Use of Cellulose Filters to Isolate Naturally Occurring *Campylobacter* spp. from Contaminated Retail Broiler Meat and Survival of *Campylobacter jejuni* and *Campylobacter coli* in Retail Broiler Meat

by

Leslie Nicole Speegle

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Approved by

Omar Oyarzabal, Chair, Assistant Professor of Poultry Science
Jong Wook Hong, Assistant Professor of Engineering
Mark Liles, Assistant Professor of Biological Sciences

Abstract

Campylobacter spp. are indicated as the most common cause of bacteria-related food-borne illness. *Campylobacter* is present at all steps of the food cycle (i.e. poultry farm, processing plant, retail store, and consumer households). Therefore, a further understanding of the factors associated with the isolation and survival of this pathogen is a necessary step for a reduction in incidence.

The first study investigated the parameters for the efficient isolation of *Campylobacter* through 0.65 µm Millipore filters on a selective medium. We determined the minimum number of *Campylobacter* cells needed to pass the filter and the effect of the status of the cells. We also determined the minimum number of cells to pass the filter from enriched food samples. Previous studies have indicated membrane filtration as an effective isolation technique for *Campylobacter* spp. from fecal samples. However, a large number of cells were required for detection.

To determine the minimum required cells that go through the filters, experiments were done with healthy (24-h under microaerobiosis), coccoid, centrifuged (20 min, 16,000 g) and non-flagellated mutant cells. We also determined the minimum number of cells needed to isolate *Campylobacter* spp. from naturally contaminated enriched retail broiler samples. Experiments included 0.65-µm-pore membrane filters (Millipore Corp.) on modified Campy-Cefex agar plates. To determine the rate of passage of *Campylobacter* through the membrane filters, inoculated filters were harvested at different time intervals and analyzed with scanning electron microscopy (SEM).

These results demonstrate that cell status may determine the minimum number of cells that can go through the filter. The use of filter membranes is an effective method to obtain pure *Campylobacter* colonies from enriched food samples.

Whereas, the second study investigated the survival of two retail chicken isolates of *C. jejuni* and two retail chicken isolates of *C. coli* on boneless, skinless broiler breast meat. Previous studies indicate the survival of *Campylobacter* spp. on chicken meat before, during, and after processing. Furthermore, its survival at retail is the primary source for at home contamination due to improper food handling techniques. Broiler meat samples were stored at 4ºC and 12ºC for 14 d and -20ºC (common food storage temperatures) for 120 d. For each run, sixteen 30 g (±1 g) pieces of broiler meat were sprayed with *C. jejuni* or *C. coli* axenically prepared inocula. Inoculation strains were previously isolated from retail broiler meat. The inoculated breast pieces were stored and sampled at specific time intervals to determine survival and presence. Counts for *C. jejuni* and *C. coli* varied significantly across all temperatures, with both species persisting and remaining viable and culturable throughout storage.

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List of Abbreviations

CFU	Colony Forming Units
SEM	Scanning Electron Microscopy
spp.	species
ATCC	American Typing Culture Collection
mCC	modified Campy-Cefex
PBS	Phosphate Buffered Saline
FCM	Fluorescent Confocal Microscope
SAS	Statistical Analysis Software

I. LITERATURE REVIEW

1.1 Poultry as a major reservoir of *Campylobacter*

Campylobacter jejuni is a microaerobic, Gram-negative, flagellated spiral bacterium that has long been recognized as a pathogen in animals. Motile campylobacters colonize the intestines of a wide range of animals. Therefore, this bacterium is found in many foods of animal origin. The lower intestines, especially the ceca, are frequently colonized by thermophilic *Campylobacter* spp (Oosterom et al. 1983; Corry and Atabay, 2001) and can lead to contamination of carcasses during processing. Thus, poultry is identified as a principal reservoir for human contamination due to the high prevalence of *Campylobacter* spp. (*C. jejuni* and *C. coli*) in retail chicken carcasses (Kramer et al. 2000). These bacteria do not cause disease in live broilers. The mishandling of raw poultry and consumption of undercooked poultry contaminated with *Campylobacter* may lead to human infection resulting in bacterial gastroenteritis (Butzler and Oosterom 1991; Tauxe 1997; Nadeau et al., 2002). The introduction of *Campylobacter* into the food supply may be linked to the consumption of contaminated drinking water from environmental reservoirs (Young et al. 2007).

In the U. S., an estimated 2.1 to 2.4 million cases of human campylobacteriosis are reported annually (Altekruse et al, 1999) with an infectious dose as low as 500 *Campylobacter* organisms (Robinson, 1981). Symptoms commonly associated with human infection include fever, headache, muscle pain, diarrhea (occasionally with blood), and abdominal cramping. Chronic sequelae associated with *C. jejuni* infections include Guillian-Barré syndrome (GBS)

and Reiter syndrome (Altekruse et al, 1999). GBS (Rhodes, 1982) and Reiter's (Keat, 1983) syndrome are not completely understood and are thought to be autoimmune responses originating from *Campylobacter* infections. Rarely, complications associated with *C. jejuni* infections may result in deaths which involve primarily infants, the elderly, and immunocompromised patients (Tauxe, 1992).

Frequently, cases of campylobacteriosis are sporadic and can be traced to a food source by the comparison of the strain causing infection and the strain isolated from the implicated food. However, the lack of a common strain typing method in the past often resulted in ambiguous links between strains found in chickens and those isolated from human cases. The identification of the food source attribution in an outbreak may provide the information to effectively improve food safety measures. A tabulation by pathogen-food vehicles of foodborne outbreaks from Australia, New Zealand, Canada, the European Union, and the US that occurred between 1988 and 2007 revealed a low specificity in the relationship between pathogen and food categories, which may indicate that cross-contamination, environmental contamination, and contamination by food handler may be common in the farm to fork chain (Greig and Ravel, 2009). Weaknesses associated with methodologies for establishing a linkage between a reported outbreak and the food source include: i) lack of a clearly defined classification standard, ii) investigation bias, and iii) bias of reporting or publication tendencies, have also been highlighted (Greig and Ravel, 2009). Recently, DNA typing methods, such as restricted fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE), have been employed to study source attribution of *Campylobacter* strains and may serve as a more uniform detection and validation method.

1.1 Poultry contamination by *Campylobacter* spp. at the farm

After ingestion, *Campylobacter* rapidly colonizes the chicken gut and is passed among chickens within a flock through the fecal–oral route, resulting in high numbers of *Campylobacter* cells in the lower intestine (Oosterom et al., 1983; Beery et al., 1988). However, the chicken gut is rarely populated by *Campylobacter* before two weeks of age during normal commercial grow-out conditions (Corry and Atabay, 2001; Berndtson et al., 1996; Jacobs-Reitsma et al., 1995). Subsequently, an internal contamination can occur with a rapid and persisting colonization (6 to 7 log CFU/g) (Corry and Atabay, 2001). Furthermore, flocks are typically infected with multiple strains (Corry and Atabay, 2001). Although the initiation of colonization has been correlated with a common bird age, the source of contamination is seldom identified. There is a lack of evidence supporting vertical transmission from breeding flocks to their progeny (Jacobs-Reitsma et al., 1995). In fact, the main source of *Campylobacter* contamination for commercial broiler flocks has been linked to the horizontal transmission from environmental sources (Berndtson et al. 1996; Berndtson et al., 1991). Pests (i.e. flies and rodents) and weak biosecurity have been indicated as risk factors (Berndtson et al. 1996; Berndtson et al., 1991).

Berndtson et al. (1996) suggested that a better understanding of the *Campylobacter* epidemiology at the farm level is important before expecting a reduction in flock colonization or the development of preventive measures. Berndtson et al. (1996) followed the colonization of two flocks throughout a grow-out program (from 0 day bird age to 5 week bird age) with an emphasis on an increased hygiene regimen. Their results suggest a delay in colonization age but not the preclusion of colonization even when improved hygiene is practiced. Therefore, *Campylobacter* persists despite improvements in farm control measures, such as i) pest control,

ii) restricted access with disinfection of vehicle and footwear, and iii) the cleaning and disinfection of houses between flocks.

