Campylobacter survival under stress conditions encountered between poultry farm and the human intestine

Yazan Alfalah

Master Degree Project in infection biology, 45 credits. 2018
Department: The Swedish National Food Agency (Livsmedelsverket)
Supervisor: Rikard Dryselius
Contents

Abstract .................................................................Page 3

Popular Scientific Summary ........................................Page 4

Keywords ........................................................................Page 5

Introduction ....................................................................Page 5
  • General view .........................................................Page 5
  • Pathogenesis, Treatment and Prevention ..................Page 5
  • Epidemiology ........................................................Page 6
  • Diagnosis and Growth conditions ..........................Page 6
  • Stress tolerance and Biofilm formation .................Page 7
  • Significance of the study ........................................Page 7

Aim of the study ........................................................Page 8

Material & Methods .....................................................Page 8

Results ...........................................................................Page 11

Discussion ......................................................................Page 20

Acknowledgments .........................................................Page 23

References .....................................................................Page 24
Campylobacter survival under stress conditions encountered between poultry farm and the human intestine.

Abstract

Campylobacter are probably the most important bacterial pathogen related to food-borne illnesses; specifically, gastroenteritis and diarrheal diseases. These bacteria can be isolated from various environments, but always originate from the intestine of warm-blooded animals. Particularly, Campylobacter are found in the intestinal tract of poultry, and due to contamination of poultry meat and also further contamination of other food they can cause human infections. Sometimes this results in larger outbreaks, such as during 2016–2017 in Sweden where thousands of persons got infected by a single strain of Campylobacter jejuni sequence type 918 (ST-918). The same strain was also identified amongst a large number of poultry farms and suspicions were directed towards dirty transport cages for poultry as a main route for transmitting the strain between different farms. Similar scenarios with large outbreaks related to one or two single strains (ST-50 and ST-257) had also been observed in previous years and this raised questions about certain strains being especially adapted to survive outside the intestine. The aim here was to examine whether outbreak strains and other strains of C. jejuni have different potential to resist different stress conditions that may be encountered between the poultry farm and the human intestine.

Results and conclusion: We observed that C. jejuni strains were able to survive up to 9 days at refrigeration temperature (4°C). Higher temperatures used in cooking (60°C, 68°C and 72°C) eliminate Campylobacter within a few minutes. Furthermore, UV-radiation is a powerful method for sterilization, at least if light of shorter wavelength is applied, although it’s not used in food decontamination processes. pH levels similar to that of the human stomach are a strong barrier for campylobacter, while bile salts have no effect on their survival. Interestingly, previously identified outbreak strains of Campylobacter (ST-918 and ST-257) showed a prolonged survival in poultry feces compared to non-outbreak strains; both under humid and dehydrating conditions. Finally most strains were able to form biofilms differentially, but we found that the outbreak strain ST-918 was not able to form biofilm.
Popular Scientific Summary

The challenges that face Campylobacter on their way from the poultry farms to our intestines

Contaminated food is an important cause behind many infections. A large number of different disease causing microorganisms can be transmitted by food and sometimes this results in large outbreaks. The bacterium Campylobacter is one of the most common causes behind foodborne illnesses and outbreaks. Infection is manifested by symptoms such as diarrhea and abdominal pain and may also lead to more severe complications, especially in individuals with weak immune systems. Particularly, contaminated chicken meat is an important cause of Campylobacter infections worldwide. During the last few years, several outbreaks of campylobacter related to poultry have been reported in Sweden. The latest outbreak occurred during 2016-2017, resulted in tens of thousands of reported human illnesses and was caused by one specific strain of Campylobacter jejuni that had spread among a large number of poultry farms across Sweden. Also in previous years, single strains of C. jejuni were widely spread among poultry farms and caused large number of infections in humans. The extensive distribution of these specific strains raises questions about them being especially apt to survive stress conditions encountered between the poultry farms and the human intestine. In this study, we compared stress tolerance for three of the outbreak strains from recent years to that of five non-outbreak strains of C. jejuni. Bacteria were subjected to temperature challenges during cold storage and cooking, exposure to UV light, the acidic media in our stomachs, bile acid concentrations similar to that in the duodenum and survival in chicken feces under humid and dehydrating conditions. Also, we examined the ability of these strains to form biofilm, since biofilm formation is an important strategy for many bacteria to resist stress.

Temperature challenges were performed by adding known amounts of Campylobacter to chicken meat before incubation at 4, 60, 68 and 72°C for different periods of time. At each time point the proportions of surviving bacteria were determined. The tolerance to UV light, low pH and bile acids was determined in a similar way except that all experiments were performed in lab media or on nutrient plates. Survival in chicken feces was tested by mixing known concentrations of bacteria with sterilized feces followed by bacterial counts at different time points. Finally, the ability for biofilm formation was tested by letting bacterial solutions stand in glass tubes before washing and quantification of cells adhering to the glass surface by staining.

All strains survived for at least nine days on poultry meat at 4°C and outbreak strains were among those with the highest bacterial counts after this time period. Among three tested strains, all survived for one minute at 72°C and up to three minutes at 60°C. Only outbreak strains were examined. UV-irradiation had a large impact on bacterial counts within a few seconds with only a tiny fraction surviving up to 20 seconds. There was no clear difference between outbreak and non-outbreak strains. Acidic media of the stomach proved to be an efficient barrier to Campylobacter with none of the strains surviving more than 30 seconds, while the presence of bile acids had no effect on bacterial counts. In chicken feces two of the outbreak strains showed better survival than all other strains, both under humid and dehydrating conditions. The ability to form biofilm differed largely between strains and some strains showed no ability at all to do so. In summary, outbreak strains were generally more tolerant to stress than non-outbreak strain.
Keywords

Campylobacter, Micro-aerobic, Survival, Dehydration, Poultry, Zoonosis, Inactivation, Biofilms.

