image at day 14 of *C. jejuni* RM1221 gfp grown at 37^{o}C. Cells grown in Bolton's broth were observed after 72, 120, and 168 h, and cells grown on solid medium were observed every 48 h for 14 days.



CHAPTER 4

ATTACHMENT AND SURVIVABILITY OF *CAMPYLOBACTER JEJUNI* AT VARIOUS TEMPERATURES IN MIXED POULTRY BACTERIAL ISOLATE BIOFILMS ON STAINLESS STEEL[1]

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[1]Sheriase Q. Sanders, Joseph F. Frank, and Judy W. Arnold. To be submitted to *Journal of Food Protection*.

ABSTRACT

Campylobacter jejuni is a thermophilic, microaerophilic pathogen that is commonly found in the intestinal tract of poultry. During the defeathering phase in poultry processing, feces can be excreted from the broiler carcass onto equipment exposing *C. jejuni* to the environment. Biofilms formed on stainless steel equipment allow opportunity for *C. jejuni* attachment. Since the temperature varies thoughout processing plants, biofilm formation and coverage may also vary along with the attachment of *C. jejuni*. In this study, the effects of temperature on biofilm coverage and *C. jejuni* attachment to biofilms formed on stainless steel were observed. Bacterial isolates collected from a saline rinse of broiler chicken carcasses were used to form biofilms at 13, 20, 37 and 42 °C over a 16 h time period on stainless steel coupons. After the 16-h biofilm formation, surface coverage of biofilms was determined. Biofilms formed at 13 and 20 °C yielded the highest percentage area coverage, 47.6 and 38.7%, respectively, but at 37 and 42 °C, surface coverage was low: 10.4 and 2.1%. *C. jejuni* attachment to pre-existing biofilms at the four temperatures were not significantly different from one another, even though biofilm surface coverage was sparse at higher temperatures (37 and 42 °C).

Culturable, viable cells of *C. jejuni* were only recovered from biofilms formed at 13 and 20 °C, which indicates that *C. jejuni* cells attached at 37 and 42 °C could be in the viable but nonculturable form or simply nonviable. This research shows that *C. jejuni* is able to attach and survive in preexisting biofilms formed on stainless steel under favorable conditions.

Campylobacter jejuni is the leading cause in food-borne illness in the United States. Approximately, 2 million cases of enteritis are caused by this organism each year (*3*). *C. jejuni* is largely associated with poultry and is known to be located in the chicken gut where it can survive at high temperatures, such as 42°C (*23*). It can also survive temperatures as low as 4°C (*5*). However, this pathogen does not survive well at 25°C. Therefore, temperature plays an intricate role in bacterial survival. This extrinsic factor is able to control many genetic and physiological activities in microorganisms (*29*). In *Pseudomonas syringae* phytotoxin corontine biosynthesis, temperature can influence promoter activity, transcript abundance and protein stability (*4*). Low temperatures have been able to affect membrane fatty acid relationship to growth (*2*). On mesophilic *Shewanella oneidensis*, long motile filaments were formed an the bacterium when grown in cold environments as opposed to cells grown close to room temperature (*1*). Sometimes at low temperatures, bacteria may also require more nutrients for survival (*24*). Other physiological properties such as growth stress (*12*), growth rate (*25*), and pH are manipulated by temperature (*14, 17*). Tienungoon *et al* (*21*) reported on the growth limits of *Listeria monocytogenes*, including temperature, pH, NaCl and lactic acid. The growth and pectinolytic activity of *Pseudomonas marginalis* were observed by Membre *et al* (*10*) for effects by temperature. Rowan *et al* (*18*) used above-optimum growth temperature to observe thermotolerance in *L. monocytogenes*.

Bacterial adhesion and biofilm development are also affected by temperature. The process of biofilm formation starts with primary reversible adhesion which leads to irreversible adhesion and the formation of the biofilm (*7*). Temperature affects adaptation of bacterial communities in the environment (*15*). The influence of temperature on bacterial adhesion has been determined (*13*). Surface physicochemical properties of bacteria correlate with adhesion

and surface colonization at different temperatures (*6*). Rogers *et al* (*16*) investigated temperature influence on *Legionella pneumophila* biofilm survivability on plumbing material. In the study, the pathogen flourished in biofilms formed on plastics at 40 °C, but was not detected at 60 °C. In other studies, temperature influenced incubation time for biofilm development and biofilm thickness (*20*).

Biofilms are a major concern in the food industry particularly in food processing plants. Stainless steel, the most prevalent material for equipment surfaces in processing plants, can enable attachment of food-borne pathogens alone or in biofilms if the temperature is favorable (*9, 27*). If biofilms are formed, they can cross-contaminate food which can lead to food spoilage or transmission of disease (*26*).

In this study, temperature effect on the attachment and survivability of *C. jejuni* on biofilms formed by poultry isolates was investigated. The *C. jejuni* strain used in the experiments was labeled with a green fluorescent protein. The results from this research will contribute to the understanding of pathogen survivability on stainless steel equipment though out the poultry processing plant.

MATERIALS AND METHODS

Test surfaces. Stainless steel (SS) used for the coupons in this study was 11 gauge (3.04 mm thick) 304 American Iron and Steel Institute SS601-477-25M-GP stainless steel plate with a 2B mill finish. Coupons (1 x 4 cm) cut from the plate were obtained from Stork Gamco, Inc., Gainesville, GA. Prior to use in experiments, stainless steel pieces were soaked at room temperature in a 2% detergent solution (Micro®, Cat # 6732, International Products, Burlington, NJ) for 5 min, followed by rinsing for 5 min under tap water to remove residual detergent.

Coupons were placed in sterile test tubes containing 5 ml of distilled water, covered, and treated by sonication for 30 min to remove extraneous metal and other material from the surface**.** The steel pieces were air dried and placed in sterile uncovered Petri plates under ultraviolet light (100 microwatt seconds per cm/2) for 48 h on each side, to eliminate bacterial contamination prior to use.

Cultures. *Campylobacter jejuni* expressing green fluorescent protein (*C. jejuni*1221gfp) and its parent strain, *C. jejuni* RM1221 (isolated from a 1 *M* NaCl wash of a retail chicken carcass), were obtained from Robert Mandrell (Food Safety and Health Research Unit, ARS-USDA, Albany, CA, U.S.A.) via Robert Phillips (USDA-FSIS, Athens, GA, U.S.A.). Characterization of these strains is provided by Miller *et al. (11)*. *Campylobacter jejuni* subsp. *jejuni* #49943 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, U.S.A.) for use as a positive control. Identification of parent, fluorescent, and ATCC strains of *C. jejuni* was confirmed in this lab by a combination of latex slide agglutination and polymerase chain reaction (PCR) assays. Cultures were initially identified as *C. jejuni*, *C. coli*, or *C. laridis* using the INDX®-Campy (jcl)™ (Panbio Inc., Columbia, MD) polyclonal antibody test. Subsequently, cultures were confirmed as *C. jejuni* by the presence of a specific 735-base pair product in agarose gels following electrophoresis of PCR products (*8*) from DNA extracts of these cultures. The whole carcass rinse (WCR) culture obtained from Judy Arnold (ARS-USDA, Athens, GA, USA) consisted of a mixed population of bacteria collected from a saline rinse of defeathered, prechilled, whole broiler carcasses collected in a commercial poultry processing facility.