1.2 Poultry contamination by *Campylobacter* spp. at the processing plant

Primary contamination of the carcass by *Campylobacter* spp. commonly occurs during processing when the carcass may come in contact with fecal material, an important source of carcass contamination (Rivoal et al., 1999; Oyarzabal, 2005), particularly during evisceration. In 1996, the Food Safety and Inspection Service of the U. S. Department of Agriculture (USDA FSIS) implemented a zero tolerance performance standard for visible fecal material as part of the final ruling for the implementation of the Hazard Analysis and Critical Control Point (HACCP) Systems into meat and poultry industry. HACCP was designed as a preventative system to help ensure food safety by identifying potential hazards associated with food production and preparation, and to develop mechanisms to eliminate or control these hazards (USDA, 1996). In response to the standard, processors increased line personnel for visual inspection and carcass wash systems as preventative measures (Bashor et al. 2004). When a carcass presents fecal contamination, the corrective action is to slow down the processing line and to remove the carcass for reprocessing (i.e. re-washing with chlorinated water, trimming contaminated area, and vacuuming off feces) (Oyarzabal, 2005).

In a commercial poultry processing operation, broiler carcasses are subjected to at least two decontamination processes i) the inside-outside-bird washer (IOBW) and ii) an immersion chill tank to reduce bacterial loads. However, secondary contamination of the carcass can result from contaminated processing equipment. To aid in the prevention of contamination, abundant chlorinated municipal water (20 -25 ppm) are directly applied to the carcass (IOBW) and

equipment as part of regular processing procedures. Furthermore, higher levels of chlorine (50 ppm) are required for the immersion chill tank (Bashor et al., 2004). Stern *et al.* (1999) reported a reduction in Campylobacter-positive carcasses, as well as 2 log CFU per carcass which was attributed to the improper chlorination of chill tank water. The goal of the chlorine addition is to control microbial growth and proliferation which increases carcass shelf life (Bashor et al., 2004) and to reduce foodborne pathogens. However, a high level of organic matter will render the chlorine ineffective for microbial reduction.

The IOBW thoroughly washes the carcass inside and out with pressurized water to remove fecal material from the carcass post-evisceration and pre-chill. Oyarzabal et al. (2004) reported a significant reduction (2.8 CFU/ml), although not consistently significant, in *Campylobacter* spp. counts (log CFU per ml of rinse) associated with the IOBW. These results are in agreement with other studies (Bashor et al. 2004) and suggest that carcass wash systems provide reductions but are ineffective at completely eliminating the presence of pathogenic organisms. However, water pressure, chlorination level, and volume are limiting factors contributing to the effectiveness of the IOBW for bacteria reduction (Oyarzabal et al., 2004).

Typically, the carcass is then transferred to a chlorinated immersion chill tank to rapidly reduce carcass temperature and retard bacteria growth. In fact, Oyarzabal et al. (2004) reported a further reduction (1.09 log CFU/ml) in the number of *Campylobacter* cells between carcasses sampled post-IOBW and carcasses sampled postchill. However, a reduction in prevalence was not observed because *Campylobacter* cells remained culturable after an enrichment step.

The use of antimicrobials (i.e. acidified sodium chlorite and trisodium phosphate) has been proven to significantly reduce bacterial loads (Oyarzabal, 2005; Bashor, 2004). However, the antimicrobials and quantities allowable by U.S. Food and Drug Administration (FDA) and

the USDA for use on raw poultry carcasses in the U.S. do not consistently reduce prevalence or *Campylobacter* cells. Due to the nature of modern processing, the complete prevention of microbial contamination is impossible without adversely affecting meat quality or compromising organoleptic characteristics.

Currently, the UDSA has not implemented a performance standard for *Campylobacter* spp. (Bashor et al., 2004). Research has shown that contamination can be reduced by the logistic processing of *Campylobacter*-negative flocks prior to *Campylobacter*-positive flocks. Rivoal *et al*. (Rivoal *et al*., 1999) compared the genotype of a *Campylobacter*-positive flock and the genotype of *Campylobacter* isolates from subsequent processed flock. A similar strain emerged which suggests cross-contamination does occur between processed flocks during processing. Potturi-Venkata *et al.* (2007) suggested that a logistic scheduling system, where the processing of known *Campylobacter*-negative flocks proceeds the processing of *Campylobacter*-positive flocks, may result in a reduction of cross-contamination during processing.

1.3 Poultry contamination by *Campylobacter* spp. at retail

In the U.S., a prevalence of retail broiler carcasses are positive for *Campylobacter* at the time of slaughter (Scherer et al., 2005; Cray et al, 2008), which correlates into a high incidence of *Campylobacter*-positive retail poultry products. Numerous reports have confirmed the presence of *Campylobacter* at slaughter and at retail. These findings further support the concept that carcasses are *Campylobacter*-positive post-processing despite intervention steps. *Campylobacter* has been shown to survive on broiler carcasses during refrigeration of retail broiler meat. Kemp and Schneider (2002) suggest that a control measure should consistently

achieve a 3.7 log CFU/ ml reduction post-processing to be considered successfully effective against *Campylobacter* contamination.

1.4 Food-handling at home to prevent *Campylobacter* spp. contamination

According to epidemiological data, the improper preparation or mishandling of contaminated food in consumers' homes has been attributed to a substantial proportion of the annually reported foodborne diseases. According to food safety observational studies, consumers frequently fail in their efforts at safe domestic food-handling practices. In fact, one observation study concerning raw chicken and consumer food-handling techniques revealed extensive *Campylobacter* spp. cross-contamination during food preparation (Redmond and Griffith, 2003). An improvement in domestic food-handling practices and behaviors is likely to reduce the risk and incidence of foodborne illness. In turn, a reduction in the economic loss associated with foodborne diseases should emerge.

1.5 Movement for a standard methodology

To fully understand the prevalence and mode of contamination, a consistent methodology for sampling, isolation, and enumeration should be introduced. A comparison of *Campylobacter* research reveals the utilization of several types of selective plate media: modified Campy-Cefex (mCC); cefoperazone charcoal deoxycholate agar (CCDA); etc., or non-selective media which may be used in combination with numerous types of enrichment broths, i. e. Bolton broth; Preston broth; cefoperazone amphotericin teicoplanin (CAT) broth; etc. Also, isolates from these experiments may have been obtained by a filtration technique or possibly a quadrant streaking method. Scherer *et al.* (2006) attempted to summarize several reports for the determination of

prevalence and counts of *Campylobacter* present at retail. However, the researchers discovered inconsistencies in the sample type (whole carcass or parts, fresh or frozen), sampling techniques (carcass rinse, surface swabbing, or stomaching sample), and enumeration technique (direct plating or MPN method) (Scherer *et al.*, 2006). The lack of a standardized method may result in the drastic under- or overestimation when directly comparing counts and frequency of *Campylobacter*. Furthermore, result comparisons should be extrapolated from studies using the same methodologies for isolation: enrichment, growth and identification.

The development of a standard methodology should consider techniques that are cost and time effective. Le Roux *et al.* (1998) consider membrane filtration as an efficient isolation method of *Campylobacter* from stools. In our studies, the objectives of the filter isolation study were to evaluate i) the action of filtration through a 0.65 µm filter, ii) the rate of cell passage through the filter, iii) influence of cell status during filtration. Findings were adapted and applied for the efficient isolation of *Campylobacter* from naturally contaminated retail broiler meat samples

The collection of *Campylobacter* researchers have failed to identify a harmonious system for the investigation of *Campylobacter* spp. Additionally, strategies based on the overall control of *Campylobacter* will depend on understanding i) the source of infection in poultry flocks, ii) reduction of contamination in processing plants and retail products, and iii) survival at retail and home. The second study assessed the duration of survival of *C. jejuni* and *C. coli* in retail broiler meat at various storage temperatures.

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II. USE OF CELLULOSE FILTERS TO ISOLATE NATURALLY OCCURRING *CAMPYLOBACTER* SPP. FROM RETAIL BROILER MEAT

2.1 Abstract

Membrane filtration has been used to isolate *Campylobacter* spp. from feces, although ~ 5 logs CFU per g must be present in the sample. Few studies have attempted to use filter membranes for the isolation of *Campylobacter* from foods. We investigated the minimum number of thermotolerant *Campylobacter* cells that pass through cellulose filters; the effect of different cells conditions on the rate of passage; and the minimum number of cells that could pass the filters from enriched broiler meat naturally contaminated with *Campylobacter* spp. Cellulose filters of 0.65 µm pore sizes retained less cells and were more effective than filters of 0.45 µm pore sizes. Scanning electron microscopy revealed that 15 minutes of contact time of the filters with agar plates allowed for the passage of most bacteria. The minimum number of bacteria required to pass through the filters was contingent to cell conditions, with non-motile cells retained more than motile cells ($P < 0.05$). The minimum number of motile bacteria from 24-h cultures and centrifuged cells were 2.2 and 2.1 log CFU, respectively, while the number of coccoid and non-motile (*fla*A/B$^-$ mutant) cells were 4.1 and 3.4 log CFU, respectively. Broiler meat samples enriched in Bolton's broth supplemented with 5% lysed blood showed that approximately 1.7 log CFU of *Campylobacter* can be filtered to pure colonies on agar plates.