Introduction

General view

Campylobacter are S-shaped gram negative bacteria, that can infect humans and animals (Anvarinejad et al., 2016; Giacomelli et al., 2014). They are motile flagellated bacteria and may contain either unipolar or bipolar flagella (Balaban & Hendrixson, 2011). Two main genes are responsible for motility; FlaA and FlaB; these genes undergo intergenic recombination which results in different serotypes of flagella with different virulence characteristics (Chaisowwong et al., 2012; Radomska et al., 2016). According to the World Health Organization (WHO) there are currently 17 species and 6 subspecies of Campylobacter, with the two species Campylobacter jejuni and C. coli mainly associated with human infections (WHO, 2018). The main known reservoir is poultry, animals usually carry Campylobacter asymptomatically (Doyle & Erickson, 2006; Johnson et al., 2014), and humans usually get Campylobacter by consuming contaminated food or by being in contact with infected animals (Fonseca et al., 2014; Sarkar et al., 2014). The infectious dose of Campylobacter is relatively low, and 500 bacterial cells may be enough to cause infection (Papic et al., 2017).

Pathogenesis, Treatment and Prevention

Campylobacter cause a gastrointestinal infection in human (campylobacteriosis) with an incubation period of 24–72. The infection is associated with; inflammatory bloody diarrhea or dysentery with abdominal cramps, fever and pain. Rarely and as late complications, bacteremia and Guillain-Barrés Syndrome (GBS) can be serious symptoms in untreated campylobacteriosis (Schnee & Petri, 2017). GBS is manifested as paralysis, and it’s believed that C. jejuni antigens cross-react with neural structures which may be the cause behind the development of Guillain-Barrés syndrome (Hahn, 1998; Lastovica et al., 1997). Many steps are required for the infection to take place. First, penetration and entry into the gastrointestinal mucosa is required, and this is established by the pathogen with the help of its high motility and helical shape (Wallis, 1994). Second, the bacteria must adhere to the enterocytes of the gut where it induces diarrhea by releasing specific toxins. C. jejuni produces many different toxins, specifically Enterotoxin and Cytotoxin, and these toxins vary between strains. There is a correlation between the toxins and the severity of the infection, but their exact role is still unclear (Crofts et al., 2018; Nielsen et al., 2010). For medication, rehydration with solutions and minerals is usually the first choice in treatment (Benoit et al., 2014; Randrianirina et al., 2014), but for severe cases antibiotics are prescribed. Azithromycin is the drug of choice in children while quinolones and tetracycline are usually used for gastrointestinal infections in adults. Other antibiotics such as, amoxicillin, ampicillin, aminoglycosides and fluoroquinolones are usually prescribed for systemic infections (Benoit et al., 2014; Randrianirina et al., 2014; Tasaka et al., 2016) but resistance development is a problem.
associated with these medications (Randrianirina et al., 2014). Infections with *Campylobacter* can be prevented and according to (WHO) there are several strategies for this including, (1) control measures at all stages in the food chain, (2) disinfection of sewage before disposal in the countries with poor sewage disposal systems, (3) reducing campylobacter in poultry by improving biosecurity, (4) applying adequate hygiene conditions during slaughtering, (5) prevention methods during cooking to avoid cross contamination and (6) heat treatment and irradiation (Golz et al., 2014; Lee et al., 1995).

**Epidemiology**

*C. jejuni*, and to some extent also *C. coli*, is considered to be one of the main causes of foodborne diseases in many developed countries, including Sweden. It can also cause extended complications for immunocompromised individuals (Edwards et al., 2014), especially AIDS patients, since it can easily spread to the blood stream and cause bacteremia (Ruiz-Contreras et al., 1997). Other species such as *C. lari*, *C. fetus* and *C. upsaliensis* have also been isolated from patients but much less frequently (Mughini Gras et al., 2013; Patrick et al., 2018). In the United States there are about 14 cases that are diagnosed annually for each 100,000 persons in the population (Batz, Hoffmann, & Morris, 2014; Guerrant et al. 1990; Tam et al., 2012), and about 200,000 cases of *Campylobacter* infections were reported in the EU during 2014 (Casanova et al., 2015; Mangen et al., 2015; Sadkowska-Todys & Kucharczyk, 2014). However, due to underreporting, The European Food Safety Authority estimated that there were a total of about nine million cases of campylobacteriosis in the EU in 2011 (Bezirtzoglou, Dekas, & Charvalos, 2011; Sadkowska-Todys & Kucharczyk, 2011). Additionally, there are high costs for public health organizations economically and in the view of individual health and productivity, which were reported to be more than 2 billion Euros per year in Europe and between 1 and 4 billion dollars in the USA (de Wit et al., 2000; Schmutz et al., 2017). These numbers reflect the importance of this pathogen and the need for studies which aim to give more understanding about its virulence and its survival.