Culture preparation and biofilm formation

A frozen cryovial of whole carcass rinse (WCR) culture was thawed, 200 µl aliquots were inoculated into a tube of 9 ml tryptic soy broth (TSB) (Difco, Sparks, MD), and incubated overnight at 37 °C. Optical density (O.D) of the overnight culture was measured on a Biotek spectrophotometer (Winooski, VT). Then a ten-fold dilution of the culture was made in TSB, in a total volume of 10 ml. The 1:10 diluted culture final concentration yielded 10^8 cells/ml. SS coupons were inserted into the diluted cultures, then incubated at 13, 20, 37 and 42 C for 16 h to allow for biofilm formation. After 16 h incubation, SS coupons were removed aseptically from tubes and rinsed with 9 ml of sterile TSB. 6

Staining of biofilms

The coupons were flooded with Hoescht DNA stain (Sigma Aldrich, Milwaukee, WI) to visualize biofilms. The Hoescht stain was diluted 1:10 from a 50 µl/ml concentration stock solution before being applied. Coupons were immersed in the stain for 30 min in a dark container before being rinsed, air dried and viewed under the epifluorescent microscope for observation of biofilm coverage.

Gfp attachment

*C. jejuni*1221gfp was thawed and 100 µl was spread onto Campy Cefex plates grown at 37°C under microaerophilic conditions (5 % O_2, 10 % CO_2, and 85 % N_2) for 48 h. Campy-Cefex agar plates were prepared by using protocol of Englen *et al*, (*8*). Cells were harvested from plates by 10 µl loop and suspended into 20 ml of TSB at room temperature to achieve an O. D. value of

approximately 0.8 – 1.0 at 410 nm. The culture was then diluted five-fold in TSB yielding a concentration of 10^7- 10^8 cells/ml.

After dilutions were prepared, SS coupons with 16 h WCR biofilm formation (prepared from previous protocol) were inserted into the *C. jejuni*1221gfp suspension and incubated for 24 h at 13, 20, 37 and 42 °C to allow for attachment. The SS coupons were then aseptically removed, rinsed with 10 ml of sterile distilled water and observed under epifluorescence microscopy.

Recoverable plate counts of *C. jejuni* from biofilm

After WCR biofilm formation and attachment of *C. jejuni*1221gfp, SS coupons were rinsed with 10 ml of sterile distilled water on each side, submerged into 9 ml of TSB broth and then scraped with a sterile 1.8 cm wide disposable cell scraper (Costar, Cambridge, MA), approximately 100 times per side into broth. After scraping, the broth containing the suspension of the detached biofilm cells was serial diluted up to 10^{-8} and plated on Campy-Cefex agar plates for culturable counts. The plates were incubated at 37 °C for 48 h. Counts were taken after incubation.

Microscopic Analysis

Attached *C. jejuni*1221gfp cells were observed by epifluorescence microscopy (Nikon Eclipse E600, Southern Micro Instruments, Marietta, GA, U.S.A.) using a fluorescein optical filter (495-nm excitation and 518-nm emission) and a 100X oil immersion objective lens. For Hoescht stained biofilm cells, UV-2E/C (DAPI/Hoescht) filter cubes (360 nm excitation and 400 nm emission) and barrier filter (460 nm) were used. Images were acquired using an Optronics Magnafire camera (Optronics, Goleta, CA, U.S.A.) and Lucius TM software (version 4.1, Image

Content Technology LLC, New Britain, CT, U.S.A.). For image analysis, ImageTool for Windows, version 3.0 (University of Texas Health Science Center at San Antonio) was used to acquire surface coverage of biofilms. Image microscopic field of view was an area of 90μm^2.

Data analysis

Data were analyzed with SAS software (Statistical Analysis System Institute, Cary, N.C.) using SAS analysis of variance (PROC ANOVA). Significant differences among means were determined by the least significant differences test (P= 0.05). Duplicate samples were prepared for each treatment and ran in three trials. *C. jejuni* cells were counted manually in at least five microscopic fields per trial. Counts were averaged per trial.

RESULTS AND DISCUSSION

Biofilms can form on any surface as long as nutrient and environmental (temperature, pH, surface, etc) conditions are favorable. In nature, biofilms are usually heterogeneous in species composition (*6*). Biofilms can be found on various surfaces. These include biotic and abiotic surfaces such as animal skin, pipelines, biomedical implants and vegetables (*7*). In this research, biofilm production from mixed population poultry isolates on stainless steel material and the attachment of *C. jejuni*1221gfp to these pre-existing biofilms were observed at temperatures 13, 20, 37 and 42 °C.

Temperature effect on WCR biofilm coverage on stainless steel coupons

Biofilms were produced, using a bacterial culture from a whole carcass rinse at four different temperatures (13, 20, 37 and 42 °C). These of the temperatures were chosen to correspond with

environmental temperatures in the processing plant. The temperature of 13 °C represents temporary storage area conditions before moving to further processing, 20 °C corresponds with the evisceration room, 42 °C represents the scald tank, and 37 °C corresponds with the defeathering environment. Biofilms were formed on stainless steel coupons over a 16 h time period to correlate with two 8 h processing shifts in the plant before the equipment is cleaned and sanitized. This time period would show how much biofilm would be produced within a 24 h period in the processing plant on the equipment if were not clean.

After 16 h of biofilm formation, the coupons were stained with Hoescht DNA stain to view surface area coverage. Biofilms formed produced similar coverage at 13 and 20 °C (47.6 and 38.7%), but were significantly greater than coverage (Table 4.1) at 42 °C. Depending on the environmental conditions, certain microorganisms can dominate and out-compete others in biofilms (*28*). At 13 and 20 °C, the biofilm consisted primarily of rod shaped cells and displayed more uniform coverage (Figure 4.1 (a) and (b)). These rods could represent psychotrophic bacteria from the chicken rinse, which found the lower temperatures to be most advantageous for survival and attachment to allow biofilm formation. The domination of these rod-shaped bacterial cells could also be attributed to competition for nutrients and possible toxic by-products produced (*7*). Biofilms produced at the higher temperatures (37 and 42 °C) displayed more sparse and patchy coverage of cells consisting of diverse morphology (Figure 4.1.(c) and (d)).

Attachment of *C. jejuni* to biofilms

Epifluorescent microscopy was used to view *C. jejuni*1221gfp attachment at different temperature treatments. A concentration of 10^7-10^8 cells/ml of *C. jejuni*1221gfp were attached to WCR biofilm formed on the coupons at 13, 20, 37 and 42 °C after 16 h. *C. jejuni*1221gfp was

allowed to attach for 24 h and then observed (Figure 4.2). Even though biofilm coverage varied between the highest (42 °C) and lowest (13 °C) temperature treatments, *C. jejuni*1221gfp attachment was not different (P= 0.05) when viewed under epifluorescent microscopy (Table 4.2). These results showed that surface coverage of pre-existing biofilms did not make a difference in the frequency of attachment of *C. jejuni*1221gfp. However, this suggests that at the higher temperatures (37 and 42 °C), *C. jejuni*1221gfp may be attaching directly to the SS coupon since biofilm coverage is low under these conditions. In addition, the morphology of attached *C. jejuni*1221gfp was difficult to determine. Spiral forms of *C. jejuni*1221gfp could not be confirmed by microscopic observation. Therefore, coupons were scraped and plated for viable *C. jejuni*1221gfp attachment to determine culturability.