These results demonstrate that the motility of the bacteria influences the passage through cellulose filters, and that 0.65 µm filter on agar plates help obtain pure *Campylobacter* colonies from enriched food samples.

2.2 Introduction

The method of choice for the isolation of *Campylobacter* from contaminated food samples is the combination of enrichment broth with selective plating or direct plating on selective agars. However, due to the slow growing attribute of *Campylobacter* spp., many isolates are lost to the competition by contaminant bacteria naturally present in foods. The use of filtration methods was first applied to the isolation of *Campylobacter fetus* (formerly *Vibrio fetus*) from bulls (*11*), and has been applied for direct isolation on agar plates of *Campylobacter* spp. from human stools (*12*), where filters are applied directly on the surface of non-selective agar plates and fecal samples from patients with diarrhea are applied on top of the filters (*6, 12*). The introduction of the membrane filtration technique has allowed for a more successful recovery of *Campylobacter* isolates from clinical samples. Cellulose nitrate, cellulose triacetate, or cellulose acetate filters of 0.45 µm or 0.65 µm pore sizes have been used for the isolation of *Campylobacter* spp. from fecal samples (*2, 8, 12, 15*).

It appears that the isolation rate is contingent on the pore size of the filter. Bolton *et al.* (1988) found that more strains of *C. jejuni* and *C. coli* were isolated by using 0.65 than 0.45 filters. In another study, Goosens and Butzler (1992) found that a filter system with a pore size of 0.45 µm resulted in less contamination than did one with 0.65 µm filters. However, bacterial concentrations of less than 5 $\log_{10}$ CFU per g of feces could not be detected by 0.45 µm filters.

Megraud (1987) and Wilson and Aitchison (2007) suggest a short enrichment step prior to filtration may increase the isolation frequency in low *Campylobacter* samples.

The filter technique has also been used for the isolation of *Campylobacter* spp. from food samples (*1*). An isolation procedure based on hydrophobic grid membrane filters applied on semisolid media that takes advantage of the differential motility of *C. jejuni* and *C. coli* has been described for the isolation of these bacteria from chicken and turkey samples (*13*). Furthermore, the direct application technique used for the isolation of *Campylobacter* spp. from feces has also been used with fresh and frozen food samples (Baggerman and Koster 1992). It is believed that a large number of bacterial cells are required for *Campylobacter* detection and that cell motility is essential for the passage of the cells through the filters (*4*, *12*, 15). Capillary action has also been suggested as a mode of action by which *Campylobacter* cells pass through cellulose filters (*3*). Yet, the parameters that influence the rate of passage of *Campylobacter* through filters have not been systematically addressed.

The aim of the present study was to determine the parameters that influence the efficient use of cellulose filters for the isolation of *Campylobacter* from retail broiler meat, and for the isolation of *Campylobacter* spp. from contaminated cultures. We tested cellulose filters because they are the most commonly cited filters for the isolation of *Campylobacter* species in the literature (*2*, *8*, *12*, *15*). Preliminary experiments evaluated the use of filters with 0.45 and 0.65 μm pore sizes and the rate of passage of cells through the filters by the visualization of scanning electron micrographs. The influence of the age of the culture, the motility of the bacterial cells and the minimum number of bacteria required for detection were studied by the direct application of filters on selective agar plates. Finally, we studied the impact of competing bacteria present in naturally contaminated enriched meat samples.

2.3 Materials and methods

2.3.1 Strain and growth conditions

C. jejuni ATCC 35918 and a non-flagellated *C. jejuni fla*A/B-mutant (strain 1543) were recovered from -80°C stock cultures and grown on modified Campy-Cefex (mCC) supplemented with 5 % sterile, lysed horse blood (*9*). Cultures were incubated at 42°C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) generated using a MACSmics Jar Gassing System (Microbiology International, Frederick, MD), for 24 h.

2.3.2 Filters

Initial experiments included 0.45 and 0.65 µm filters from GE Water & Process Technologies (Fisher Scientific, Trevose, PA, catalogue number E06WP04700), Millipore Corporation (Fisher Scientific, Billerica, MA, catalogue number DAWP04700), and Whatman (Fisher Scientific, Dassel, Germany, catalogue number 10-401-512). However, filters with 0.65 µm pore diameter from Millipore were more readily available; had the lowest cost; and were therefore used throughout the experiments.

2.3.3 Enrichment of retail broiler samples

Broiler retail samples (boneless breast meat) were bought from local retail stores and were enriched by stomaching 25 g of chicken meat with 100 ml of Bolton broth supplemented with 5% sterile, lysed horse blood in Whirl-Pak bags (*10*). Samples were incubated at 42 °C under microaerobic conditions for 48 h. Broiler meat samples were screened for *Campylobacter* in advance, and only 12 positive samples were used in these experiments. Enriched samples were

transferred to mCC plates using the filters as described for spike samples. An isolate from each sample was stored at -80°C.

2.3.4 Effect of cell conditions and calculation of the minimum number of cells that pass through the filter

Five cell treatments were used in these experiments. One treatment included very motile, 24-h growth of *C. jejuni* ATCC 35918. Cells were grown on mCC plates under microaerobic conditions and at 42 °C. Cells were dissolved in phosphate buffer solution (PBS) to achieve an OD_{600} of 0.14-0.15. An aliquot of this cell suspension was also used for the second treatment, in which cells were centrifuged to reduce their motility. Cells were centrifuged in PBS at 25°C for 10 min at 16,000 g; the supernatant was discarded, and the pellet was resuspended in fresh PBS. This centrifugation step was repeated three times (*14*). Another treatment included the induction of coccoid cells of *C. jejuni* ATCC 35918. This cell status was achieved by leaving 24-h cultures on mCC at 25°C under aerobic conditions for 24 h. The fourth was comprised of 24-h growth of a non-flagellated *fla*A/B$^-$ mutant dissolved in PBS to an OD_{600} of 0.14-0.15. The fifth treatment included enrichment of broiler meat samples spiked with *C. jejuni* ATCC 35918. Samples were enriched at 42 ºC under microaerobic conditions for 48 h.

Each cell treatment was viewed under a phase-contrast microscope to corroborate that the cells from the first and second treatments were indeed spiral and motile, although the centrifuged cells were less motile; the cells from the third treatment were coccoid and non-motile in more than 90% of the cells; and the cells from the fourth treatments were spiral, yet completely non-motile. The broth from *Campylobacter*-positive enriched food samples showed very motile *Campylobacter* cells in comparison with the other treatments.

The broiler meat samples were screened for *Campylobacter* in advance, and only 12 positive samples were used in these experiments. Enriched samples were transferred to mCC plates and isolates from each sample were collected and stored at -80°C.

To standardize the dryness of the mCC plates, all plates were dried in a biological hood for five h prior to use. For each treatment, a 10-fold serial dilution in sterile PBS was performed and spread-plated, while five 20 µl drops (100 µl total) were applied to the filter surface. The filter was allowed to remain in contact with the surface of the agar plate for 15 min. mCC plates were incubated at 42°C under microaerobic conditions for 48 h.

Each treatment was performed in triplicate and plated in duplicate. The CFU per ml was recorded for the last countable spread plate and filter plate from each plating set of dilution. Only the data for the minimum number of cells was recorded from each treatment. The equation used to determine the minimum number of cells that went through the filter was:

Log of Spread Plate – Log of Filter Plate = Minimum Number of Cells Needed for Detectable Filter Plate

2.3.5 Scanning electron microscopy (SEM) studies to calculate the rate of cell passage

Four bacterial cell treatments were used for SEM: 24-h growth, centrifuged cells, coccoid cells, and cells from enriched cultures. For each treatment, filters were placed on the surface of an empty Petri dish and also directly onto the surface of several mCC agar plates. The treatment specific inoculum was applied as five 20 µl drops (100 µl total) per filter. Filters inoculated with sterile PBS were used as controls. At 0, 5, 10 and 15 min a filter was removed from a Petri dish and one from a mCC plate. These filters were fixed in osmium tetroxide vapor for 2 h. This step was repeated for each treatment. Segments of each filter were mounted on to aluminum support

stubs with double-stick carbon tape and coated with gold using an EMS 550X Auto Sputter Coating Device (Electron Microscopy Sciences, Hatfield, PA). Samples were analyzed with a Zeiss EVO 50 Variable Pressure SEM (Carl Zeiss SMT, New York) operated at 20 kV. For standardization purposes, all scanning electron micrographs shown were captured at 6.50 K magnification.