**Diagnosis and Growth conditions**

*Campylobacter* are oxidase positive and catalase positive (Nakajima et al., 2016; van Vliet et al., 1999) and for diagnosis, cultivation of stool specimens or body tissues and fluids is the standard method to isolate different strains (Khoshbakht et al., 2015; Kirk, Nielsen, & Nielsen, 2015). Media used for cultivation of *Campylobacter* are either blood containing agar, such as Skirrow and campy CVA medium or blood free agar, such as Charcoal-Cefoperazone Deoxycholate Agar (CCDA) and charcoal-based Selective Medium (CSM) (Jokinen et al., 2012; Omurtag et al., 2011). Several biochemical tests are applied for species determination and diagnostic tests like PCR or whole genome sequencing are used as well to differentiate between strains and to learn more about genetics (Buchan et al., 2013; Price, Huygens, & Giffard, 2006). There are specific growth requirements for *C. jejuni*, since these bacteria are considered to be thermo-tolerant and they grow optimally within the range 40-42 °C (Di Giannatale et al., 2010; Rosenquist et al., 2013). Furthermore, they are microaerophilic (5% O₂ concentration is optimal) and capnophilic (require 10% CO₂ to grow optimally), (Mace et al., 2015; Nachnani et al., 1992). In spite of these requirements, *C. jejuni* are able to survive different stress conditions in the environment and show high prevalence worldwide compared to many other pathogenic microorganisms (Alpigiani et al., 2017; Oh, McMullen, & Jeon, 2015). *Campylobacter* persistence can be due to the development of several adaptation
mechanisms to overcome stresses which may be encountered all the way from the poultry slaughter house to the human intestine (Bereswill & Kist, 2002; Kreuder et al., 2017).

Stress tolerance and Biofilm formation

It has been noticed that *C. jejuni* transforms into a coccal form upon exposure to atmospheric oxygen (Harvey & Leach, 1998). Campylobacter can also survive at refrigeration temperatures up to 14 days (Eideh & Al-Qadiri, 2011; Gruntar et al., 2015; Sampers et al., 2010), but are poorly viable at room temperature (Rogol et al., 1990; Thormar et al., 2006). Fortunately, heating can destroy *Campylobacter* cells due to its sensitivity to temperatures above 48˚C (Solis-Soto et al., 2011; Sung, Hiett, & Stern, 2005). Similar to most other organisms, *Campylobacter* are highly sensitive to UV light although UV radiation is not used as a sterilization method in many countries’ food safety management including Sweden (Butler et al., 1987) (Kentson et al., 2018; Klionsky et al., 2016). In the human stomach where the pH is relatively low (1.5 - 2) (Ahirwar et al., 2014), *Campylobacter* are not able to survive for longer times, while in the duodenum where the pH becomes higher (5 – 6) due to secretion of bile acids and bicarbonate from the gall bladder and the pancreas, *Campylobacter* can survive longer, and also cause infections (Santini et al., 2010). Bile acids contain deoxycholate which has been found to enhance survival and virulence of campylobacter through the stimulation of the flaA promoter (Mohan et al., 2017; Ugarte-Ruiz et al., 2013); FlaA is the major component of the Flagella and it’s an important factor for colonization (Svensson et al., 2009; Q. V. Tu et al., 2008; Wosten et al., 2004). Interestingly, *Campylobacter* can survive outside the human intestine under different humidity conditions (Kalupahana et al., 2018; Smith et al., 2016); it has been found that *Campylobacter* can survive on poultry feces and resist the environmental changes for about six days (Kalupahana et al., 2018; Smith et al., 2016). Biofilm formation is one of the important strategies used by many bacteria in order to survive different stress conditions like those mentioned previously (Efimochkina, Bykova, et al., 2017; Efimochkina et al., 2018). We can define biofilm as a complexation of layers of bacterial cells which are attached together within a matrix of EPS (Extracellular Polymeric Substances) (Beech et al., 2006; Lin et al., 2018). *C. jejuni* is one of these microorganisms which are able to form biofilms. Different types of biofilms with *Campylobacter* have been identified such as (1) surface attached structures, (2) at the surfaces of liquids as a pellicle and (3) in liquid cultures as floating aggregates (Efimochkina, Stetsenko, et al., 2017; Kassem et al., 2012; Rajashekara et al., 2009).

Significance of the study

In 2016 during the summer, the number of domestically acquired *Campylobacter* infections in Sweden increased (The Swedish Public Health Agency, 2016), and at the end of the year thousands of additional cases were reported (EFSA, 2011). In 2017 the high number of infections continued and soon a large producer of poultry identified problems with the cleaning of their transport cages. Whole genome sequencing of isolates from poultry meat from retail and from patients revealed that one single *Campylobacter* strain of sequence type 918 was both present in meat from a large number of farms and dominated among patient samples from across the country (The Swedish National Food Agency, 2018). *Campylobacter jejuni* strain ST-918 was therefore considered the main and only cause for the increased number of cases during 2016-2017 and contaminated transport cages was considered to be a main factor behind the spread of the strain between farms. A similar scenario is also thought
to have led to outbreaks in 2014-15 and in late 2015, but these were smaller and instead involved strains of sequence type 50 and 257, respectively.

In this study, several methods were set up for quantification of *Campylobacter* subjected to different types of stress including refrigeration and cooking temperatures, pH similar to that of the human stomach, UV-irradiation and survival in poultry feces under humid and dehydrating conditions. Also the ability to form biofilms was examined. Eight different strains were compared including non-outbreak strains, the outbreak strain *C. jejuni* ST-918, and two strains responsible for other recent outbreaks (ST257 and ST50).

**Aim of the study**

- To test whether outbreak strains of *C. jejuni* (ST-918, ST257 & ST-50) are more tolerant to stress conditions encountered between the poultry farm and the human intestine compared to non-outbreak strains.