Culturable *C. jejuni* from biofilms

*C. jejuni*1221gfp, allowed 24 h for attachment to WCR biofilms grown at 13, 20, 37 and 42 °C, were detached and plated to determine culturability. Trachoo *et al* (*22*) previously has shown recovered *C. jejuni* attached to pre-existing biofilms incubated at 12 and 23 °C for 4 days. In this study, colony forming units were recovered only from 13 and 20 °C (Table 4.3), albeit *C. jejuni*1221gfp was attached on the stainless steel at all the temperature treatments. *C. jejuni*1221gfp could have been in the VBNC form at the 37 and 42 °C forms at the end of the 24h incubation during attachment. At these temperatures, cells may be nonviable but still fluorescing. A bacterial cell labeled with green fluorescent protein does not have to be viable in order to fluoresce (*19*). These results indicate that under these poultry processing plant conditions, *C. jejuni* can attach to preexisting biofilm, survive, and recontaminate carcasses in storage areas and in the evisceration room.

In conclusion, this study indicated that *C. jejuni* is able to attach to and survive in pre-existing biofilms on stainless steel in the poultry processing environment at various temperatures. At high temperature environments such as the kill and scald tank areas, *C. jejuni* may be able to attach to stainless steel equipment, not needing a pre-existing biofilm. However, attached *C. jejuni* at these temperatures are most likely to be in the VBNC form. Contrarily, due to more profuse and uniform biofilm coverage on stainless steel, cooler areas such as the evisceration and storage areas may harbor greater numbers of more culturable *C. jejuni*. Therefore, temperature is a critical factor in the survivability and attachment of *C. jejuni* in poultry processing environments.

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Table 4.1 Surface area coverage of biofilms formed at different temperatures[a]

Treatment	Percent area coverage[b]
13 °C	47.6 A
20 °C	38.7 A B
37 °C	10.4 B C
42 °C	2.1 C

[a] Means of percent area coverage of whole carcass rinse biofilms from three trials.

[b] Means in columns with the same letters not significantly different ($P < 0.05$).

Table 4.2 *C. jejuni*1221gfp attachment to preexisting biofilms on stainless steel[a].

Treatment	No. of attached *C. jejuni*[b]
13 °C	2.8 A
20 °C	1.9 A
37 °C	4.3 A
42 °C	1.7 A

[a] Means of attached *C. jejuni*1221gfp values from three trials.

[b] Means in columns with the same letters not significantly different ($P < 0.05$).

Table 4.3 Culturable counts (cfu/cm^2) of attached *C. jejuni*1221gfp from stainless steel coupons after 24 h incubation in TSB at different temperatures.[a]

	13	20	37	42
Trial 1	7.70×10^4	1.13×10^5	$< 10^2$	$< 10^2$
Trial 2	3.40×10^4	2.19×10^4	$< 10^2$	$< 10^2$

[a] Means from two trials with duplicate samples.

Figure 4.1 Biofilm formation grown for 16 hours on stainless steel coupons at temperatures (a) 13, (b) 20, (c) 37 and (d) 42 °C. Biofilm cells were stained with Hoescht DNA stained. Bar = 10μm.

