

INFLUENCE OF TEMPERATURE ON *CAMPYLOBACTER JEJUNI* SURVIVAL RATES IN PORK MEAT

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Summary

Vashin, I. T. & T. T. Stoyanchev, 2011. Influence of temperature on *Campylobacter jejuni* survival rates in pork meat. *Bulg. J. Vet. Med.*, 14, No 1, 25–30.

The effect of low temperatures on *C. jejuni* in artificially contaminated pork meat was studied. It was established that the microorganisms did not grow in chilled or frozen meat, but were able to survive during the storage period. At 1–4°C campylobacteria could be detected up to the 25th day, while at –18 to –20 °C: up to the 45th day. During the process of storage, a large part of the campylobacteria were killed, and 89.5% of those that survived were sublethally damaged (stressed) and could not grow in or on selective nutrient media. The cultivation of samples in broth without a selective supplement at 37°C for 4 to 5 h allowed recovery of the sublethally damaged cells.

Key words: *Campylobacter jejuni*, freezing, meat, survival

INTRODUCTION

Over the last years, *Campylobacter jejuni* (*C. jejuni*) became one of the most commonly detected bacteria causing gastrointestinal diseases in humans and animals (Pebody *et al.*, 1997; Gendrel & Cochen, 2008; Lowe *et al.*, 2008). The microorganism has been isolated from animals and birds, milk, beef, pork and poultry meat (Adak *et al.*, 2005; Sulonen *et al.*, 2007). Food of animal origin contaminated with *C. jejuni* is a primary source of *Campylobacter* infection in man (Franchin *et al.*, 2007; Mataragas *et al.*, 2008).

During processing of foods of animal origin, a significant part of the present microflora on the raw products is eliminated or left as sublethally damaged (stressed) by the high or low temperatures, drying, salting and pH value changes. Bacterial damage has been observed in *Staphylococcus*, *Salmonella*, *Streptococ-*

cus, *Pseudomonas*, yeasts, moulds, and spore-forming bacteria. Jang *et al.* (2007) found out that the morphological change of *C. jejuni* from spiral into coccoid or VBNC (viable but non-culturable) form occurred more rapidly under aerobic conditions at 4, 25, and 37°C.

The goal of our research was to determine the influence of low temperatures on *C. jejuni* survival in pork meat, the extent of sublethal damage and the possibility for recovery of damaged bacterial cells.

MATERIALS AND METHODS

Strains

C. jejuni 1128 (provided by the National Bank for Industrial Microorganisms and Cell Cultures, Sofia) and 4 laboratory

C. jejuni isolates were kept in peptone water (1%) with glycerol (30%) at -21 ± 1.0 °C. Immediately before their usage, the strains were cultivated for 48 h in nutrient medium containing growth factors (0.025% ferrous sulphate, sodium metabisulphite, sodium pyruvate) and 0.16% agar, in microaerophilic atmosphere (gas-generating kit, Merck&Co).

Samples

Immediately after slaughtering, pork meat samples were collected under aseptic conditions from the depth of the carcass muscle, cut into small cubes, 10 g each. In the centre of each cube, 1 mL of the 48-hour culture of the studied *C. jejuni* strains was injected and then wrapped in sterile foil. Half of the contaminated samples were left at -18 to -20 °C, while the rest: at 1 to 4 °C. The samples were studied immediately after contamination and at 5-day intervals. After homogenizing the sample (1:10) in enrichment broth, 10-fold serial dilutions were prepared and 0.1 mL of each dilution was inoculated onto Campylobacter agar base with growth factors (CAB) and Campylobacter agar base with growth factors and a selective supplement (CABS). The selective supplement contained polymyxin B, rifampicin, trimethoprim lactate, actidione. Part of the sample homogenate in the broth was cultivated for up to 6 hours at 37 °C or 42 °C, with inoculations at 1-hour intervals onto CAB and CABS. The inoculated agar media were cultivated for 24–48 h at 42°C in microaerophilic conditions. Bacterial growth of *Campylobacter* was estimated in colony forming units per gramme (CFU/g), transformed in log₁₀ of CFU/g.

Data were statistically analyzed by one-way ANOVA using Statistica 6® software at a level of significance $P < 0.05$.

RESULTS

In the samples kept at 1 to 4 °C, the number of viable *C. jejuni* gradually decreased. After 5 days, the amount of these bacteria on CAB (non-selective agar medium) was nearly 84% of the initial counts (before chilling), while on the 10th, 15th, and 20th day of storage the values were 68%, 56.2% and 35.5%, respectively (Table 1). On the 30th day of the meat storage at refrigerator temperature, no campylobacteria capable to grow on non-selective agar medium could be detected.