**Materials & Methods**

**1. Strains, sample preparation and growth conditions:**

Eight strains of *Campylobacter jejuni* were used in the study (Table 1); they were prepared as glycerol cultures and stored at -70°C. Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) plates (OXOID/ CM0739) and Bolton broth (OXOID/ CM0983) were used as selective media for Campylobacter and Blood Agar (BA) plates (OXOID/ CM0331 + horse blood/ Håtuna-lab AB) were used for non-selective propagation of the bacteria. Cultures were incubated in air-tight jars in a micro-aerobic conditions obtained by gas generating sachets (2.5L CampyGen™ sachets / Thermo-Scientific) at 41.5°C for 48 hours. Bacterial inoculums were prepared by dissolving pre-grown bacteria from BA plates in 2 ml Buffered Peptone Water (BPW) (OXOID/ CM0509) to obtain an OD<sub>600</sub> (The Optical Density at a wavelength of 600nm) = 0,5 ± 0,01, which corresponds to an approximate cell density of 10<sup>9</sup> CFU/ml. OD<sub>600</sub> was measured using a spectrophotometer (GeneQuant Pro/ Boule Nordic AB). Most experiments were performed in technical replicates to ensure reproducibility, and the final values in the results were taken from the performed three experiments.
2. Chicken meat preparation:

Chicken fillet of a brand that usually doesn’t contain *Campylobacter* was purchased from the store, and the meat was cut into pieces of 10g using a sharp knife. Pieces were put in freezing bags and stored at -20˚C until usage. To ensure the absence of *Campylobacter* in the meat; 10g of leftover pieces of meat were added to a 100ml flask together with Bolton broth, and incubated micro-aerobically at 41.5˚C for 48 hours. After incubation, 100µl culture was spread on mCCDA. The meat was considered free of *Campylobacter* if no growth was observed on mCCDA after micro-aerobic incubation at 41.5˚C for 48 hours.

3. Survival of Campylobacter on poultry meat at 4˚C:

Initially, survival at 4˚C was examined as this is a common temperature used for refrigeration storage of fresh chicken meat. For this, chicken pieces were thawed, put in stomacher bags (WHIRL-PAK®/Nasco) and inoculated with 100µl bacterial solution corresponding to approximately 10^8 CFU. Initial concentrations of bacteria were determined by addition of 90 ml BPW to the bags, stomaching for 30 seconds and spreading of dilution series onto mCCDA-plates. Plates were incubated as mentioned before, and the colonies were counted. Experimental samples were stored at 4˚C and harvested at day 2, day 4 and day 9 by addition of BPW, stomaching, dilution, spreading onto mCCDA, incubation and colony count. Experiments were performed in technical triplicates with all eight strains of *Campylobacter* listed in Table 1.

4. Survival of campylobacter on poultry meat at elevated temperatures:

Chicken pieces of 10g were thawed, put in smaller bags, and infected with 100µl bacterial solution (approximately 10^8 CFU). After removal of air bubbles the bags were tightly sealed. The experiment was done at 72˚C, 68˚C and 60˚C using a water bath, temperatures that chicken meat may obtain during cooking. For the 72˚C experiment, samples were removed

---

### Table 1: *Campylobacter jejuni* strains used in the study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST-45</td>
<td>Sporadic, isolated from poultry meat</td>
</tr>
<tr>
<td>ST-47</td>
<td>Sporadic, isolated from poultry meat</td>
</tr>
<tr>
<td>ST-50</td>
<td>Main cause of 2014-2015 outbreak in Sweden</td>
</tr>
<tr>
<td>ST-257</td>
<td>Main cause of winter-2015 outbreak in Sweden</td>
</tr>
<tr>
<td>ST-586</td>
<td>Sporadic, isolated from poultry meat</td>
</tr>
<tr>
<td>ST-918</td>
<td>The cause of last occurred outbreak 2016-2017 in Sweden, with the largest number of infections.</td>
</tr>
<tr>
<td>ST-1003</td>
<td>Sporadic, isolated from poultry meat</td>
</tr>
<tr>
<td>ST-4875</td>
<td>Sporadic, isolated from poultry meat</td>
</tr>
</tbody>
</table>

ST stands for (Sequence Type), outbreak strains are highlighted.
from the water bath at time points between (0.25 and 2.5 minutes), for 68°C, time points 
between 0.5 and 7 minutes were selected and for 60°C time points were chosen between 1 and
10 minutes. After heat treatment the bags were cut open and put into stomacher bags along 
with 90ml BPW. Stomacher treatment, dilution, spreading on mCCDA plates and incubation 
of the plates were performed as described above and colonies were counted. Experiments 
were performed with three different strains (ST-50, ST-257 and ST-918) and repeated at three
different occasions.

5. UV effect on campylobacter survival:
Survival of Campylobacter strains was tested when exposed to UV light. After spreading of 
the prepared bacterial dilutions on mCCDA plates (100μl, approximately 10^8 CFU), the plates 
were exposed to either UV light subtype-A (365nm/Thermo-Scientific) or subtype B 
(302nm/Gel Doc™ XR+/Bio-Rad) for different time periods. For UV-A time points between 
5 and 60 seconds were tested and for UV-B time points between 3 and 20 seconds. Plates 
were incubated at the same conditions mentioned previously and colonies were counted. 
Experiments were performed in technical triplicates with eight strains of Campylobacter. For 
UV-A, experiments were performed in technical triplicates with the outbreak strain ST-918.

6. Survival of campylobacter in acidic media:
The survival of Campylobacter strains was examined at pH 2, which is similar to that of the 
human stomach. For this, 900 μl samples of BPW adjusted to pH 1.85 with 1 M HCl were 
prepared before addition of 100 μl bacterial culture (approximately 10^8 CFU) resulting in a 
bacterial solution in pH 2.0. Upon exposure between 15 and 60 seconds the low pH 
environment was neutralized by transfer of 100 μl experimental culture into 9.9 ml BPW at 
neutral pH (7.32). Samples were spread onto mCCDA plates and the plates were incubated 
micro-aerobically using the same conditions as before and the colonies were counted. 
Experiments were performed in technical triplicates with eight strains of Campylobacter.