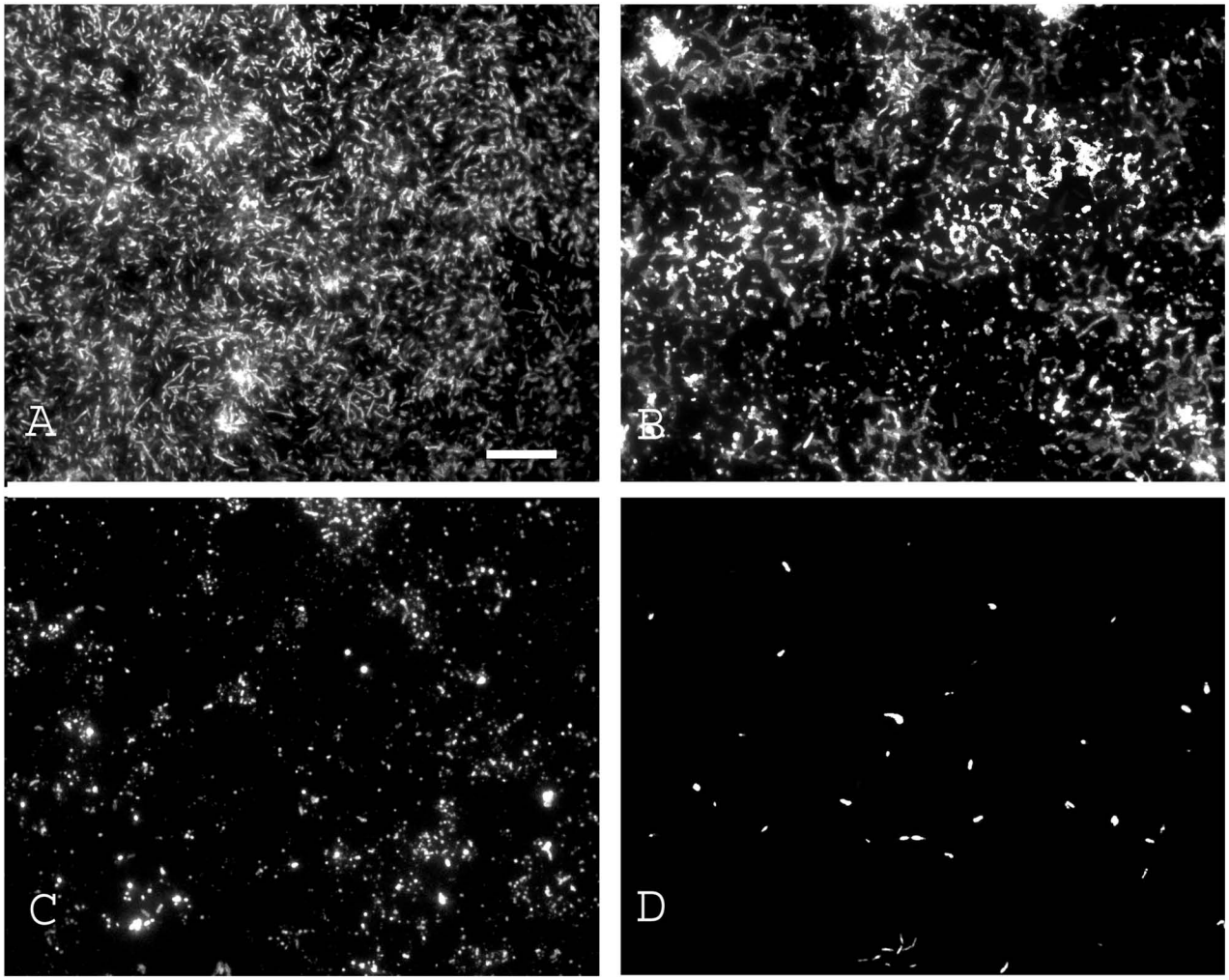
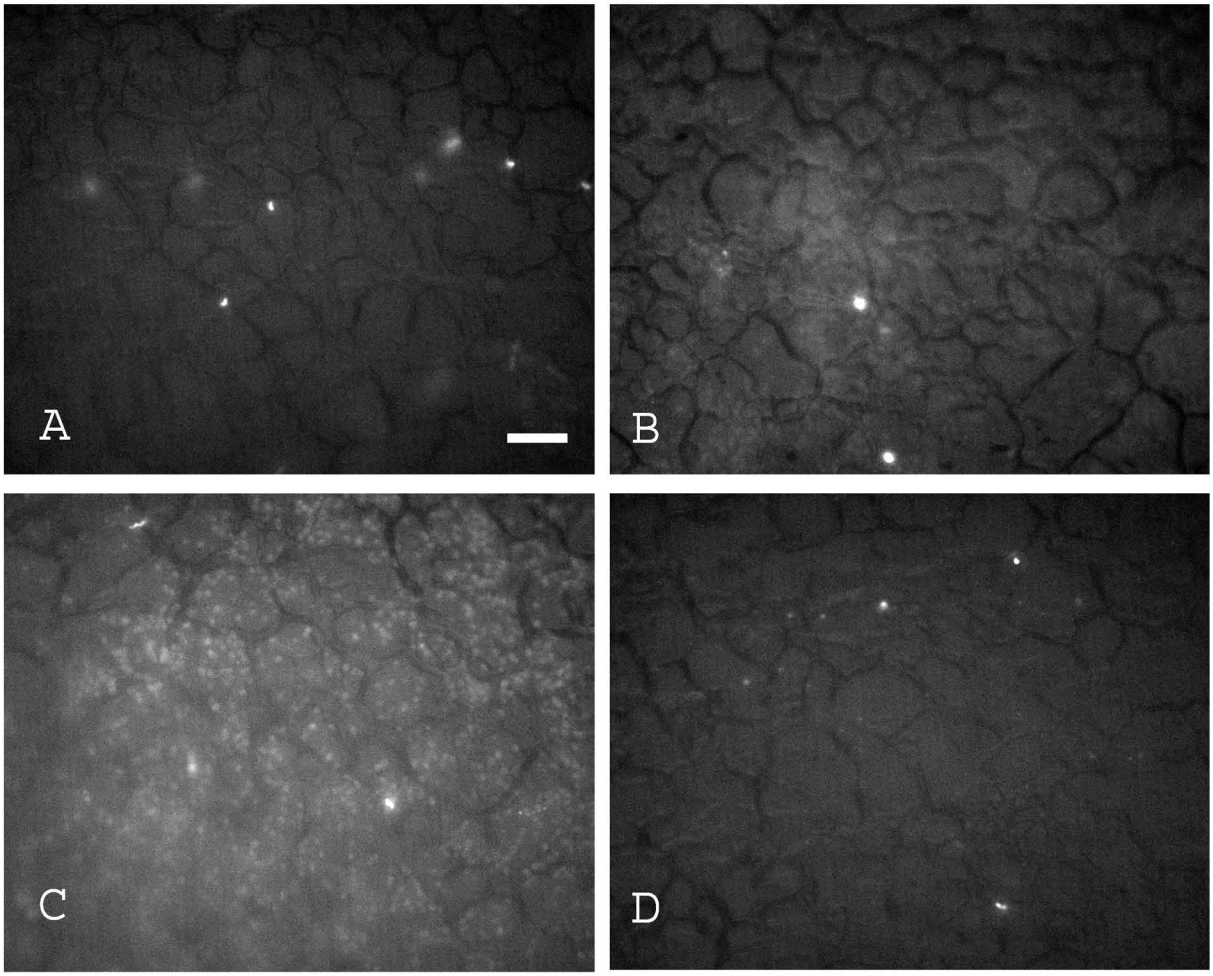

A
B
C
D

Figure 4.2 *C. jejuni*1221gfp attached biofilms formed on stainless steel coupon after 24 hours. Attachment assay temperatures were (a) 13, (b) 20, (c) 37 and (d) 42 °C. Bar = 10μm


A
B
C
D

CHAPTER 5

NUTRIENT LIMITATION EFFECTS ON *CAMPYLOBACTER JEJUNI* ATTACHMENT AND SURVIVAL IN MIXED BIOFILMS ONSTAINLESS STEEL[1]

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[1]Sheriase Q. Sanders, Joseph F. Frank, and Judy W. Arnold. To be submitted to *Journal of Food Protection*.

ABSTRACT

C. jejuni is a gram-negative, microaerophilic bacterial pathogen that can cause gastroenteritis. *C. jejuni* can be excreted into the poultry processing plant environment during defeathering and in the evisceration area (approximately 20 °C) where it could rinse off, come in contact with stainless steel equipment and form biofilms. Due to water flow, various nutrients levels could be present in the environment. In this study, *C. jejuni* attachment and survivability in biofilms on stainless steel were assessed in relation to nutrient limitation. Tryptic soy broth (TSB) was diluted ten- and fifty-fold and then inoculated with an overnight mixed bacterial culture collected from a whole carcass rinse (WCR). Stainless steel coupons were inserted into the tubes of diluted broth and allowed to form biofilms incubated at 20 and 37 °C for 48 h. Biofilm surface area coverage on coupons was the highest in both concentrations of TSB in 37 °C (approximately 2%). *C. jejuni* was allowed to attach to coupons with a 48 h biofilm already existing on the surface for 48 h at 20 and 37 °C. Numbers of attached *C. jejuni* differed between treatments of 1:10 TSB (20 °C) and 1:50 TSB (37 °C): numbers from 1:10 TSB (20 °C) were the highest of the two. Also, after 48 h *C. jejuni* attachment to the coupons with preexisting biofilms, *C. jejuni* were scraped off and detached cells were suspended in TSB, serial diluted and plated on Campy Cefex agar for counts. Counts were recovered only from coupons incubated at 20 °C. Epifluorescent micrcoscopy showed *C. jejuni* attached directly to coupons instead of biofilm areas on the coupons in both broth concentrations and treatments. These results show that *C. jejuni* can attach to and survive on stainless steel equipment in low nutrient environments and that temperature combined with nutrient availability affect attachment of *C. jejuni*.

Campylobacter jejuni is one the leading causes in gastroenteritis in the United States. This bacterium is commonly found on both live and processed chickens. Since *C. jejuni* has been found on retail chicken, it could possibly be surviving in the poultry processing plant environment. Bacterial biofilms have the potential to develop on stainless steel equipment in processing plants especially in the evisceration area where equipment is frequently sprayed with water during shifts and come in contact with bacteria and nutrients from the chicken carcasses during processing. Biofilms formed on the equipment, could harbor attached pathogens, such as *C. jejuni,* which could recontaminate the chicken during or after processing if the carcass comes in contact with the equipment. The evisceration area would also vary in concentration of nutrients available to bacteria in the environment due to some locations being flushed with more water than others.