The number of *C. jejuni*, determined on CABS in all studied periods after the 5th day of sample storage in chilled condition was relatively lower than that established on CAB. On the 5th day, an average of 96.4% of the campylobacteria on the non-selective medium could also grow on CABS, while on the 10th, 15th, and 20th day the respective values were 68.1%, 44.3% and 48.2%. After 20 days of pork meat storage at 1 to 4 °C, there was no growth of *C. jejuni* on the selective agar medium.

During storage at freezer temperatures (-18 to -20 °C) the number of viable *C. jejuni* was progressively decreasing, and established count by CAB were 1.75, 3.77, 4.57, 5.27 and 5.57 log₁₀ CFU/g on the 10th, 20th, 30th, 40th and 45th day, respectively. After 45 days of storage, no *C. jejuni* could be detected in the samples.

Detected *Campylobacter* counts on CABS were lower, compared to those obtained on CAB. On the 25th day, for frozen meat, only 16.4% of the bacteria capable of growing on the non-selective medium (CAB) were able to form colonies on CABS. After 30 days storage, no *C. jejuni* positive samples could be detected by cultivation on CABS.

The results of our studies indicated that after low temperatures exposure, the

Table 1. Survival of *C. jejuni* in chilled and frozen pork meat (mean \pm SD, n=12)

Storage duration (days)	Number of <i>C. jejuni</i> (\log_{10} CFU/g)			
	Chilled meat		Frozen meat	
	CAB	CABS	CAB	CABS
0	6.91 \pm 0.14	6.91 \pm 0.14	6.57 \pm 0.21	6.57 \pm 0.21
5	5.81 \pm 1.27	5.60 \pm 0.81	5.98 \pm 0.20	4.92 \pm 0.89
10	4.70 \pm 1.34	3.20 \pm 1.34	4.82 \pm 0.84	3.83 \pm 0.98
15	3.88 \pm 1.48	1.72 \pm 0.84	3.82 \pm 0.94	2.51 \pm 0.81
20	2.45 \pm 0.68	1.18 \pm 0.48	2.80 \pm 1.32	1.04 \pm 0.41
25	1.95 \pm 1.39	0	2.50 \pm 1.15	1.41 \pm 0.38
30	0	0	2.00 \pm 0.85	1.21 \pm 0.34
35	0	0	1.70 \pm 1.00	0
40	0	0	1.30 \pm 0.54	0
45	0	0	1.00 \pm 0.24	0
50	0	0	0	0

CAB – Campylobacter agar base with growth factors; CABS – Campylobacter agar base with growth factors and selective supplement.

major part of *C. jejuni* cells died or stayed alive but with damaged cell functionality (up to 89.5%). However, the growth of colonies on CABS selective agar medium was insufficient. In the effort to revitalize stressed bacteria using enrichment broth at 37 °C and 42 °C we established that after 1 hour of cultivation at 37 °C, 4.89% of the *C. jejuni* damaged by freezing during storage were able to grow on the selective Campylobacter agar. After 2, 3, and 4 h of incubation, the counts of recovered cells increased to 28.09%, 58.22% and 82.30%, respectively. After 5 hours of cultivation in enrichment broth, the counts established with CABS were higher (121.2%) than those established with CAB. The *C. jejuni* cells damaged by freezing temperatures would also recover their functionality after short incubation in enrichment broth at 37 °C. After 5 hours of incubation, their counts on CABS were 1.66 times higher than on CAB.

DISCUSSION

The results of our research pointed out that refrigerator temperature (1 to 4°C) completely suppressed the growth of *C. jejuni* in artificially contaminated samples of pork meat. At such low temperatures *C. jejuni* counts constantly decreased, with no campylobacteria detectable in the meat after the 25th day. These data confirm the statements of other authors that chilled meat does not provide suitable conditions for *C. jejuni* growth (Christopher *et al.*, 1982; Abram & Potter, 1985; Beuchat, 1985). Ray & Johnson (1984) reported that 49–93% of *C. jejuni* population died in meat samples stored at 4°C for 2 days, which is explained with the microorganism's high sensitivity to low temperatures.