7. Bile Acids effect on Campylobacter survival
Since the concentration of bile acids in the human intestine usually ranges between 0.2 to 2%, 
bile acid concentrations of 0.5 and 2 % were tested to examine the survival of Campylobacter. 
For this two stock solutions were prepared by dissolving bile acid in BPW adjusted to pH 5.5 
which corresponds to the pH of the human duodenum where bile acids enter. Bacterial 
solutions were prepared as mentioned above and 100μl of each bacterial solution 
(approximately 10^8 CFU) were added to 900μl of each bile acid solution. After incubation 
between 30 seconds and 10 minutes, 100μl experimental culture was added to 9.9 ml BPW 
(pH = 7.31) to interrupt exposure to high concentrations of bile acids. Samples were spread 
onto mCCDA plates and colonies were counted upon micro-aerobic incubation. Experiments 
were performed twice with two strains of Campylobacter according to the limited time during 
the project.

8. Campylobacter survival in poultry feces with and without dehydration
Fecal material from poultry was mixed with water using a ratio 1:2 (water: fecal material) and 
then sterilized by heating at 80°C for 10 minutes using a water-bath. Test samples were spread 
onto mCCDA plates to ensure the absence of Campylobacter. For the experiments, 1 g (±
0,01g) mixture was added into small petri dishes (SARSTEDT/ 82.1194.500). Thereafter, 100μl of prepared bacterial solutions (corresponding to about 10⁸ CFU/ml) were added to the dishes and the content was homogenized properly using plastic inoculation loops. All dishes were weighed and subsets of plates were either sealed with par-film (American National Can™) to avoid loss of moisture or left unsealed to examine the effect of dehydration before incubation under aerobic conditions at 18 °C for 24, 48, 72 and 144 h. Primary concentrations of bacteria were determined by immediate plating, incubation and colony count. At each time point for harvest, petri dishes were taken out and weighed before addition of BPW up to a final sample weight of 10 g. Samples were mixed, spread onto mCCDA plates and incubated under the same conditions as above before colonies were counted. An initial test was performed with all eight strains and the experiment was repeated with three strains.

9. Biofilm Formation

By using the protocol provided in (Reuter, Mark et al., 2010) with some modifications. In brief, bacterial strains pre-grown on BA-plates were dissolved in BHI (OXOID/ CM1135) to obtain a density of OD₆₀₀= 0.5 ±0.01. Volumes of 1 ml bacterial solutions were added to glass tubes. BHI alone was used as negative controls. Upon static micro-aerobic incubation for 48 hours tubes were washed with water and then dried at 60°C for 30 minutes. Remaining water drops inside the tubes were pipetted out carefully before addition of 1 ml Crystal violet 0.3% (BD/ 212525). Tubes were incubated with shaking at room temperature for 30 minutes before removal of the Crystal violate solution and washing five times with water. After drying at 37°C, bound crystal violet inside the tubes was dissolved in 1 ml (Acetone 20% + Ethanol 80%) and OD₅₉₀ was measured on a spectrophotometer (Cary 100 UV-Vis/ Agilent).

Experiments were performed in technical triplicates on all eight strains and results for clearly deviating replicates were removed.

Results

(A) Campylobacter survival at 4 °C

As mentioned above, fresh poultry meat is a main source for Campylobacter infections in humans and an enhanced ability to survive on poultry meat during refrigeration could be an important property among strains involved in outbreaks. To test this, we compared the ability of three outbreak strains and five other strains of *C. jejuni* to survive on chicken meat stored at 4 °C for up to nine days. The results indicate an average decline in bacterial counts to half after two days and one third after four days although there were large differences between the tested strains (Figure 1). After nine days, all strains were still alive and, interestingly, the three outbreak strains (ST-50, ST-257 and ST-918) were among those with highest bacterial counts.
Figure 1: Survival on poultry meat at 4°C. Declining of bacterial concentrations (as percentages %) overtime; Blue (the primary concentrations), Red (after 2 days incubation), Green (after 4 days incubation) and Purple (after 9 days incubation). The outbreak strains are: ST-918, ST-257 and ST-50.

(B) *Campylobacter survival at elevated temperatures; 72°C, 68°C and 60°C*

During this experiment, we examined the survival of three outbreak *Campylobacter* strains (ST-918, ST-257 and ST-50) on poultry meat under several temperatures used in cooking (72°C, 68°C and 60°C). We exposed the meat pieces to different temperatures which are mentioned above for different time periods (shown in Figure 2). Not surprisingly, incubation at a higher temperature resulted in a faster bacterial killing for all three strains tested.
However, there were slight differences in killing rates between the strains with ST-918 surviving for 1 min at 72°C, for 1.5 min at 68°C and for 3 min at 60°C. ST-257 survived for as long as 5 min at both 60 and 68°C while ST-50 survived slightly longer than the other strains at 72°C. In summary, ST-918 appeared more sensitive to elevated temperatures compared to the other strains.

**Figure 2: Survival on poultry meat at elevated temperatures.** Comparison between outbreaks strains, ST-918 (Red), ST-257 (Blue) and ST-50 (Green). Bacterial concentrations (CFU/ml) overtime upon exposure to different temperatures (72°C, 68°C and 60°C). The initial CFU was set to be approximately $10^8$ CFU/ml for the samples using by measuring OD$_{600}$. 
(C) **Campylobacter tolerance to UV-irradiation**:

Although the atmosphere absorbs most of the harmful UV light, sunlight still represents an important stress to many microorganisms. In this experiment, we examined the bactericidal effect on *Campylobacter* exposed to two subtypes of UV (UV-A & UV-B) which differ in their wavelengths; (320-400 nm and 290-320 nm, respectively). When exposed to much less harmful UV-A (365nm), strain ST-918 showed no decline in bacterial counts even after one minute (Table 2). When exposed to more harmful UV-B light (302nm), however, numbers of ST-918 declined more than $10^5$ fold after just 20 seconds of exposure (Figure 3). A similar result was also observed for strains ST-50, ST-4875 and ST-586, while the other strains tested were even more sensitive to UV-B irradiation (Figure 3).