Nutrient limitation and starvation can cause diverse responses from various microorganisms. Nutrient limitation can lead to reduced cell size and cross-protection to environmental stress (*2, 13*), affect expression of major outer membrane porins (*18*) and change bacterial protein synthesis and morphology (*1, 25*). Phylogeneticaly different bacteria are able to outcompete and survive under harsh conditions with low nutrients (*17*). Cell division can also be reduced if essential nutrients are depleted from the growth environment (*5*). Changes in physiology occur in order to insure survival. Notely (*22*) reported *E. coli* shifting into a protective stationary phase response when nutrient levels of glucose fell below a sufficient point. Some bacteria are able to shift to extreme competitive survival modes. For example, sporulating *Bacillus subtilus* can trigger cannibalism when nutrient-deprived (*11*). In this case, cells that have begun the sporulation process produce a killing protein which blocks sporulating sister cells and lyses them. After sister cells are lysed, the original sporulating cells consume the released

nutrients to continue growth. Bacteria genetics are also affected by nutrient limitation. Griffioen *et al* (*10*) demonstrated how nutritional changes determined ribosomal protein gene transcription in *Saccharomyces cerevisae*.

Bacterial adhesion is affected by nutrient availability. Decrease in attachment ability can occur (*8*) if cells are starved. In some cases, nutrient limiting media can produce higher expression of adhesion proteins in bacteria when compared to bacteria grown in nutrient rich media (*14*). Therefore, nutrient deprivation does not produce the same effects in bacterial adhesion.

There are various ways bacterial communities are affected by nutrient limitation. Matz *et al* (*19*) observed changes in phenotypic structure of some lakewater bacterial communities when carbon and phosphorus were limited. Carbon limitation produced small and motile cells while phosphorus limitation resulted in bacteria having large, elongated and capsulated characteristics. Allan *et al* (*3*) also demonstrated how lactose limitation over a period time altered *Citrobacter* sp. cell and biofilm morphology. Biofilm bacteria can build up resistance to antibiotics when exposed to nutrient limitation. In one study, *Klebisella pneumoniae* biofilm bacteria entered into stationary phase when nutrients were limited in the environment (*4*). This depression of the growth allowed resistance against the killing of ampicillin and ciprofloxacin antibiotics. Bacterial transport and colonization can be negatively or positively affected by in low nutrient environments (*21*) as well as biofilms detaching under these conditions (*7*). Trachoo *et al* (*24*) has reported that *C. jejuni* is able to attached to biofilms already formed on inanimate surfaces such as polyvinyl chloride, and this bacterium was reported (chapter 1) to attach to stainless steel with and without other biofilm constituents. Temperature also plays a critical role in biofilm formation, however, *C. jejuni* attachment was observed in nutrient rich environments (reported in

chapter two). Therefore, the objective in this study was to observe the ability of *C. jejuni* to attach and survive on biofilms formed on stainless steel in a nutrient limited environment.

MATERIALS AND METHODS

Test surfaces. Surfaces used in this study were 11 gauge (3.04 mm thick) 304 American Iron and Steel Institute SS601-477-25M-GP stainless steel (SS) plates with a 2B mill finish. Coupons (1 x 4 cm) cut from the plate were obtained from Stork Gamco, Inc., Gainesville, GA, U.S.A. Prior to use in experiments, stainless steel pieces were soaked at room temperature in a 2% detergent solution (Micro®, Cat # 6732, International Products, Burlington, NJ, U.S.A.) for 5 min, followed by rinsing for 5 min under tap water to remove residual detergent. Coupons were placed in sterile test tubes containing at least 5 ml of distilled water, covered, and treated by sonication for 30 min to remove extraneous metal and other material from the surface**.** The steel pieces were air dried and placed in sterile uncovered Petri plates under ultraviolet light (100 microwatt seconds per cm/2) for 48 h on each side, to eliminate bacterial contamination prior to use.

Cultures. *Campylobacter jejuni* expressing green fluorescent protein (*C. jejuni* 1221 gfp) and its parent strain, *C. jejuni* RM1221 (isolated from a 1 *M* NaCl wash of a retail chicken carcass), were obtained from Robert Mandrell (Food Safety and Health Research Unit, ARS-USDA, Albany, CA, U.S.A.) via Robert Phillips (USDA-FSIS, Athens, GA, U.S.A.). Characterization of these strains is provided by Miller *et al* (20). *Campylobacter jejuni* subsp. *jejuni* #49943 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, U.S.A.) for use as a positive control. Identification of parent, fluorescent, and ATCC strains of *C. jejuni* was

confirmed in this lab by a combination of latex slide agglutination and polymerase chain reaction (PCR) assays. Cultures were initially identified as *C. jejuni*, *C. coli*, or *C. laridis* using the INDX®-Campy (jcl)™ (Panbio Inc., Columbia, MD, U.S.A.) polyclonal antibody test. Subsequently, cultures were confirmed as *C. jejuni* by the presence of a specific 735-base pair product in agarose gels following electrophoresis of PCR products (*9*) from DNA extracts of these cultures. The whole carcass rinse (WCR) culture obtained from Judy Arnold (ARS-USDA, Athens, GA, USA) consisted of a mixed population of bacteria collected from a saline rinse of defeathered, whole broiler carcasses collected in a commercial poultry facility.

Broth preparation

Two concentrations of tryptic soy broth (TSB) (Difco, Sparks, MD, U.S.A.) were prepared, 1:10 (2.75g/L) and 1:50 (0.55g/L). Total volume of 9 ml was dispensed into 16 X 150 mm sized borosilicate glass culture tubes.

Culture preparation and biofilm formation

A frozen cryovial of whole carcass rinse (WCR) culture was thawed then 200 µl were inoculated into a tube of 9 ml TSB and incubated overnight at 37 °C. Optical density (O.D) values were measured on a Biotek spectrophotometer (Winooski, VT, U.S.A.) and then 2 ml of the overnight WCR culture was added to 8 ml of the diluted TSB. The 1:10 diluted culture yielded 10^8 cells/ml. Coupons were inserted into sample tubes and were incubated at 20 and 37 °C for approximately 18-24 h. After incubation, SS coupons were remove aseptically from tubes, rinsed with 10 ml of sterile distilled water on each side, inserted into fresh diluted broth and incubated for an additional 24 h to allow further biofilm formation. After the second 24 h incubation, the

coupons were rinsed with 10 ml of sterile distilled water per side and were ready for *C. jejuni*1221gfp attachment. Duplicate samples were prepared for each temperature.

C. jejuni attachment

*C. jejuni*1221gfp was thawed and 100 µl was spread onto Campy Cefex plates grown for 48 h at 37°C under microaerophillic conditions (5 % O_2, 10 % CO_2, and 85 % N_2). Campy-Cefex agar plates were prepared by using the protocol of Englen *et al* (9). Cells were harvested from plates and suspended in TSB at room temperature until an O. D. value of approximately 0.7 – 1.0 read at 410 nm was reached. The concentration of cells determined by aerobic plate was 10^9 cfu/ml. Coupons with 48 h WCR biofilm formation were inserted into the *C. jejuni*1221gfp suspension and incubated for 24 h at 20 and 37 °C to allow for attachment. After incubation, coupons were removed and rinsed with 10 ml sterile distilled water on each side, and reinserted into fresh sterile diluted TSB for an additional 24 h incubation. The SS coupons were then aseptically removed and rinsed with 10 ml of sterile distilled water.