2.3.6 Fluorescent confocal microscopy (FCM) studies

FCM was used to corroborate the passage of cells through the filters and to validate SEM findings. Cells from *C. jejuni* ATCC 35918 (24 h growth) were dissolved in PBS to achieve an OD_{600} of 0.14-0.15. One ml of this cell suspension was combined with 100 µl (0.4-0.5 mg of protein) of biotinylated polyclonal antibody from rabbit serum (AbD Serotec, Raleigh, NC) and 50 µl (1/100 working stock) of streptividin labeled tetramethyl rhodamine isothiocyanate. The mixture was incubated for 10 min at 42°C. Ten µl of the sample were applied to a filter and mounted on a glass slide with a cover slip. For controls, 10 µl of the sample and a piece of sterile filter were similarly mounted. The transfer time between the preparation lab and the equipment lab was approximately 10 min. Samples were analyzed with a MRC 1024 Confocal Scanning Laser Microscope (Carl Zeiss, Inc., New York).

2.3.7 Statistical analysis

Experiments to determine the minimum number of cells that pass through the filter were run in triplicates. CFU counts were transformed into log CFU values. The analysis of variance was done with SAS version 9.1 (SAS Institute Incorporated, Cary, NC), with separation of means using Duncan test. Statistical differences were set at $P < 0.05$.

2.4 Results and discussion

2.4.1 Filters

An initial comparison of three brands of filters revealed that the filters by Millipore and Whatman had consistently similar results. However, the third brand of filters was inconsistent with inoculated PBS and its use was discontinued from the experiments. It is important to mention that the filters by Whatman (ME 26) were formerly manufactured by Schleicher & Schuell, and is the filter described in the Cape Town protocol (*6*). However, this filter has to be imported to the US, and it is more expensive. Therefore, the filter by Millipore appears to be a good alternative, at a reasonable price, for the US market.

In our first set of experiments, we found that filters with pores of 0.45 µm retained too many bacterial cells and their sensitivity for efficient *Campylobacter* isolation was low. In general, more strains of *C. jejuni* and *C. coli* have been isolated by using 0.65 µm than by using 0.45 µm filters (*2*). To offset the limited sensitivity of 0.45 µm filters, a short enrichment step prior to filtration has been suggested to increase the isolation frequency in samples with low numbers of *Campylobacter* spp. (*8, 15*). Steele and McDermott (*12*) calculated that 90% of the cells are retained by filters with a pore diameter of 0.45 µm. The large retention rate by these filters and the large amount of competing microflora may explain the low sensitivity found with these filters when trying to isolate *Campylobacter* from feces (*7*).

We also noticed that filters placed on agar plates with high moisture content soaked in the moisture and did not allow for the inoculum placed on top of the filter to dry in less than 20 minutes. We also noticed that any volume of liquid above 100 µl would take longer to go through the filter. Whenever liquid was still present on top of the filters at the time of the removal of the filter from the agar plates, we increased the chances of mishandling the filters,

and allowing for unfiltered liquid to end up on the surface of the agar, which could lead to contamination of the plate. Amounts of liquid larger than 200 µl flooded the filter and spilled over the filter onto the agar. Therefore, we standardize the drying of the agar plates for five h in a laminar flow. We also found that 100 µl distributed in five 20 µl drops on top of the filter provided with a practical solution to obtain isolated *Campylobacter* colonies.

It is pertinent to mention that dried agar plates will absorb any liquid much more quickly, and that a shorter time, perhaps 10 min, would suffice for the liquid (and the cells) to pass through the filter. Yet, very dried agar plates may not be conducive for *Campylobacter* growth and may result in lower sensitivity when using cellulose filters. The exact drying time for the plates is difficult to predict. In general, the storage time and the handling of the plates influence the time needed to obtain the right dryness of the plates. Because these parameters vary from laboratory to laboratory but can be standardize in a given environment, the best is to establish first the practice to provide for a plate that is dry enough and that can absorb the liquid from the filter within 15 min.

2.3.2 Effect of cell conditions on the minimum number of cells needed to pass through the filter

The minimum number of cells required to pass through the filters was dependent of the conditions of the cells. Non-motile cells (coccoid and flaA$^-$ mutants) were retained more than motile cells (24-h and centrifuged) by the filters ($P < 0.05$). Surprisingly, the lowest number of cells needed to go through the filters came from enriched broiler meat naturally contaminated with *Campylobacter* cells and enriched in Bolton broth. Table 2.1. shows the calculated number of cells needed to pass the filters for each of the cell conditions tested.

In studies with the enriched broiler meat samples, several components of the broth (meat, blood, etc.) collected on top of the filter, blocked the pores and hampered the visualization of the *Campylobacter* cells by SEM (Figure 2.2.). Yet, the rate of passage of *Campylobacter* cells was not affected by the presence of these substances. In fact, enriched broiler meat samples showed the highest sensitivity for isolation of *Campylobacter* using filters (Table 2.1.). The enriched samples were not prone to contamination from the naturally occurring competing bacteria. After filtration, the enriched samples were pure and often appeared as isolated colonies atypical from this swarming-type bacterium.

The centrifuged bacterial cells were less motile, the coccoid were non-motile in more than 90% of the cells, and the mutants were spiral-shaped, but entirely non-motile. From observations under phase-contrast microscope, the centrifugation process used in our experiments decreased the activity of *Campylobacter* cells. However, they were still more active and motile than the coccoid bacteria, or the non-flagellated mutants. Remarkably, we have found that naturally occurring *Campylobacter* strains in enriched broiler samples exhibited the highest rate of motility of the different groups described in our experiments. Therefore, cell motility appears to play a crucial role in the passage of *Campylobacter* through the filter, but the absence of motility does not completely hinder cell passage. These assessments are somewhat surprising and we are continuing our research to further investigate these findings.

2.3.3 SEM studies to calculate the rate of cell passage

SEM studies revealed that 0.65 µl cellulose filters presented a large variation in pore sizes (Figure 2.3. A). The spiral morphology of *Campylobacter* was difficult to distinguish from the fiber background of the filters. Despite these limitations, SEM micrographs allowed us to

determine the rate of emptying of the filter pores, and that was used as an indication that *Campylobacter* cells transferred from the filter surface to the agar plates (Figure 2.3.). A 15-min contact between the inoculated filters and the agar plates was found to be the best time to allow for the majority of the cells to go through the filter. This time was used as the standard contact time for the rest of the experiments. No differences in the minimum number of cells needed to go through filters were found between 15 and 20 min, and SEM micrographs showed that less than 15 min may not be sufficient for the passage of the bacterial cells (Figure 2.3., compare D with E, B and C).

2.3.4 FCM studies

The studies with FCM showed fluorescently labeled *Campylobacter* cells immediately after the inoculation with the filters. At 15-20 minutes after the inoculation, most of the *Campylobacter* cells already passed the filter (data not shown). The nitrocellulose filter used as control also yielded a small amount of background fluorescence, as it has been reported by the manufacturer (http://www.millipore.com/faqs/tech1/69vtv9). With this technique, we corroborated that 15-20 minutes is enough for the inoculated cells to transverse through filters.

2.4 Conclusions

In summary, we have defined some of the parameters pertaining to the use of direct plating filtration. Filters with 0.65 µm pore diameter are the best choice when trying to isolate the highest number of *Campylobacter* from food samples. The motility of the cell plays a role in the sensitivity of the filter. However, a cell can be stressed or injured and still pass through the filter and grow on selective media. We also observed isolated colonies when samples were plated

on a dry media plate, which seems to hinder the cells capacity for motility across the plate surface. The main advantage of using the filtration technique was that *Campylobacter* isolates were pure, and the success of retaining and storing the pure culture was higher. Therefore, the samples were not out competed by naturally occurring bacteria which were retained in the filter.

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Table 2.1. Minimum number of *Campylobacter* cells needed to pass the filter for detection on the agar plate.

Treatment	Mean Log_{10} CFU (± SEM)[1]	Cell Number (CFU)[2]
Intact	2.2 ± 0.16 [A]	158
Centrifuged	2.1 ± 0.12 [A]	125
Enriched	1.7 ± 0.35 [A]	50
Coccoid	4.1 ± 0.49 [B]	12,589
Mutant	3.4 ± 0.32 [B]	2,511

[1] Different letters within the column means significant difference ($P < 0.05$).