<table>
<thead>
<tr>
<th>ST-918/365nm</th>
<th>Time points</th>
<th>Bacterial concentrations CFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0s</td>
<td>$123 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>5s</td>
<td>$135 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>10s</td>
<td>$127 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>30s</td>
<td>$150 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>60s</td>
<td>$134 \times 10^3$</td>
</tr>
</tbody>
</table>

*Table 2: Tolerance to UV irradiation; UV subtype A (365nm).* Bacterial counts of *C. jejuni* strain ST-918 upon exposure to UV-A (365nm) at different time points (between 0 and 60 seconds).
Figure 3: Tolerance to UV irradiation; UV subtype B (302nm). Bacterial counts percentages of different strains of *C. jejuni* upon exposure to UV-B, different time points (between 0 and 20 seconds). The outbreak strains are: ST-918, ST-257 and ST-50.
(D) Campylobacter survival at low pH

In the human stomach, the pH usually ranges between 1.5 and 2, which makes it a highly acidic environment that plays an important role in protection against different microorganisms. We hypothesized that one reason behind the previously occurred outbreak could be that some strains of Campylobacter were more tolerant to the acidic media of the human stomach. To test this, all eight strains were challenged to low pH (pH=2) for different periods of time (15, 30, 45 and 60 seconds). The results indicate that none of the strains survived longer than 30 seconds and that highest bacterial count were found for the non-outbreak strain ST-586 (Table 3 and Figure 4). However, differences in survival for the eight strains were only very small.

<table>
<thead>
<tr>
<th>Strain</th>
<th>bacterial counts</th>
<th>15s</th>
<th>30s</th>
<th>45s</th>
<th>1min</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST-45</td>
<td>53* 10^6 CFU/ml</td>
<td>1.37%</td>
<td>0.08%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>ST-47</td>
<td>37* 10^6 CFU/ml</td>
<td>4.51%</td>
<td>0.16%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>ST-50</td>
<td>31* 10^6 CFU/ml</td>
<td>1.42%</td>
<td>0.12%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>ST-257</td>
<td>44* 10^6 CFU/ml</td>
<td>5.69%</td>
<td>0.1%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>ST-586</td>
<td>40* 10^6 CFU/ml</td>
<td>7.23%</td>
<td>0.4%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>ST-918</td>
<td>34* 10^6 CFU/ml</td>
<td>1.9%</td>
<td>0.21%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>ST-1003</td>
<td>35* 10^6 CFU/ml</td>
<td>6.56%</td>
<td>0.05%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>ST-4875</td>
<td>34* 10^6 CFU/ml</td>
<td>1.71%</td>
<td>0.25%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table 3: Campylobacter survival at low pH similar to that of the human stomach. Bacterial concentrations (CFU/ml) of different strains of C. jejuni at pH=2, different time points between 0 (Primary) and 1 minute. The outbreak strains are: ST-918, ST-257 and ST-50.
Figure 4: Campylobacter survival at low pH similar to that of the human stomach. Bacterial counts percentages of different strains of *C. jejuni* at pH=2, different time points (between 0 and 1 minute). The outbreak strains are: ST-918, ST-257 and ST-50.

**Effect of bile acids on the survival of campylobacter**

In the duodenum, the first part of the human intestine, bile salt concentrations are within the range of 0.2-2%. Together with other compounds such as bicarbonate, the bile salts help increasing the pH to 5-6. As *Campylobacter* species infect and colonize the intestine of poultry and humans, they should be able to tolerate high concentrations of bile salts at pH 5-6. To test this survival of two strains (ST-918 and ST-45) was examined in two different concentrations of bile salts, 0.5, 2%, at pH 5.5. As predicted, the results revealed that bile salts
had no effect on the survival of campylobacter as there was no observed decline in bacterial counts after up to 10 minutes exposure (data not shown).

(F) Campylobacter survival in poultry feces under humid and dehydrating conditions

Campylobacter are able to survive in poultry feces outside the intestine for some time (Kalupahana et al., 2018; Smith et al., 2016) if poultry farms are not properly cleaned after an infected flock, there is therefore a risk of transfer of a strain to the next. There is also a risk of transfer of bacteria between poultry farms with dirty transport equipment, which has been repeatedly observed in outbreaks during the past few years. To test whether outbreak strains survive better than non-outbreak strains we examined the survival of Campylobacter in poultry feces under humid and dehydrating conditions at 18°C for 6 days. Interestingly, the outbreak strains survived generally better than the non-outbreak strains in both conditions (Table 4 and Figure 5). All tested strains could be detected after 3 days while outbreak strains ST-918 and ST-257 could be detected after up to 6 days incubation.

Table 4: Survival in poultry feces under humid and dehydrating conditions. Bacterial concentrations (CFU/ml) overtime (between 1 and 6 days) for different strains of C.jejuni in poultry feces during humid or dehydrating conditions. Red (more than 10⁶CFU/ml), Orange (more than 10⁵ but less than 10⁶CFU/ml), Light orange (more than 10⁴ but less than 10⁵CFU/ml), Yellow (more than 10³ but less than 10⁴ CFU/ml), Blue (more than 10 but less than 10² CFU/ml), Grey (between 0 and 10 CFU/ml) and White (0 CFU/ml). The outbreak strains are: ST-918, ST-257 and ST-50.
Figure 5: Survival in poultry feces under humid and dehydrating conditions. Bacterial concentrations percentages of the outbreak strains (ST-257 in red, ST-918 in blue and ST-50 in green) overtime (up to 6 days) in poultry feces, dehydrating conditions (above) and humid conditions (below).
(G) *Ability to form biofilm*

Biofilm formation is an important strategy used by many bacteria in order to resist different types of stress circumstances. We examined the ability for biofilm formation using all eight strains of *Campylobacter*. Most strains could form biofilms, but to different extent (Table 5). However, some of the strains could not, including the outbreak strain ST-918 and also strains ST-45 and ST-4875.