Extracellular polymeric substance (EPS) staining

Peanut agglutinin (PNA) from *Arachis hypogea* Alexa Fluor® 568 conjugate was (Molecular Probes, Eugene, OR) used to label EPS associated with the biofilm. A stock solution of 1 mg/ml (wt/vol) was prepared in sterile distilled water, then divided into 50 µL aliquots. To prepare the working solution, a stock solution was diluted 1:200 with a total volume of 2 ml. Coupons were flooded with lectin solution for 45 min and rinsed with sterile distilled water. After rinsing, coupons were placed onto glass slides and covered with coverslips to view under fluorescent microscopy.

Enumeration of *C. jejuni* from biofilm

After WCR biofilm formation and attachment of *C. jejuni*1221gfp, SS coupons were rinsed with 10 ml of sterile distilled water on each side, submerged into 9 ml of TSB broth and then scraped approximately 100 times per side into broth. After scraping, the broth containing the suspension of the detached biofilm cells was serial diluted, plated on Campy-Cefex agar and incubated at 37 °C under microaerophilic conditions for culturable counts.

Microscopy

Attached cells and biofilms were observed by using epifluorescence microscopy (Olympus model BX60, Melville, N.Y., U.S.A.). To view gfp fluorescence, a filter cube with excitation of 470-490 nm, barrier filter of 515 nm and dichoic mirror of 500 nm was used. To observe PNA stained EPS and biofilm cells, a filter cube with excitation of 510-550 nm, barrier filter of 590 nm and dichoic mirror of 570 nm was. An Olympus UPlanFl 100X oil immersion, phase 3, objective lens was used with a numerical aperature of 1.30 for both filters. Images were acquired using a QImaging2 camera and version 1.1 software (British Columbia, Canada). UV light source was provided by an Olympus mercury burner. Microscopic field of view of image is an area of 90μm^2.

Data analysis

Data were analyzed with SAS software (Statistical Analysis System Institute, Cary, N.C., U.S.A.) using SAS analysis of variance (PROC ANOVA). Significant differences among means were determined by the least significant differences test (P= 0.05) and Dunnetts t-test was used to compare controls to treatment samples by percent surface area coverage (P= 0.05). Three trials

were performed with duplicate samples. *C. jejuni* cells were manually counted in at least five microscopic fields per trial and averaged.

RESULTS

Surface coverage of biofilm on stainless steel coupons

Each tube of broth (8 ml of 1:10 or 1:50 TSB) was inoculated with 2ml of overnight WCR culture. Stainless steel coupons then were inserted into tubes for 48 hs to form biofilms at temperatures 20 and 37 °C. After biofilm formation, PNA lectin conjugate was used to stain the EPS of the biofilm to visualize surface coverage (Figure 5.1). A control of biofilms formed at 20 and 37 °C in full strength TSB was used to compare surface coverage. Biofilms formed on coupons in 1:10 and 1:50 TSB at both temperatures were significantly lower than each of their controls (Table 5.1). When treated biofilms were compared by temperature for percent surface coverage of biofilms, samples of biofilm grown at 37 °C in diluted TSB covered more of the coupon than at 20 °C. However, differences among dilution concentrations within each temperature set were the same (P = 0.05).

Attachment of *C. jejuni* in diluted broth

A concentration of 10^9 cells/ml of *C. jejuni*1221gfp was allowed to attach to 48h biofilms formed on coupons at 20 and 37 °C. The number of attached *C. jejuni*1221gfp only showed significant differences between treatments of 1:10 TSB at 20 °C (7.20) and 1:50 TSB at 37°C (2.83) (Table 5.2). Surface coverage of the biofilm did not effect the attachment of *C. jejuni*1221gfp. There was no correlation between biofilm surface area coverage and *C. jejuni* attachment. Many *C. jejuni*1221gfp cells attached directly to the coupons rather than to the biofilm (Figure 5.2). Therefore, nutrient levels alone within each temperature set did not affect

*C. jejuni*1221gfp attachment. However, when different temperatures are combined with different nutrient levels, variances are seen in attachment.

Culturability of *C. jejuni* after attachment

After 48 h attachment of *C. jejuni*1221gfp to coupons, cells were scraped off into 9 ml TSB, serial diluted up to 10^{-8} and plated onto Campy Cefex agar to determine culturabiltiy. Cells were recovered only from the 20 °C 1:10 and 1:50 TSB treatments (See Table 5.3). *C. jejuni*1221gfp in biofilms at 37 °C could not be cultured ($<10^2$ cfu/cm^2). A combination of temperature and nutrient levels had an effect on the culturability of *C. jejuni*1221gfp attached to the coupons. *C. jejuni*1221gfp was observed by microscopy to attach to coupons at both 20 and 37 °C; however, at 37 °C these cells may be nonviable or in the viable- but-nonculturable (VBNC) form.

DISCUSSION

Biofilms are a major concern in the food industry. In poultry processing plants, the evisceration area atmosphere is very moist with wet equipment and floors caused by frequent spraying of water. This environment provides an optimal environment for biofilm formation of chicken microflora and pathogens to occur on stainless steel equipment. Pathogens, such as *Salmonella* and *Campylobacter*, can be found regularly on chicken carcasses after reaching retail, therefore it is possible that these pathogens exist in the poultry processing plant. Studies have already reported detection of *Salmonella* on equipment from the slaughter and evisceration area (*12, 15*), while Trachoo *et al* (*24*) reported how *C. jejuni* isolated from chicken houses survived within bioflilms at 12 and 23 °C over a 7 day period. *C. jejuni* can also attach to chicken skin and survive (*6*). Therefore, with the combination of water sprays and contact of chicken

carcasses on stainless steel equipment in the evisceration room, there is an opportunity for biofilms to form with *C. jejuni* attached to them. Water flow in the evisceration area fluctuates; therefore, nutrient levels may vary, too.

In this study, *C. jejuni* attachment and survival in biofilms formed on stainless steel was measured to determine effects from nutrient limitation. Biofilms were formed on coupons for a total of 48 h and then stained with PNA to visualize surface coverage. Biofilm surface coverage barely reached 2% on the coupons when formed in 1:10 and 1:50 TSB at 20 and 37 °C. In full strength TSB, biofilms formed at the same temperatures consisted of percent area coverage of 14.92 % (20 °C) and 3.96 % (37 °C), which are higher (P = 0.05) than biofilms formed in the diluted broths (Table 5.1). This indicates that lower levels of nutrients can retard effect on biofilm growth. Because dextrose was the carbohydrate source available in TSB and was diluted ten and fifty-fold, it could have impacted biofilm development of the mixed WCR culture. Biofilm coverage as reported in chapter two, was also higher at lower temperatures such as 13 and 20 °C than at 37 and 42 °C in full strength TSB. However, availability of different nutrients in the environment can both negatively and positively affect biofilm formation on stainless steel (*16*).