[2] Assuming an estimated retention of 90% by the filter.

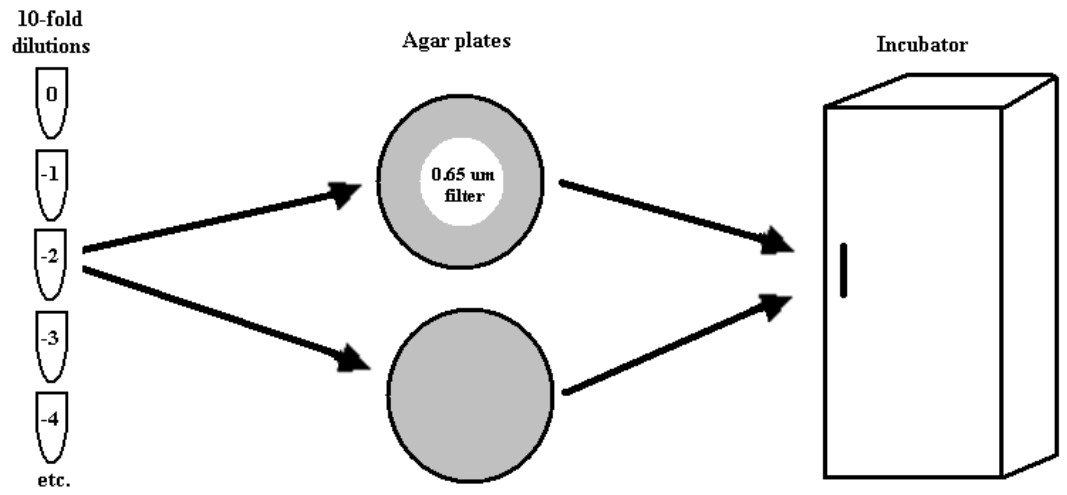

Fig. 2.1. A schematic illustrating the technique used for the direct plating method to determine the minimum number of cells needed for passage.10- fold dilutions were performed in PBS. From each dilution, Five 20 µl drops (100 µl total) were distributed on top of the filter and 100 µl was spread plated for counts. The two sets of plates were incubated at 42°C for 48 h under microaerobic conditions.

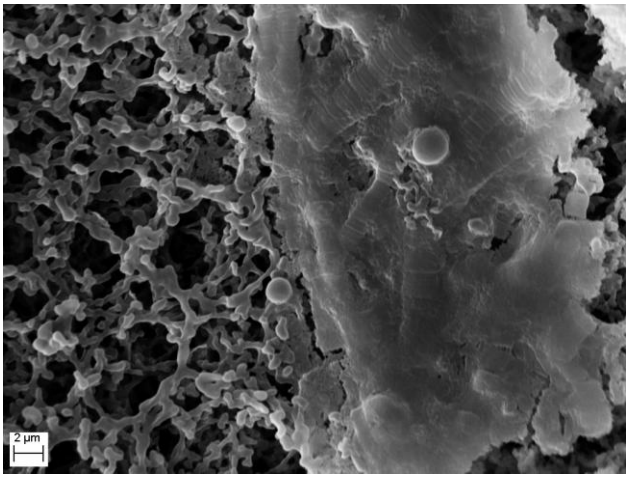

Fig. 2.2. A SEM image reveals the diversity of components present in an enriched food sample applied to a filter.

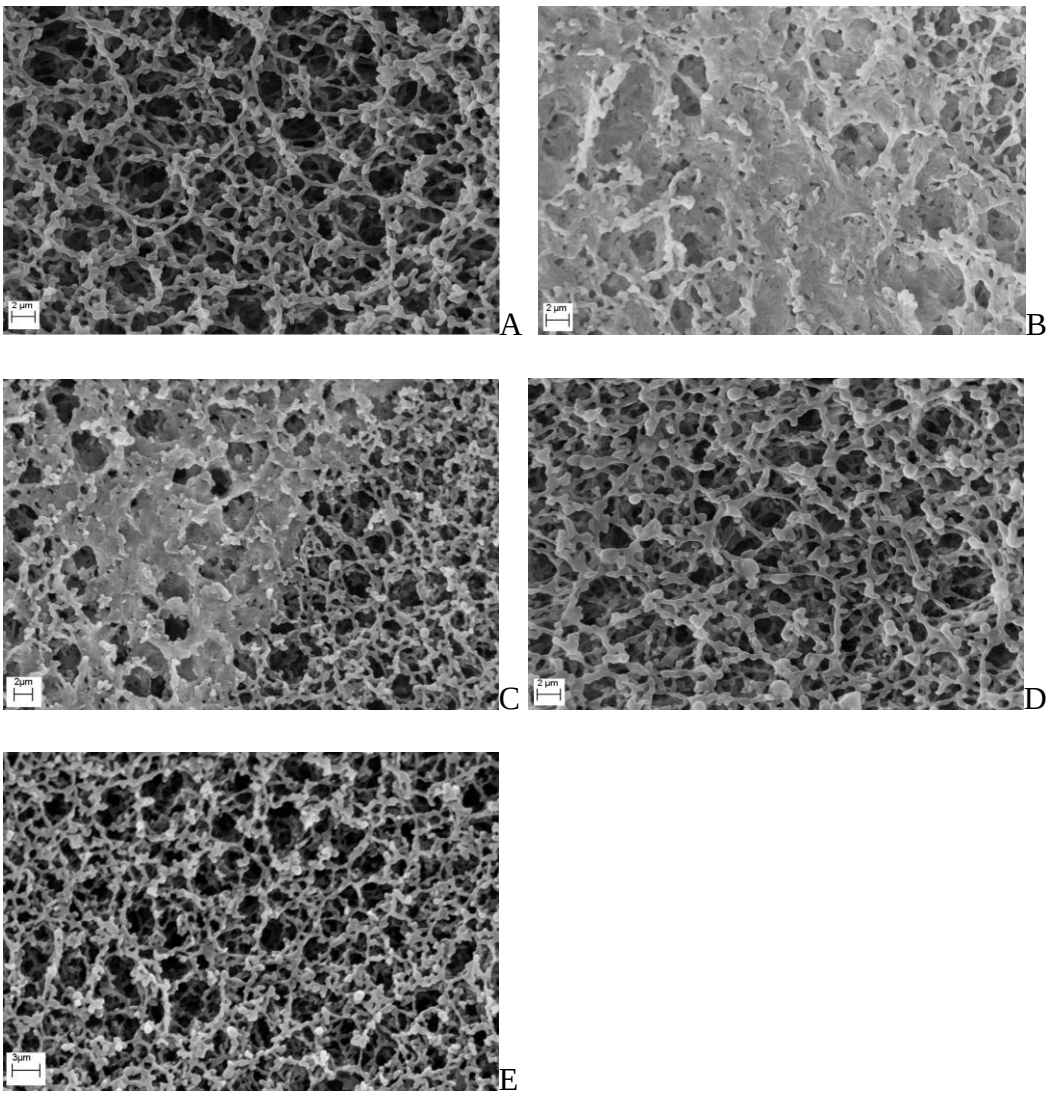

Fig. 2.3. SEM images of 0.65 µm pore filters (Millipore Corporation) which collectively demonstrate the clearing of pores throughout a course of time. A: Filter control. B: Intact cells air dried for 0 min. C: Intact cells air dried for 5 min. D: Intact cells air dried for 15 min.

III. SURVIVAL OF *CAMPYLOBACTER JEJUNI AND CAMPYLOBACTER COLI*

3.1 Abstract

The survival of two *C. jejuni* and two *C. coli* strains isolated from broiler meat was studied in inoculation experiments using boneless, skinless retail broiler breast meat. Inoculated meat was stored at -20ºC for 84 d or at 4 or 12ºC for 14 d. Storage at -20ºC yielded a reduction of 2.9 ± 0.59 log CFU/g and 2.8 ± 0.51 log CFU/g for *C. jejuni* and *C. coli*, respectively. Storage at 4°C yielded a reduction of 0.9 ± 0.29 log CFU/g for *C. coli* and 1.7 ± 0.48 log CFU/g for *C. jejuni*, while storage at 12°C resulted in a reduction of 2.1 ± 0.16 log CFU/g for *C. coli* and 5.3 ± 0.84 log CFU/g for *C. jejuni*. The survival of *C. jejuni* and *C. coli* was similar at -20°C, but *C. coli* had higher survival rates than *C. jejuni* at 4 and 12°C ($P < 0.05$). It appears that *Campylobacter* spp. survive better in broiler meat than on chicken skin and therefore more studies should be performed with retail broiler meat to provide more accurate survival data for risk assessment purposes.

