SURVIVAL OF CAMPYLOBACTER JEUNI AND CAMPYLOBACTER COLI IN CHICKEN LIVER AT FROZEN STORAGE TEMPERATURES

Snezana Ivić-Kolevska¹*, Biljana Miljković-Selimović², Branislava Kocić², Goran Kolevski³

¹Institute of Public Health of the Republic of Macedonia, 50 Divizija 6, 1000 Skopje, Macedonia
²Referent Laboratory for Campylobacter and Helicobacter, Institute for Public Health, Dr Zoran Djindjic 50, 18000 Niš, Serbia
³Clinic of Neurology, Bul. Vodnanjska nn, 1000 Skopje, Macedonia

*e-mail: snezanaivickolevska@hotmail.com

Abstract

The aim of this study was to determine the survival of Campylobacter jejuni and Campylobacter coli in chicken liver samples at low and frozen temperatures after different times of incubation, to assess the effect of the type of plate, to determine the difference in survival of C. jejuni and C. coli at different temperatures and to determine the impact of aerobic bacteria on the survival of C. jejuni and C. coli.

Chicken liver samples were inoculated with C. jejuni NCTC 11351 suspensions and stored in bags at temperatures of -20 °C and -70 °C, same time, other samples of chicken liver were inoculated with C. coli ATCC 33559 suspensions and stored in the same conditions. After the incubation period, every sample was left to defrost spontaneously. 0.1% of peptone water was added to the sample to obtain dilution of 1:10 and pummeled for 2 min. at 400 rpm/min. Then, from the homogenized mixture, serial dilutions (1:10 to 1:10⁷) were made with 0.1% of peptone water. 0.1 mL of every dilution was cultivated in duplicate, on two selective (Modified charcoal cefoperazone deoxycholate agar and Campylobacter agar with 5% sheepblood) and two non-selective media (Tryptic soy blood agar with 5% sheepblood and Columbia blood agar with 5% sheepblood) for C. jejuni and C. coli isolation. Plates were incubated at 42 °C for 48 h in microaerobic atmosphere with 9 - 10% of CO₂. For detection of aerobic mesophilic bacteria, 0.1 mL of every sample were cultivated on plate (Plate Count Agar) in serial dilutions 1:10 to 1:10⁷ and incubated at 30 °C for 72 hours.

The mean value of C. jejuni from liver samples decreased from 7.38 log10 CFU/g after 30 minutes of incubation at ambient temperature, while at freezing temperatures the value in the 10-th week decreased to 3.41 log10 CFU/g at -20 °C and 3.97 log10 CFU/g at -70 °C. The mean value of C. coli from liver samples decreased from 6.29 log10 CFU/g after 30 minutes of incubation at ambient temperature, while at freezing temperatures the value in the 10-th week decreased to 4.38 log10 CFU/g at -20 °C and 4.40 log10 CFU/g at -70 °C.

The presence of aerobic mesophilic bacteria did not influence the survival of C. jejuni and C. coli in chicken liver samples. Keeping poultry liver at freezing temperatures is important for reducing the number of C. jejuni and C. coli which has a strong influence on the prevention of campylobacteriosis occurrence in humans.

Key words: Campylobacter jejuni, Campylobacter coli, Campylobacteriosis, Chicken liver.

1. Introduction

Campylobacter jejuni (C. jejuni) and Campylobacter coli (C. coli) are the most common causative agents of enteritis in the modern world, with about 400 million patients each year [1]. In England and Wales, as well as in other parts of the developed world, primarily C. jejuni is still a very important pathogen which can be found in food and water. Laboratory Service of Public Health (Public Health Laboratory Service, PHLS) reported that in 2001 reported 60,000 cases of campylobacteriosis in England and Wales [2].