The studies of Meldrum *et al.* (2005) demonstrated no significant differences regarding *Campylobacter* contamination of fresh and frozen poultry meat. On the

contrary, Park *et al.* (2002) found out that the number of *Campylobacter* dropped by approximately 1.0 log₁₀ CFU/g after freezing and remained relatively constant during storage in refrigerator for 31–220 days. In this study on *C. jejuni*, a progressive decline in the number of living cells during the period of storage, with a reduction of 5.57 log₁₀ CFU/g for 45 days in frozen meats was evidenced. Georgsson *et al.* (2006) indicated that freezing of broiler chickens before distribution to retail stores led to reduction of the present contamination with *Campylobacter*, which is of significance to public health.

Three different types of poultry meat surfaces (skin, muscle without skin, and muscle cut) were contaminated with *Campylobacter jejuni* in order to trace its ability to survive 5 weeks after freezing at –20 °C (Ritz *et al.*, 2007). In general, the survival was short on the skin, longer on muscle without skin, and the longest in muscle cuts. In pork meat without fat and skin used by us, *C. jejuni* survived for 45 days in frozen state. On the contrary, Bracewell *et al.* (1985) reported that at –20 °C *C. jejuni* died very fast after 8 days in storage and could not be detected on the artificially contaminated meat samples. These variations in the resistance of bacteria could be explained with the different initial *C. jejuni* counts in the meat, as well as with differences in the used methods and nutrient growth media. Many authors (Hanninen, 1981; Svadhem *et al.*, 1981; Christopher *et al.*, 1982; Stern *et al.*, 1984) pointed out that a higher initial number of *C. jejuni* prolonged the duration of survival in meat at low temperatures.

At low temperatures, some campylobacteria die, while the surviving organisms were significantly damaged and lost their ability to grow on the routinely used

selective nutrient media (Christopher *et al.*, 1982; Hanninen, 1981; Palumbo, 1984). Cold-induced damage to the cytoplasmic membrane and the cellular wall impairs the permeability of the bacterial cell, related to the efflux of low-molecule substances and the influx of harmful substances (Calcott *et al.*, 1976). Stressed by refrigerator temperatures, *C. jejuni* are more sensitive to some antibiotics included in the routinely used agar media (Beuchat, 1985). According to Beuchat (1985) and Humphrey (1984) selective antimicrobial substances (rifampicin, vancomycin, polymyxin B, cefalotin, amphotericin) suppress the recovery and development of sublethally damaged *C. jejuni* cells. These data explain the observed differences in the number of colonies grown on CABS and CAB. Throughout the entire experimental period, the number of *C. jejuni* in chilled or frozen pork meat, determined on CABS, was lower as compared to the number on CAB.

Palumbo (1984) did not find any differences in the recovery time of sublethally damaged *C. jejuni* at 37 °C and 42 °C. Our results showed that the recovery process was slowed down at 42 °C and was completed within 7–8 hours, whereas at 37 °C it occurred for about 4 h. These differences could be explained with the increased sensitivity of sublethally damaged bacteria to higher temperatures. Slowed recovery at 42 °C creates conditions for fast growth of accompanying microflora, which further impedes the isolation of campylobacteria (Abram & Potter, 1985; Stern *et al.*, 1984).

A number of authors (Svadhem *et al.*, 1981; Christopher *et al.*, 1982; Abram & Potter, 1985; Beuchat, 1985) showed that damaged *C. jejuni* in food products could cause illness in humans, identical with campylobacteriosis, whose etiology is

related to undamaged cells. We determined that the damaged, yet living cells of *C. jejuni* were a potential threat, and under suitable conditions they could recover their vitality. In order to maintain efficient laboratory microbiological control and to determine the real number of *C. jejuni* it is recommended that sublethally damaged bacterial cells be recovered first, and then detected in a selective growth medium. The presence of damaged, yet vital *Campylobacter* cells in frozen meat products is often underestimated and thus becomes a significant factor in the assessment of the risk of campylobacteriosis in humans.

In conclusion, chilling or freezing temperatures affected artificially inoculated *C. jejuni* in pork meat, and the count of viable bacterial cells was significantly reduced during the period of storage. *Campylobacter* were detected up to the 25th day during storage at 1–4 °C, and up to the 45th day at –18 to –20°C. From the surviving bacteria up to 89.5% were damaged (stressed) and could not grow on or in selective media used routinely for *Campylobacter* isolation.

Campylobacter agar base with growth factors and selective supplement (CABS) is a suitable growth medium for sublethally damaged (stressed) *C. jejuni*, although it required recovery of the stressed bacteria. Preincubation of homogenized samples in enrichment broth with growth factors under microaerophilic atmosphere at 37°C for 4–5 hours, created suitable conditions for recovery of damaged *C. jejuni* cells.

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Paper received 25.05.2009; accepted for publication 20.11.2009

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