3.2 Introduction

Although commercially-processed broilers undergo a wide variety of steps during processing to reduce microbial contaminants (*15*), several studies demonstrate that retail broiler meat is frequently contaminated with *Campylobacter* spp. (*16*). This contamination occurs during processing, when carcasses come in direct contact with fecal matter and commingle in the chiller tank (*9,13,24*).

In an attempt to reduce contamination and improve the shelf life of broiler carcasses, rapid chilling methods have been developed by the poultry industry. In the US, immersion chilling is the typical method used to reduce the carcass temperature. However, air chilling and evaporative air chilling are used more regularly in other countries (*22*). All three cooling methods are effective for rapidly reducing the temperature of the carcasses, which display similar prevalence of microbial contamination (*11*,*8*). However, El-Shibiny et al. (2009) found that these methods may improve the survival of foodborne bacterial pathogens, including *Campylobacter*, throughout the shelf life of broiler meat.

Beyond the rapid chilling methods, retail broiler meat also undergoes variable freezing and refrigeration temperatures during storage, transportation, display in retail outlets and in consumers' refrigerators. Due to the relatively high prevalence of *Campylobacter spp.* found in retail broilers and the low infective dose required to cause human disease (*21*), studying the ability of *Campylobacter spp.* to survive refrigeration and freezing is directly relevant to designing new strategies to improve food safety and public health.

Several studies have reported the effects of refrigeration and freezing on the survival of *C. jejuni* (*10*,*20*,*2*) but all of these studies have used chicken skin as the product to determine survival. In one study, broth medium was used instead of poultry meat (*4*). Therefore, there are very few studies carried out in the last 20 years that address the survival of *C. jejuni* in broiler meat. Similarly, the survival of *C. coli* has been studied only in inoculated chicken skin (*7*). In addition, no study has included the development of predictive models to address the survival of *C. jejuni* and *C. coli* as it relates to storage temperature and time.

The survival of *Campylobacter* spp. on broiler meat may be an important source of at-home contamination due to improper food handling. The objective of the present study was to

investigate the survival rate of retail broiler isolates of *C. jejuni* and *C. coli* inoculated on boneless, skinless broiler breast meat and stored at 4, 12 and -20º C for various time intervals. Survival rates were then used to develop a predictive model for determining *Campylobacter* counts at the aforementioned conditions.

3.3 Materials and methods

3.3.1 Bacterial strains and culture conditions

C. jejuni 971 and 1065 and *C. coli* 947 and 956, isolated from retail broiler meat and identified using described multiplex PCR assays (*16*) were recovered from stock cultures (-80ºC in Brucella broth supplemented with 30% glycerol and 5% lysed horse blood) by filtration through a 0.65µm Millipore filter (Fisher Scientific, Billerica, MA) onto modified Campy-Cefex (mCC) supplemented with 5 % lysed horse blood (*15*). Cultures were incubated at 42°C for 48 h under microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2; Airgas, Radnor, PA) provided by an evacuation-replacement system (MACSmics Jar Gassing System; Microbiology International, Frederick, MD) in anaerobic jars. All strains were typed using a pulsed-filed gel electrophoresis protocol (PFGE) described elsewhere (*17*). During the trials, isolates were collected at the initial, middle and final sampling points and were typed using the same PFGE protocol.

3.3.2 Retail broiler meat and inoculum preparation

Boneless, skinless broiler breast meat was purchased from a local retail store. The meat was aseptically cut into 30 g (± 1 g) pieces and grouped into runs consisting of 16 pieces. Groups were spread onto sanitized trays and allowed to dry in a biological safety II laminar flow cabinet for 20 min. Inocula were prepared using colonies grown on mCC plates for 24 h at 42ºC under

microaerobic conditions and then dissolved into 4.5 ml of phosphate buffered saline (PBS). Suspension concentrations were standardized to an optical densities at 600_{nm} of 1.5 (± 0.2) and transferred into a sanitized spray bottle. The inoculum was supplemented with 15.5 ml of sterile PBS to obtain a final volume of 20 ml, with a final concentration of approximately 7 log CFU/ml.

Meat samples were evenly inoculated on all sides until the inoculum was exhausted, and samples were allowed to dry in a biological hood for 60 min before being transferred to individual Ziploc® freezer bags (The Glad Products Company Oakland, CA). These bags where then stored at the required test temperatures.

3.3.3 Survival experiments

Samples stored at 4 and 12°C were placed in a MIR 252 Incubator (Sanyo North America Corporation, San Diego, CA) and two samples were removed from each trial for enumeration at 0 d and every 2 d for up to 14 d (Figure 3.1.). Samples stored at -20°C were initially stored at 4 °C for 24 h and then placed in a freezer (Thermo-Kool, Laurel, MS). Two samples were then removed from each run for enumeration at 0 d and every 14 d for 84 d. Samples removed from -20ºC storage were allowed to thaw at room temperature (~25ºC) for 1 h. All samples were then aseptically transferred to individual sterile plastic bags (Whirl-Pak®, Nasco, Fort Atkinson, WI) and stomached for 1 min in a 1:2 (w:v) ratio of Bolton broth supplemented with 5% lysed horse blood. For survival at -20°C, three replicate experiments were run with *C. jejuni* 1065, and three replicates with *C. coli* 947. For survival at 4°C, three and one replicate experiments were run with *C. jejuni* 971 and 1065, respectively, and three and one replicate experiments were run with *C. coli* 947 and 956, respectively. For survival at 12°C, three and one replicate experiments were

run with *C. jejuni* 1065 and 971, respectively, and three and one replicate experiments were run with *C. coli* 947 and 956, respectively.

3.3.4 Bacterial counts

Surviving *Campylobacter* were enumerated by direct plating. Samples were serially diluted in sterile PBS (1:9) and spread-plated on mCC agar in duplicates. The average of two duplicate plates and the average of two samples were used to calculate the surviving number of cells per replicate. Enrichment samples and plates were incubated at 42ºC under microaerobic conditions for 48 h and CFUs for the last countable spread plate were recorded. If the enriched sample was positive and no *Campylobacter* colonies were found during enumeration, a value of 10 CFU/g of meat was assigned for that sample.

3.3.6 Statistical analysis

CFU counts of surviving cells were transformed into log CFU values. The log CFU values from duplicate samples at each time point were averaged and the standard error of the mean (SEM) calculated for each set of measures using Excel. Within temperature, effects of *Campylobacter* species, time and their interaction on log Δ were evaluated by two-way analysis of variance using the Prism software program. When a significant effect ($P < 0.05$) of species was observed, means among species within storage times were compared using Bonferroni's post-test.

3.4 Results and discussion

3.4.1 Survival of C. jejuni and C. coli at -20, 4 and 12ºC

Storage of inoculated meat at -20°C for 86 days resulted in a reduction of 2.9 ± 0.59 log CFU/g and 2.8 ± 0.51 log CFU/g for *C. jejuni* and *C. coli*, respectively (Figure 3.4.). Storage of inoculated meat at 4°C for 14 days resulted in a reduction of 0.9 ± 0.29 log CFU/g of *C. coli* and 1.7 ± 0.48 log CFU/g of *C. jejuni*. The reduction when meat was stored at 12°C, however, was much higher with 2.1 ± 0.16 CFU/g for *C. coli* and 5.3 ± 0.84 log CFU/g for *C. jejuni* (Figure 3.3.). The reduction at -20°C and 4°C was similar ($P > 0.05$) between *C. jejuni* and *C. coli* for each of the temperatures, although a higher numerical reduction was recorded for *C. jejuni* at 4°C (Figure 3.2.). The reduction between *C. jejuni* and *C. coli* at 12°C was different ($P < 0.05$), with *C. coli* exhibiting a higher survival rate at 14 days. Most of the samples had a number of cells that could be counted by direct plating at the end of the experiment, except for *C. jejuni* at 12°C on day 14, for which some of the samples were negative by direct plating and after enrichment.

The experiments were aimed at collecting survival data for *C. jejuni* and *C. coli* inoculated on boneless, skinless breast meat. This report may be one of the first publications evaluating the survival of *C. coli* in broiler meat. Inoculated products were stored at -20°C for up to 84 days, or at 4 or 12°C for up to 14 days. We are not aware of any survival publication at -20°C for more than 56 days in chicken skin fragments (*10*), or of any survival studies with inoculated product held at 12°C for up to 14 days. Most of the products held at 12°C were spoiled by the end of the study. Therefore, we believe these trials represented the "worst case scenario" of temperature/time abuse for these products.