The main reasons for frequent occurrence of enteritis caused by Campylobacter are a small infectious dose, from 100 to 800 microorganisms (usually 500) [3], the way of entering the body through contaminated food, and the main reason for its expression is reduced immunity [4]. The infective dose is even smaller in children and immunocompromised persons than in adult healthy people [4, and 5].
Clinical course of campylobacteriosis can be ranged from mild enteritis with spontaneous remission to very serious conditions with extraintestinal forms of the disease. The most common symptoms of campylobacteriosis in humans are: enteritis, diarrhea, fever, abdominal pain, pseudoapendicitis, cell exudate and blood in the stool [6]. However, the extraintestinal forms of disease can manifest as bacteremia or myocarditis, pancreatitis [7], septicemia [8], hemolytic uremic syndrome [9], meningitis [10], cholecystitis [11], arthritis and erythema nodosum. In addition, some patients will suffer from postinfectious sequelae such as Guillain-Barré syndrome (GBS) [12], Miller Fisher syndrome [13], Reiter’s syndrome, reactive arthritis [14], and ulcerative colitis [15, and 16].

Reservoirs of campylobacters are domestic and wild animals and they provide the circulation of bacteria in nature. Poultry is colonized by a large amount of Campylobacter in feathers, skin and intestinal tract, which can confer contamination by this microorganism during slaughter, as well as its maintenance in retail stores [17]. Although poultry can be colonized in 50 - 90% from the second to third week of age, in chickens, clinical symptoms occur relatively rarely [1, 18, and 19].

Most common sources of Campylobacter infections are: food (raw and undercooked chicken and raw and pasteurized milk) and untreated drink water from the cold mountain streams. Mode of transmission may be indirect contact with infected animals or infected person [20]. In addition, cross-contamination of kitchen surfaces or direct hand-to-mouth contact after handling raw poultry can occur [17].

Freezing has been reported to reduce the number of campylobacters in chicken carcasses [21], and has been proposed as a method for reducing contamination levels on poultry meat [22]. The rate of chilling could have a significant effect on the survival rates of microorganisms, since rapid chilling will reduce the formation of ice crystals [23]. On the other hand, these temperatures maintain a small number of live bacteria, which means that single or in combination, refrigeration and freezing are not a substitute for safe handling and proper storage of poultry [24].

Given that products derived from chicken often are the source of infection with C. jejuni, following goals are set for this research: to determine the survival of Campylobacter jejuni and Campylobacter coli in chicken liver samples at frozen temperatures after different times of incubation; to assess the effect of the type of plate; to determine the difference in survival of C. jejuni and C. coli at different temperatures and to determine the influence of aerobic bacteria on the survival of C. jejuni and C. coli on the given temperatures and given length of incubation.

2. Materials and Methods

2.1 Bacterial strains and culture conditions
C. jejuni NCTC 11351 (Microbiologics, USA) and C. coli ATCC 33559 (Microbiologics, USA) strain was resuscitated at non-selective medium (Columbia Sheep Blood Agar (CBA), Liofilchem, Italy), at 42 °C for 48 h in microaerobic atmosphere with 9 - 10% CO₂ (thermostat - BINDER, USA). For inoculation onto liver, strain was grown as a lawn on CBA, and incubated at 42 °C for 48 h under microaerophilic conditions. Immediately before use as an inoculum, the lawn was washed off each plate with saline solution (0.9% NaCl) at room temperature and mixed using the vortex (EV102, Tehtnica, Železniki, Slovenia), in order to make the final concentration of bacterial suspension, of 10⁷ CFU/mL, which was confirmed on the densitometer (Vitek Densichek, BioMérieux, France).

2.2 Liver preparation and packaging
All chicken carcasses were purchased at a local retail market and were previously tested for the presence of Campylobacter species according to ISO 10272-1:2006 and ISO 10272-2:2006 [25, and 26]. Specimens did not contain any Campylobacter species and were further processed.