C. jejuni attachment to coupons was about the same for almost all the treatments. Only coupons treated 1:10 TSB at 20 °C and 1:50 TSB at 37 °C showed any significant differences from one another (P= 0.05) (Table 5.2). Higher numbers of *C. jejuni*1221gfp were attached on coupons from biofilms formed under 1:10 TSB (20 °C) treatment. Therefore, nutrient limitation combined with temperature can reduce attachment of *C. jejuni*1221gfp, if temperatures are at least 37 °C and nutrient availability is extremely low. At all of these temperatures,

C. jejuni seemed to be directly attaching to the coupon when viewed under epifluorescence microscopy. This could be due to the length of time biofilms were allowed to formed, along with temperature and diluted broth conditions contributing to low biofilm surface coverage. Previous chapters (chapter one) indicated that *C. jejuni* was able to attach directly to stainless steel material without pre-existing biofilms. Therefore, it could be displaying the same property of attachment under these particular conditions.

After *C. jejuni*1221gfp was allowed to attach to coupons with 48h-biofilms formed on them,the same coupons were scraped to detach *C. jejuni*121gfp to determine if culturable cells of this bacterium were recoverable. Counts were only recovered from coupons incubated at 20 °C, even though attachment of *C. jejuni*1221gfp was observed at both temperatures and all nutrient levels (Figure 5.2). *C. jejuni*1221gfp is able to display green protein fluorescence without being viable (*23*). Therefore, *C. jejuni*1221gfp cells seen under the microscope at 37 °C could have been in the nonviable or VBNC state when attached at this temperature. In the temperature study previously reported (chapter two), *C. jejuni*1221gfp was not recovered when cells were allowed to attach at 37 °C in full strength TSB after 24 hours. These results indicate that temperature is more of a factor than nutrient limitation in determining if *C. jejuni*1221gfp is in culturable state when attached by itself or to biofilms on stainless steel.

In conclusion, this study showed that *C. jejuni* could attach to stainless steel equipment without attaching to biofilms in low nutrient areas. Low nutrient levels did not produce enough biofilm surface coverage on stainless steel in 48 hours to allow *C. jejuni* attachment to biofilms. Therefore, nutrient limitation negatively affected biofilm formation under these particular conditions. However, at temperatures such as 20 °C, which is a common temperature of the evisceration area, *C. jejuni* may find this environment more favorable for survival as opposed to

higher temperature rooms such as the scald and defeather areas of the plant. Chances of *C. jejuni* attachment and survival on equipment and recontamination onto broiler carcasses during the evisceration process are highly possible. At higher temperatures, this organism may be in the VBNC or nonviable state that could cause it to be undetectable in the poultry processing plant environment.

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Table 5.1 Surface area coverage (%) of whole carcass rinse biofilms on stainless steel coupons in tryptic soy broth grown at 20 and 37 °C.[a]

Dilution Concentration	20 °C	37°C
Full strength	14.92 [b] A	3.69 A
1:10	0.20 B	1.94 B
1:50	0.22 B	1.56 B

[a] Means of three trials.

[b] Means in each column with different letters differ significantly from control mean ($P < 0.05$) (Dunnetts t-test).

Table 5.2 Number of *C. jejuni*1221gfp attached on stainless steel coupons in diluted tryptic soy broth grown at 20 and 37 °C.[a]

Dilution Concentration	20 °C		37°C	
1:10	7.20	A[b]	4.60	AB
1:50	4.75	AB	2.83	B

[a] Means of three trials.

[b] Means in both columns and rows with different letters differ significantly at $P < 0.05$ (least significant difference).

Table 5.3 Culturable counts (cfu/cm^2) of attached *C. jejuni*1221gfp from stainless steel coupons after 48 h incubation in diluted typtic soy broth.

	20 1:10	20 1:50	37 1:10	37 1:50
Trial 1	3.10×10^4	2.30×10^5	$< 10^2$	$< 10^2$
Trial 2	2.89×10^4	1.22×10^5	$< 10^2$	$< 10^2$

[a] Means from two trials with duplicate samples.

Figure 5.1 Lectin stained EPS of biofilms formed at different temperatures and diluted TSB concentration, (a) 20 °C, 1:10TSB (b) 20 °C, 1:50TSB,
(c) 37 °C, 1:10TSB and (d) 37 °C, 1:50TSB. Bar= 10 μm