Campylobacter spp. in retail broiler meat are usually at low numbers (approximately 0.7-0.8 CFU/g of meat), and therefore the enrichment of the samples is necessary for the isolation of the contaminating strains (*16*). Because our inoculation resulted in a countable number of *Campylobacter* cells per g of meat (6-7 log), we were confident that the isolates retrieved by

direct plating were indeed the inoculated strains. However, we also used PFGE to corroborate the genetic profile of the collected isolates. In addition, although we enriched the samples throughout the experiment the collection of *Campylobacter* cells was done by direct plating and no sample was negative by direct plating but positive after enrichment.

Survival experiments at 4^{o}C usually do not extend beyond 9 days (*7*), although a report exists for survival up to 18 days in cooked (autoclaved) meat and 24 days in raw chicken drumsticks (*3*). We decided to test up to 14 days to extend beyond most of the published survival studies in broiler skin and meat. For all practical purposes, any survival beyond 8 days is outside the shelf life of commercial broiler meat stored at 4^{o}C (*5*). In the US, product dating is not required by Federal regulations, but stores and processors voluntarily date packages of chicken or chicken products with a "sell by" date.

Storage of inoculated meat at -20^{o}C for up to 84 days resulted in a reduction of less than 3 log CFU/g of *C. jejuni* and *C. coli* in meat products, with the most important reduction appearing in the first day and a relatively constant survival up to 44 days (Figure 1). This decrease in the first 24 h of freezing also appears to be consistent with experiments using chicken meat (*12*), chicken skin (*2,7,10,23*) and culture media (*4*). Storage at 4^{o}C for 14 days resulted in a reduction of less than 1 log CFU/g for *C. coli* and approximately 1.7 log CFU/g for *C. jejuni*. The results from the experiments at 4^{o}C are in agreement with previous reports (*18*), and it appears that storage at 4^{o}C, or at -1.5^{o}C (*6*), do not yield any major reduction of *Campylobacter* spp. during the shelf life of the product. However, a much higher reduction was seen for *C. jejuni*, compared to *C. coli*, when inoculated meat was stored at 12^{o}C. The trend noticed at 4^{o}C, in which *C. coli* survived more than *C. jejuni*, was highly amplified at 12^{o}C.

We could not find previous reports comparing the survival rate of *C. jejuni* and *C. coli* in broiler breast meat. *C. jejuni* and *C. coli* have been reported to have similar survival rates on inoculated chicken skin. Both *Campylobacter* spp. exhibited a reduction of more than 3 log CFU when skin was stored at 4^{o}C for 9 days (*7*), and a reduction of 2 log CFU or more when skin was stored at -20^{o}C for 7 to 9 days (*2,7,10*). However, the survival rate of *C. jejuni* on retail broiler meat appears to be different than the survival on chicken skin, with *C. jejuni* surviving at a high number for the shelf life of the product in raw meat (*3,18*). It is surprising that few publications have assessed the survival of *C. jejuni* in retail broiler meat, with no study addressing the survival of *C. coli*. However, there are several publications dealing with the survival of *C. jejuni* on chicken skin. It is important to mention the difference in the food matrix (skin vs. meat) because the use of survival data from chicken skin may result in the underestimation of *Campylobacter* survival in raw meat.

According to epidemiological data, a failure by the consumer to properly prepare or handle contaminated food accounts for a significant proportion of the reported food borne diseases (*19*). Presently, commercial broiler processing facilities do not apply control measures that completely guarantee the elimination of these human pathogens (*15*). Therefore, the consumer is responsible for the utilization of proper food-handling techniques.

3.5 Conclusions

In summary, data results indicated that the survival was affected by storage temperature and species of *Campylobacter*. Survival of *C. jejuni* and *C. coli* was similar at -20°C, but at 4 and 12°C *C. coli* had a higher survival rates than *C. jejuni*. Therefore, although the survival of *C. coli* and *C. jejuni* may be similar at freezing temperatures the survival at refrigeration temperatures may be different. It appears that *Campylobacter* spp. may survive better on broiler

meat than on chicken skin. Therefore, more survival studies should be carried out with actual retail broiler meat, instead of chicken skin, to provide more accurate data for risk assessment studies.

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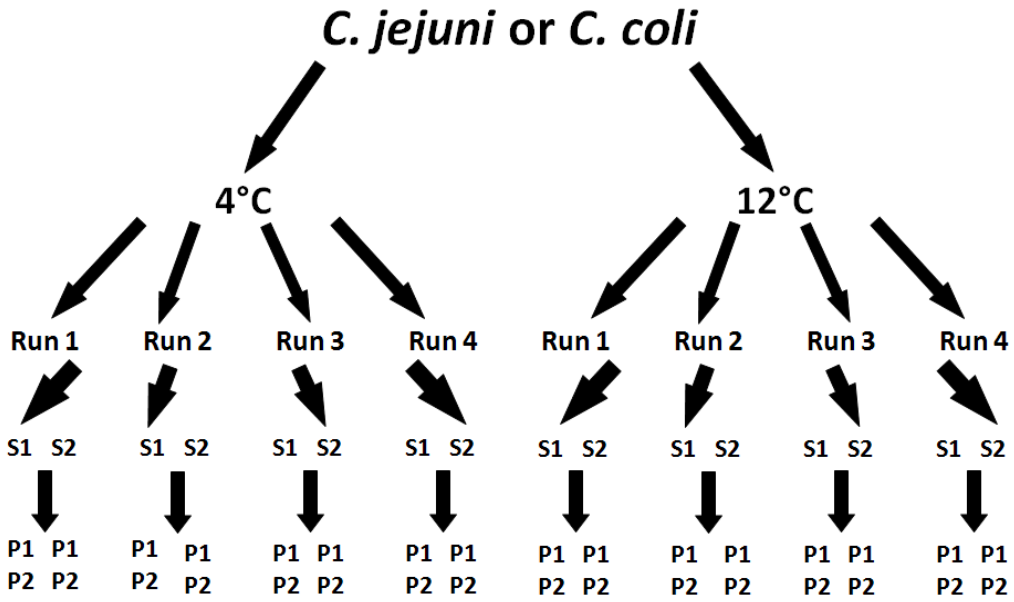

Figure 3.1. Flow chart illustrates the system used during 4°C and 12°C storage trails. Each species, *C. jejuni* and *C. coli*, was subjected to 4 runs per storage temperature. At each sample (s) time (every 48 h), two pieces of broiler meat were enriched and plated (p) in duplicate.