<table>
<thead>
<tr>
<th>Samples/ Strains</th>
<th>OD&lt;sub&gt;590&lt;/sub&gt; (Average value)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHI</td>
<td>0.177</td>
<td>Control</td>
</tr>
<tr>
<td>ST-45</td>
<td>0.146</td>
<td>(-)</td>
</tr>
<tr>
<td>ST-47</td>
<td>0.271</td>
<td>(+)</td>
</tr>
<tr>
<td>ST-50</td>
<td>0.36</td>
<td>(++)</td>
</tr>
<tr>
<td>ST-257</td>
<td>0.239</td>
<td>(+)</td>
</tr>
<tr>
<td>ST-586</td>
<td>0.287</td>
<td>(+)</td>
</tr>
<tr>
<td>ST-918</td>
<td>0.146</td>
<td>(-)</td>
</tr>
<tr>
<td>ST-1003</td>
<td>0.357</td>
<td>(++)</td>
</tr>
<tr>
<td>ST-4875</td>
<td>0.186</td>
<td>(-)</td>
</tr>
</tbody>
</table>

Table 5: Biofilm formation. Strong biofilm forming strains (++), weak biofilm forming strains (+) and negative biofilm forming strains (-). Eight strains of *C. jejuni*, BHI (Blood-Heart Infusion) as control. OD<sub>590</sub> (the optical density at wavelength 590nm for crystal violet). The outbreak strains are: ST-918, ST-257 and ST-50.

**Discussion**

In this study, we found several strong correlations between our results and those of previous studies within the area. Interestingly, repetition of our experiments showed a reliable interference statistically, the mean and the standard variations were stabilized. Additionally, we performed a new approach when we compared the observed outbreak strains of *C. jejuni* with the non-outbreak strains of the same species, this could build the basics for additional wider studies with more species and strains and thus limits the occurrence of outbreaks connected with these bacteria. Furtherly, we could have done our experiments by testing naturally contaminated meat instead of artificially inoculated one, anyway we consider our work as a beginning to perform more modifications in futuristic research. In specific, we found that all eight tested strains of *Campylobacter* were able to survive at refrigeration temperatures, 4°C specifically, for more than 9 days. This means that campylobacter in general are relatively tolerant to refrigeration temperatures and thus can survive for long periods on stored poultry meat and later cause infections, especially during poor hygiene practices. Previous studies have shown that different strains of *C. jejuni* are cold tolerant for up to 14 days of storage at 4°C and the degree of tolerance is strain specific. This suggests a
genetic basis for differences in tolerance and resistance development (Chan, Le Tran, Kanenaka, & Kathariou, 2001). Additionally, we found that the outbreak strains (ST-918, ST-257 and ST-50) survived slightly better with higher bacterial counts after 9 days storage at 4°C compared to most other strains examined. Another study have found that Campylobacter strains isolated from the clinic are more resistant and tolerant under cold storage at 4°C (Chan et al., 2001). As conclusion, outbreak strains seem to be generally more resistant and tolerant to low temperatures used for refrigeration storage.

Similar to previous findings, elevated temperatures like those used in cooking showed high efficiency in eliminating Campylobacter. We saw some differences between the three outbreak strains examined where ST-257 could survive longest (5 minutes) at 60 and 68˚C while ST-50 survived longest (1.5 minutes) at 72˚C. However, the survival of all three examined C. jejuni strains were decreasing with increased temperature. In similar studies, the effect of thermal stress on C. jejuni and C. coli strains were examined and bacteria were able to survive for up to 5 minutes at temperatures ranging between 56.6 - 62.5˚C (J.E. Moore & R.H. Madden, 2000). Other studies have also tested the survival of Campylobacter in milk to investigate the efficiency of pasteurization process shown that different temperatures such like 50˚C and 60˚C are effective in eliminating and inactivating Campylobacter species within 4 and 1 minute respectively (Gill et al., 1981). However, it’s not unlikely that matrix effect from the milk contributes to this shortened survival. As conclusion, elevated temperatures commonly used in cooking or pasteurization are effective in food sterilization and management with a long enough time of heat treatment.

UV-irradiation has a strong influence on living cells and it can be used as a bactericidal to kill most microorganisms. Yet it’s not used in many countries, including Sweden, during food hygiene practices. UV affects biological pathways and the protein synthesis mechanisms inside living cells, it might cause peptide bonds to be altered and thus DNA will be destroyed or changed (J. A. Parrish et al., 1978; Heinrich et al., 2016; J. C. Tu, 2002). When we examined the influence of UV irradiation on campylobacter, we found that the UV-subtype is important for the efficiency of bacterial killing. There are three subtypes of UV radiation which are classified according to their wavelengths; UV-A (320-400 nm), UV-B (290-320 nm) and UV-C (100-290 nm) (Dardalhon et al., 2008; National Toxicology, 2002). We found that UV-A had no influence on the survival of the Campylobacter outbreak strain ST-918 after 1 minute exposure, while a large reduction was observed within 20 seconds for all eight strains tested when exposed to UV-B light. However, we have an idea that UV-A which is close to the visible light has a very low or even no effect on bacterial killing (E. R. Kashket and A. F. Brodie, 1962), so the experiments were performed just withLastly observed outbreak strain ST-918 to save time and material. Other studies have also indicated that UV-B light is effective in bacterial elimination (Isohanni & Lyhs, 2009). However, they indicated that there is less effect when it’s applied on inoculated poultry meat than on bacterial cultures directly. Another study found that the artificial sunlight has a smaller effect on Campylobacter as bacteria were able to survive up to 30 minutes of exposure (Obiri-Danso et al., 2001).