Chicken liver was divided into portion samples of 10 g (measured on digital balance, SCALTEC, Germany). Samples were placed in bags (Becton Dickinson, New York, USA) and inoculated with 800µl/g of C. jejuni NCTC 11351 and C. coli ATCC 33559 suspensions with concentration of 10⁶ CFU/mL. After removing the excess of air, the whole amount of the inoculums with samples were stored in closed bags, to prevent drying. For homogenous dispersion of inoculums, contaminated samples were mixed thoroughly.

Inoculated chicken liver samples were stored at temperatures of -20 °C and -70 °C and the survival of C. jejuni NCTC 11351 and C. coli ATCC 33559 were investigated after: the 7th day of incubation period, on the 2nd week, 4th week, 8th week and 10th week. Also, survival of C. jejuni and C. coli ATCC 33559 in one inoculated chicken liver sample was determined after 30 minutes of incubation at ambient temperature.

2.3 Microbiological and statistical analysis
After the incubation period, every sample incubated at -20 °C and -70 °C was left to defrost spontaneously. 0.1% of peptone water (Torlak, Serbia) was added to the sample to obtain dilution of 1:10 and pummelled for 2 min. at 400 rpm/min., (Stomacher lab blender, MIX2, AES Laboratories, Seward, London, UK). Then, from the homogenized mixture, serial dilutions (1:10 to 1:10⁷) were made with 0.1% of peptone water. 0.1 mL of every dilution
was cultivated in duplicate, on two selective (Modified charcoal cefoperazone deoxycholate agar (mCCDA), and Campylobacter agar with 5% sheepblood (CA) (Liofilchem, Italy), and two non-selective media (Tryptic soy blood agar with 5% sheepblood (TSBA), and Columbia blood agar with 5% sheepblood (CBA) (Liofilchem, Italy) for \( C. \text{jejuni} \) and \( C. \text{coli} \) isolation. Plates were incubated at 42 °C for 48 h in microaerobic atmosphere with 9 - 10% of CO\(_2\) (thermostat BINDER, USA). Thereafter, the growth of \( C. \text{jejuni} \) and \( C. \text{coli} \) was confirmed based on typical colony and bacterial cells morphology, standard biochemical tests and API Campy identification system (bioMérieux, France).

For detection of aerobic mesophilic bacteria, 0.1 mL of every sample were cultivated on plate (Plate Count Agar (PCA) (Liofilchem, Italy) in serial dilutions 1:10 to 1:10\(^7\) and incubated at 30 °C for 72 hours.

The counting of confirmed \( C. \text{jejuni} \) and \( C. \text{coli} \) colonies was performed in duplicate, by colony counter (Colony Counter, Japan). The number of grown microorganisms was expressed according to ISO 10272-2:2006, as the logarithm to the base of the 10\(^{th}\).

Each plate count was multiplied by the appropriate dilution factor to estimate the total CFU per gram for each sample. The average count of \( C. \text{jejuni} \) and \( C. \text{coli} \) on the selective plates was determined as mean value (\( \bar{X} \)) by calculating the number of bacteria on mCCDA and CA, and for non-selective plates it was obtained by calculating the number of bacteria on the TSBA and CBA. The mean value of Campylobacter on selective and non-selective media plate were obtained by calculating the number of bacteria on mCCDA, CA, TSBA, CBA.

Comparison of the number of surviving bacteria was carried out in relation to the number of bacteria obtained after incubation of the inoculated material for 30 minutes at room temperature.

Quantitative statistical analysis and statistical calculations were performed using the SPSS program, version 10.0. To compare the mean count of bacteria isolated on different temperatures and on different types of plate as well as to test the effects of incubation temperature, presence of aerobic mesophilic bacteria and the incubation period to the number of survived bacteria, analysis of variance for repeated measures (Repeated Measures ANOVA) was carried out, using Greenhouse-Geisser correction for sphericity control, and significance was based on level of 5% (\( p < 0.05 \)).

3. Results and Discussion

Campylobacter spp. is very sensitive to extraintestinal environment. \( C. \text{jejuni} \) is hard to survive the high and low temperature, low pH, dry conditions and seems much sensitive to outer surrounding against the bacteria of the genus Salmonella (4, 17, and 18).