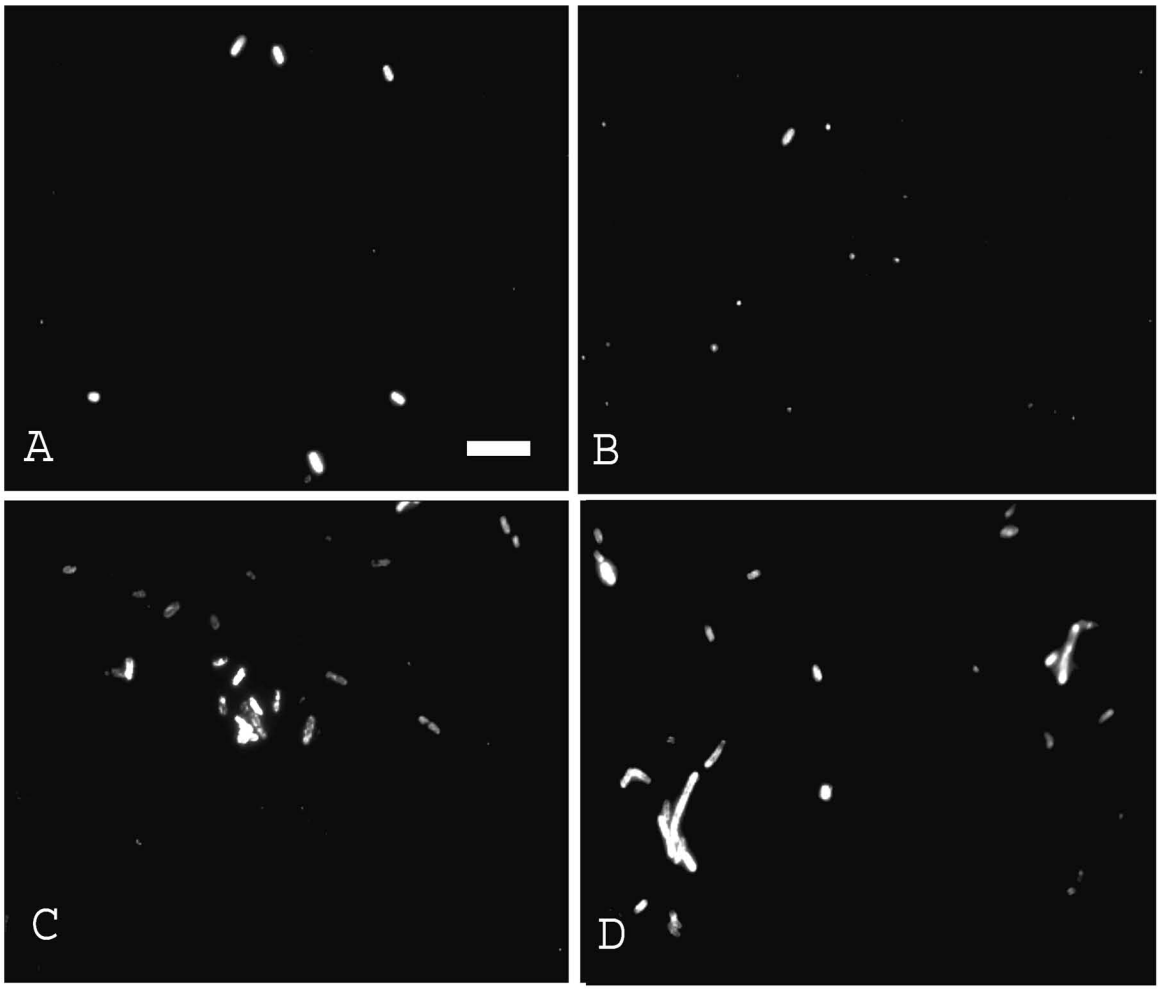
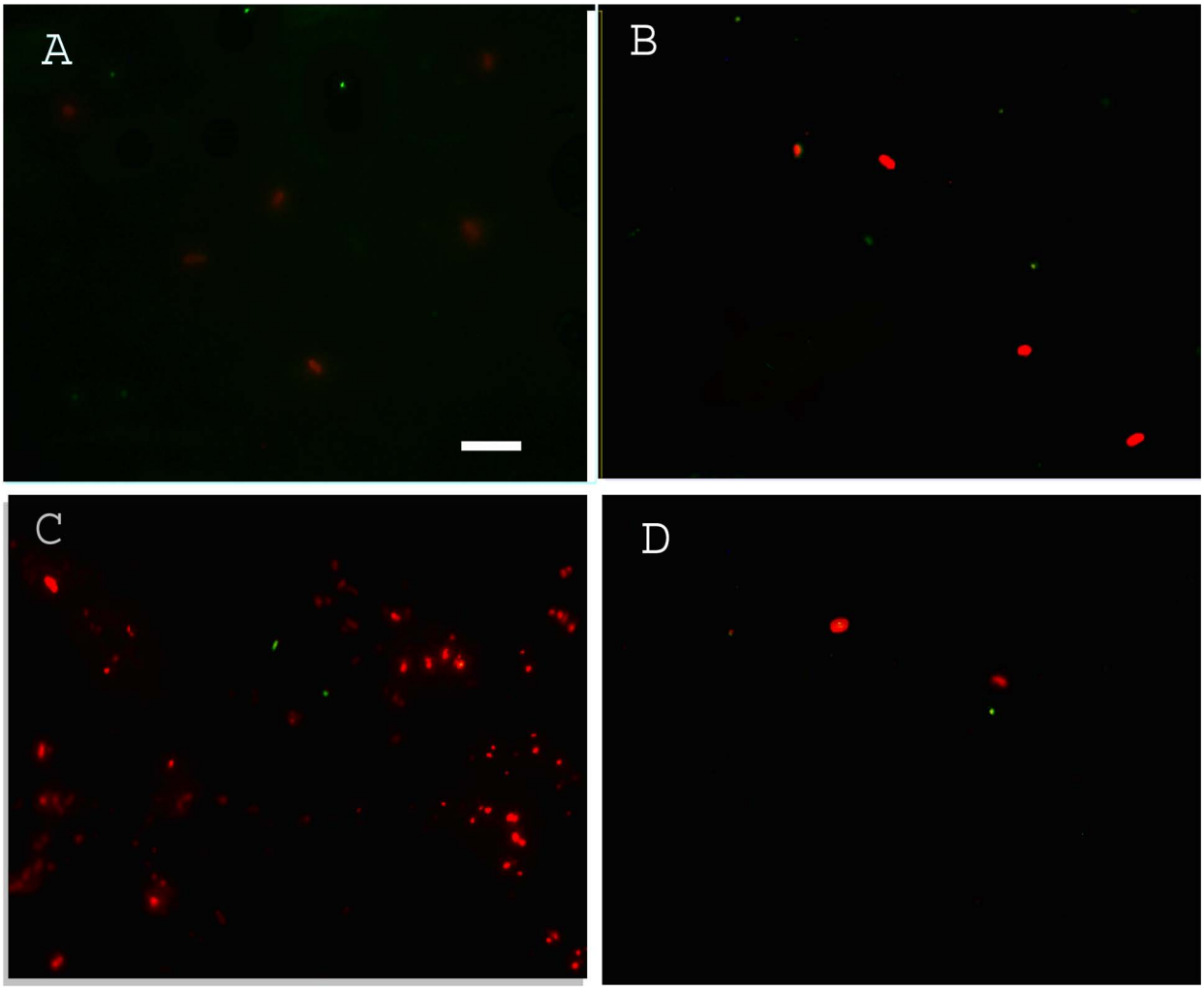

A
B
C
D

Figure 5.2 *C. jejuni*1221gfp attached directly to stainless steel coupon surface and not to biofilm. All temperatures and TSB concentrations are shown, (a) 20 °C, 1:10TSB (b) 20°C, 1:50TSB, (c) 37 °C, 1:10TSB and (d) 37 °C, 1:50TSB. Bar= 10 μm


A
B
C
D

CHAPTER 6

CONCLUSIONS

Campylobacter jejuni can attach and survive in or without biofilms on stainless steel coupons. Confocal scanning laser microscopy (CSLM) and epifluorescent microscopy showed visualization of gfp-labeled *C. jejuni* used in this study. Biofilms containing mixed populations of poultry bacteria and *C. jejuni* were produced on stainless steel for 7 days at 37 °C. In this study, we observed that pre-existing biofilms on stainless steel surfaces are not necessary for attachment of *C. jejuni*. *C. jejuni* is able to attach to stainless steel by itself, however its coverage is enhanced when mixed with other poultry bacteria to form biofilms. Due to CTC (5-cyano, 2, 3-ditolyl tetrazolium chloride), *C. jejuni* in biofilms could be seen as maintaining viability for 7 days.

Temperature (13, 20, 37 and 42°C) did not make a difference in *C. jejuni* attachment to biofilms. The poultry bacterial biofilms were, however, affected by temperature. Lower temperatures such as 13 and 20 °C produce more biofilm coverage on stainless steel than higher temperatures such as 37 and 42 °C. Temperature does effect survival of attached *C. jejuni* due to culturability only being observed in cells from lower temperatures (13 and 20 °C). If *C. jejuni* is attached on stainless steel equipment in higher temperature environments (37 °C and above) in the poultry plant, it may be nonviable or in the VBNC form.

Nutrient limitation (diluted TSB to 1:10 and 1:50) combined with temperature (20 and 37 °C) did show some effect in attachment of *C. jejuni*. Even though biofilm coverage was sparse, *C. jejuni* still attached directly to the stainless steel material under nutrient deprived

conditions as seen under the microscope. *C. jejuni* attachment is decreased when temperature is increased and nutrient availability is decreased (20 °C, 1:10TSB to 37 °C, 1:50TSB treatment). Temperature also has the same effect on culturability when combined with nutrient limitation. Attached *C. jejuni* was only cultured from nutrient treatment incubated at 20 °C.

In conclusion, this research did show that *C. jejuni* can attach to stainless steel equipment with and without other bacterial biofilms being present. Attached *C. jejuni* can also survive in biofilms or directly on stainless steel equipment in lower temperature areas such as the evisceration (20 °C) area in poultry processing plant.