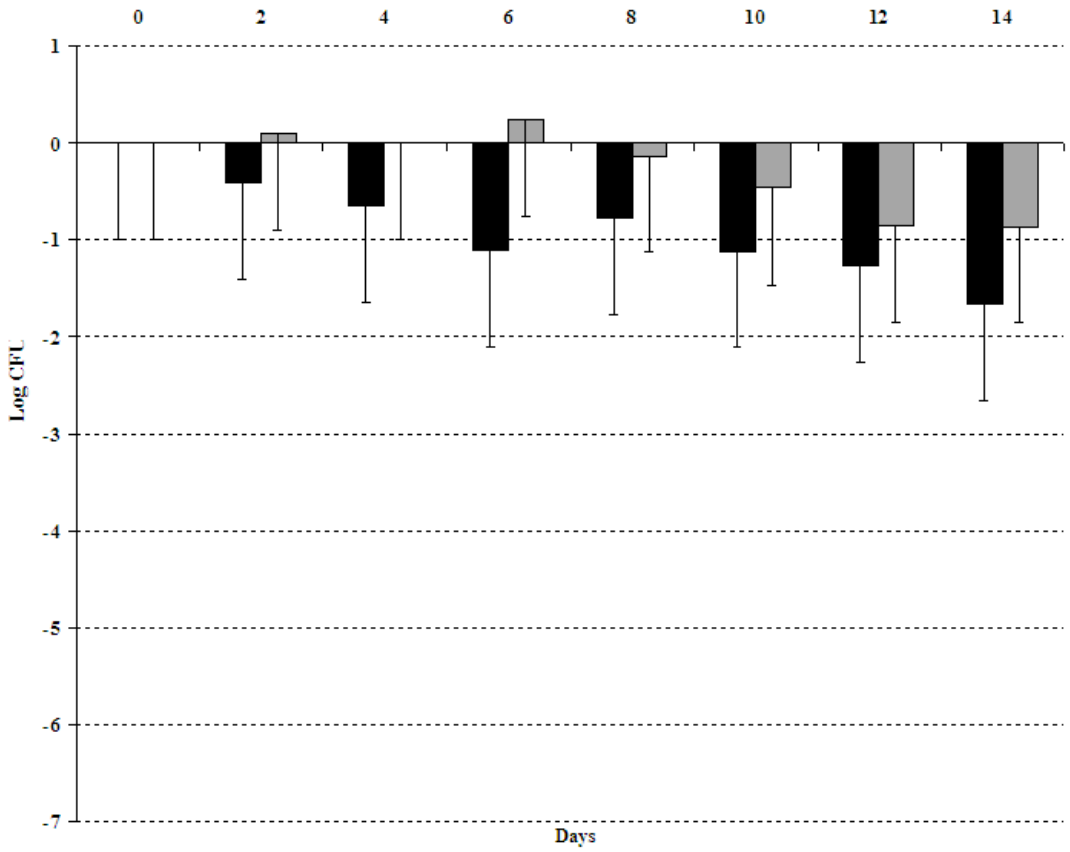

Figure 3.2. Mean log CFU/ml (±SEM) reduction of *C. coli* (gray) and *C. jejuni* (black) by day during storage at 4°C. Mean of four replicates.

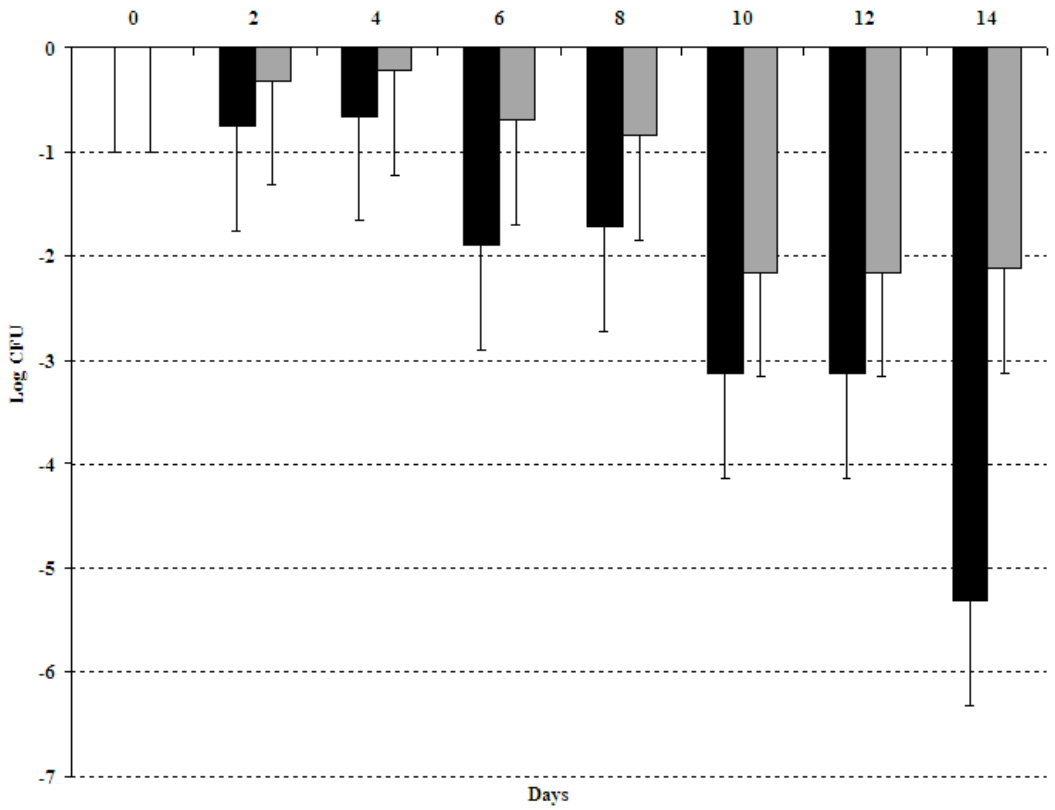

Figure 3.3. Mean log CFU/ml (±SEM) reduction of *C. coli* (gray) and *C. jejuni* (black) by day during storage at 12°C. Mean of four replicates.

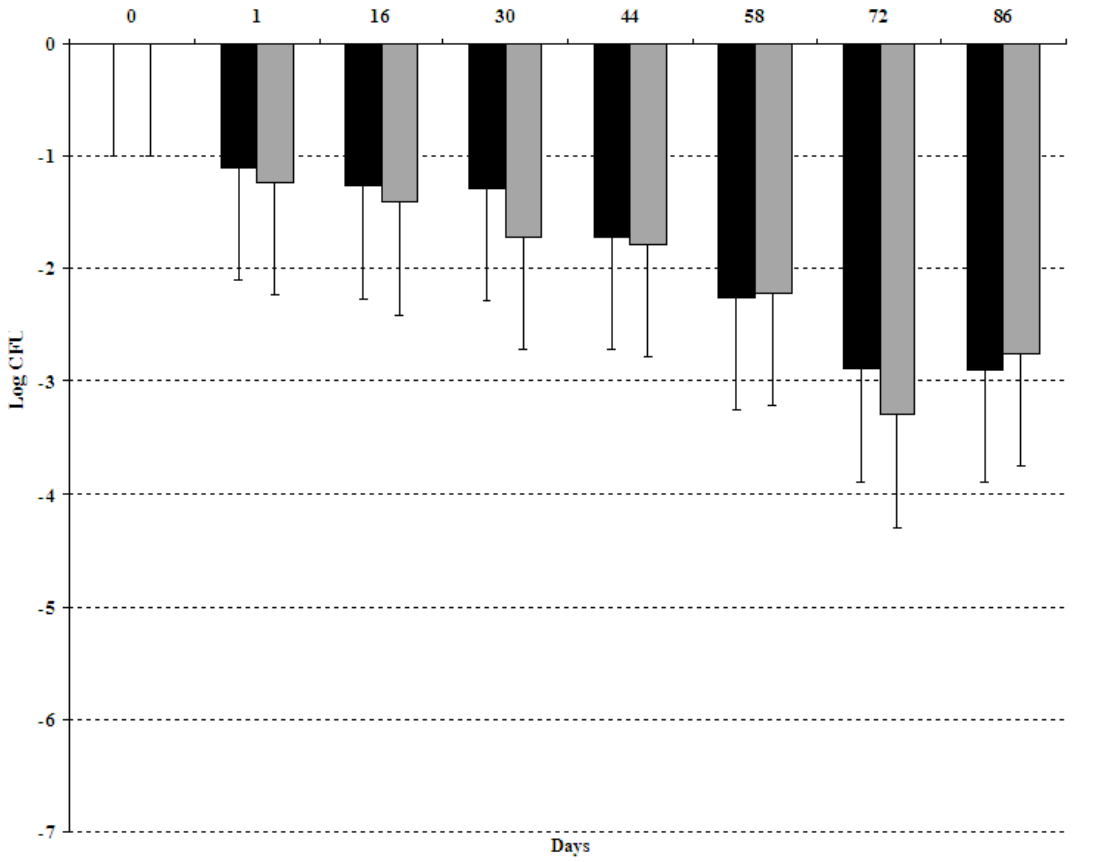

Fig 3.4. Mean log CFU/ml (±SEM) reduction of *C. coli* (gray) and *C. jejuni* (black) by day during storage at -20°C. Mean of three replicates.

Table 3.1. Results of two-way analysis of variance for effects of *Campylobacter* species and time on survival of *C. jejuni* and *C. coli* on raw broiler breast meat stored at three temperatures.

Temperature	Source of Variation	Df	Sum-of-squares	Mean square	F	P value
-20°C	Interaction	6	0.3681	0.06135	0.08667	0.9972
	Species	1	0.2002	0.2002	0.2829	0.599
	Time	6	20.46	3.41	4.817	0.0017
	Residual	28	19.82	0.7079		
4°C	Interaction	6	1.027	0.1712	0.4245	0.8583
	Species	1	7.07	7.07	17.53	0.0002
	Time	6	7.864	1.311	3.25	0.0107
	Residual	40	16.13	0.4033		
12°C	Interaction	6	11.42	1.903	1.845	0.1134
	Species	1	16.72	16.72	16.21	0.0002
	Time	6	67.98	11.33	10.99	< 0.0001
	Residual	42	43.32	1.031		

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