We found that our tested Campylobacter strains are not able to survive for long at pH levels similar to that of the human stomach. None of the eight examined strains was detected after 45 seconds incubation in BPW (pH=2), which suggests that campylobacter have extremely
limited opportunities to infect and colonize the stomach in humans. Other studies have mentioned that there is no evidence of the isolation of *C. jejuni* from the stomach (Sahay et al., 1995). Yet, *Campylobacter* must be able to pass the low pH in the human stomach, and maybe transport with food could help them avoid the low pH levels. In contrast, *C. jejuni* strains are able to survive in the duodenum where bile acids play an important role in pH neutralization (pH= 5-6) (Castillo-Lopez et al., 2014; Matsuha & Tsukui, 2012; Zeller et al., 2015). In agreement with this, we didn’t observe a reduction in bacterial counts when *Campylobacter* strains were tested with different concentrations of bile salts that exist normally in human body (0.5 and 2%). This observation is in line with that *Campylobacter* species infect and colonize the intestine in humans and in poultry (Pielsticker et al., 2012). Furthermore, it has been found that deoxy-cholate, which is a part of bile acid salts, enhances the expression of the flaA gene which is important for colonization (Malik-Kale et al., 2008).

Contaminated transport cages are believed to have been a major contributing factor for the spread of *Campylobacter* between poultry farms during the outbreaks in Sweden the past few years. We found that the outbreak strains of *C. jejuni* (ST-918, ST-257 and ST-50) are able to survive better and for longer periods on chicken feces compared to most non-outbreak strains. Only small differences were determined between humid and dehydrating conditions, however, ST-257 and at one occasion also ST-918, could survive for 6 days in chicken feces under humid conditions. These findings could explain why these strains became widely spread between poultry farms, remained at specific farms for extended time periods and could cause the large outbreaks. It has been noted by others, though, that there are some differences in survival between naturally and artificially contaminated feces (Ahmed et al., 2013). For example Ahmed et al. did not detect any bacteria after 6 days of incubation of artificially inoculated feces while they detected bacterial survival after 6 days in naturally contaminated feces. With this in mind, further studies should be performed using naturally contaminated feces to complement our results obtained here.

Interestingly, most of the tested strains were able to form biofilms, although there were some differences. For example, the outbreak strain ST-918 couldn’t form biofilm, which suggests that there are different mechanisms or strategies that ST-918 use to survive for extended time outside the intestine. Other strains such as ST-45 and ST-4875 couldn’t form biofilm either. However, our experiments for biofilm formation were done micro-aerobically which is considered to be the standard condition for *Campylobacter* growth (Davis & DiRita, 2008). Another study have shown that campylobacter species form biofilm better in the aerobic conditions (Reuter et al., 2010). The same authors also found that the strain ability to form biofilms is influenced by specific characteristics of bacteria such as motility. It would therefore be interesting to perform further examinations of such characteristics and conditions on the strains we have used here.

In summary, *C. jejuni* strains examined here show some differences in resisting different stress conditions. They showed a decline in colony count overtime at refrigeration (4°C), although they were all able to survive more than 9 days. Cooking (60°C, 68°C or 72°C) can kill the bacteria within a relatively short period of time. Further, UV-irradiation is destructive for *Campylobacter* although it’s not used in food hygiene practices in many countries around the world including Sweden. Actually, the gastric pH in humans inhibits the survival of *Campylobacter* in less than 45 seconds, while it survives in the lower parts of the GI tract.
especially in the presence of bile salts which helps the survival and improves colonization and cause enteritis rather than gastritis. Additionally, *C. jejuni* strains are able to survive outside the intestine, specifically in poultry feces from where they transmit between poultry flocks within a farm as well as between farms during transportation. Finally, biofilm formation is a characteristic for a majority of the studied strains except for ST-918, ST-45 and ST-4875 which could be able to develop resistance to extra-intestinal stress using other strategies and mechanisms.

Interestingly, during most examined stress conditions, the outbreak strains of *C. jejuni* were more resistant and survived better than the other studied strains. This supports the hypothesis that outbreak strains are more fit to survive and spread outside the intestine and therefore cause more infections. Actually, this creates new hypothesizes that describe the mechanisms behind being an outbreak strain. Genetic basis, stress tolerance or different natural conditions could play essential roles in being an outbreak strain. To test this, more studies are needed on genetic mechanisms behind stress tolerance beside our experiments and findings presented here. These to more easily identify strains of *C. jejuni* that pose larger risks and be able to eliminate them from poultry production at an early stage. It’s also important to include more strains of *C. jejuni* and maybe also *C. coli* to confirm the results obtained here, especially that the experiments seem to be reproducible according to our obtained results. Finally, it would be important to perform experiments in biological triplicates as well to determine the variations between the biologically distinct samples.

**Acknowledgements**

I would like to mention that this work was supported by different institutions, first of all; The Swedish National Food Agency (Livsmedelsverket) which has provided the materials and the workplace during this project. Many thanks as well to all biology department members who work there for being good helpful work partners. Secondly, I also want to acknowledge the Swedish National Veterinary Institute (SVA) for kindly providing strains ST-50 and ST-257.
References


EFSA. (2011). Scientific opinion on Campylobacter in broiler meat production: control options and performance objectives and/or targets at different stages of the food chain. *EFSA Journal* 9(4); 2105