There are very few studies dealing with the study of the survival of the type Campylobacter in chicken liver. Whyte \textit{et al.}, (27), concluded that the speed of freezing has more influence on the survival of Campylobacter than the length of storage of chicken parts at low temperatures. Slow freezing is deadly for Campylobacter than rapid freezing due to osmotic stress cells, which is more pronounced in slow freezing. Very high difference in the freezing rate (over 10 °C/min.) can reduce cell survival Campylobacter and intracellular ice crystal formation, which in turn leads to mechanical damage to the cells, although these differences in the temperature of freezing are difficult to achieve in industry. However, the results indicate that Campylobacter is tolerant to chilling, although the conditions under which achieves a significant reduction in the number of Campylobacter cells, the optimal freezing chicken at low temperatures (-10 °C) for a long enough period of time, are useful for reducing the number of bacteria in the samples (27).

The same group of researchers has found that there is a significant reduction in the number of Campylobacter-emerged in chicken parts that were inoculated with the bacteria and stored at lower temperatures, which in their experiment was freezing, at -10 OC. This effect is most likely due to a longer cooling time necessary to reach a temperature of -10 OC (19 h and 40 min.), compared to the time to achieve a temperature of -2 OC (4 h and 20 min.) (27).

During this investigation it was determined that, after 30 minutes of samples incubation at ambient temperature (day 0, initial number), the mean count (\( \bar{X} \)) of \( C. \text{jejuni} \) on both types of plates was 7.38 log10 CFU/g.

In our study, the mean count of \( C. \text{jejuni} \) stored at -20 OC decreased from initial number (7.38 log10 CFU/g) for 2.42 after 1 week, and additionally for 0.78 log10 CFU/g after 8 weeks of incubation, so in the 10 week of incubation survival rate was 3.41 log10 CFU/g (Figure 1). At this incubation temperature, changes in the number of bacteria according to the incubation periods were statistically significant (\( p = 0.011 \)).

The number of surviving bacteria tends to decline with prolonged preservation of products at freezing temperatures, although there is usually stabilization after a few months when further reduction is minimal. The types of bacteria present in the frozen products, depend on the initial population (28).

Some were killed, others only sublethaly damaged and can be recovered during thawing, especially if the storage temperature above -10 OC (below -10 OC sublethal damaged bacteria tend to die over time, and therefore recommendations are that the frozen meat should be stored at or about -18 OC). Usually, the process of freezing is more lethal to bacteria than keeping the product in the frozen state (29).
In our study, the mean count of *C. jejuni* stored at -70°C decreased from initial number (7.38 log$_{10}$ CFU/g) for 2.66 after 1 week, and additionally for 0.15 log$_{10}$ CFU/g after 8 weeks of incubation, so it is in the 10 week of incubation that survival rate was 3.97 log$_{10}$ CFU/g (Figure 1). At this incubation temperature, changes in the number of bacteria according to the incubation periods were statistically significant (p = 0.004).

The number of survived aerobic mesophilic bacteria in samples with *C. jejuni* stored at -20°C was 8.85 log$_{10}$ CFU/g and increased to 9.16 log$_{10}$ CFU/g after 10 weeks of incubation (Figure 2). Changes were not statistically significant (p = 0.457), which shows that those changes are not related with the previously evidenced significant changes of the number of *C. jejuni*.

The number of survived aerobic mesophilic bacteria at the same specimens stored at -70°C after 10 weeks decreased from 0.24 log$_{10}$ CFU/g and was 8.61 log$_{10}$ CFU/g (Figure 2). Changes were not statistically significant (p = 0.612), which shows that those changes are not related with the previously evidenced significant changes of the number of *C. jejuni*.

Freezing works deadly to *Campylobacter* spp. [30, and 31]. Although *C. jejuni* can be destroyed by storing at freezing temperatures, however, one of these bacteria can be sublethally damaged and cannot recover to favourable environmental conditions [32].

In our study, the mean count of *C. coli* stored at -20°C decreased from initial number (6.29 log$_{10}$ CFU/g) for 2.39 after 1 week, and additionally for 0.15 log$_{10}$ CFU/g after 8 weeks of incubation, so it is in the 10 week of incubation that survival rate was 6.55 log$_{10}$ CFU/g (Figure 3). At this incubation temperature, changes in the number of bacteria according to the incubation periods were not statistically significant (p = 0.055).

The number of survived aerobic mesophilic bacteria in samples with *C. coli* stored at -20°C was 6.55 log$_{10}$ CFU/g and increased to 8.87 log$_{10}$ CFU/g after 10 weeks of incubation (Figure 4). Changes were not statistically significant (p = 0.062), which shows that those changes are not related with the previously evidenced significant changes of the number of *C. coli*.

Analysis of variance for repeated measurements did not confirm that the difference in the average number of *C. jejuni* and *C. coli* isolated from liver samples storage at -20°C was statistically significant (p = 0.290). Changes in the average number of *C. jejuni* and *C. coli* in time were significant (p < 0.001), and occurred at significantly different way with the examined bacteria (p < 0.001).
Analysis of variance for repeated measurements confirmed that the difference in the average number of *C. jejuni* and *C. coli* isolated from liver samples storage at -70 °C was statistically significant (p = 0.048). Changes in the average number of *C. jejuni* and *C. coli* in time were significant (p < 0.001), and occurred at significantly different way with the examined bacteria (p < 0.001).

The number of survival aerobic mesophilic bacteria at the same specimens stored at -70 °C after 10 weeks increased from 2.74 log10 CFU/g and was 9.29 log10 CFU/g (Figure 4). Changes were not statistically significant (p = 0.131), which shows that those changes are not related with the previously evidenced significant changes of the number of *C. coli*.

Mai [33], concluded that organisms associated with poultry products in the processing environment, such as the pseudomonads, micrococci and staphylococci, inhibit the growth of *C. jejuni*, in a range from log10 1.05 to 5.77. Diverse microflora in microenvironmental niches such as those on the surface of meat may reduce the oxygen concentration and to protect associated pathogen from cold stress [34] which might be important for viability and cultivability of fastidious *C. jejuni* sensitive to various stress conditions. However, the presence of competitive microflora may be unfavourable to *Campylobacter* maintenance and survival in chicken meat [33]. Therefore, the complex mechanisms which determine the extent of survival of *Campylobacter* on poultry products await further clarification.

4. Conclusions

- Although freezing is not the method that can sterilize chicken products, neither can it be the substitute for safe handling or proper cooking of poultry, it substantially reduces the initial number of campylobacters present in the samples before the freezing. Both freezing temperatures, -20 °C and -70 °C, decreased the number of campylobacters. According to our results, after the first day of incubation at -20 °C there was a larger decline in the number of surviving *C. jejuni* as compared to samples incubated for the same period at -70 °C. However, there was no significant difference in bacterial count reduction after 10 weeks of incubation on both temperatures.

- Type of the medium did not influence the survival, neither the presence of aerobic mesophilic bacteria, suggesting the favourable milieu for campylobacter survival. Although the rate of reduction of *Campylobacter* spp. can be influenced by temperature, storage and freezing conditions, the decreasing rate of the number of survived *C. jejuni* in this investigation was similar at both temperatures.

- However, keeping poultry liver at freezing temperatures is important for the reduction of the number of *C. jejuni*, which has a strong influence on the prevention of occurrence of campylobacteriosis in humans, having in mind the infectious dose. Although freezing of samples contaminated with *C. jejuni* should not be the only preventive measure in prevention of food contamination and human infection, it can substantially influence on the number of potentially ingested bacteria and on the reduction of occurrence of campylobacteriosis in humans.

5. References


