Retail liver juices enhance the survivability of *Campylobacter jejuni* and *Campylobacter coli* at low temperatures

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The high prevalence of *Campylobacter* spp. in retail liver products was previously reported and has been linked to several outbreaks of campylobacteriosis. The main objective of this study was to investigate the influence of retail liver juices on the survivability of several strains of *C. jejuni* and *C. coli*, which were previously isolated from various retail meats at 4 °C. All tested *Campylobacter* strains showed higher survival in beef liver juice (BLJ) and chicken liver juice (CLJ) as compared to beef and chicken juices (BJ and CJ) or Mueller Hinton broth (MHB) at 4 °C. Overall, *C. jejuni* strains showed greater survival in retail liver and meat juices as compared to *C. coli*. CLJ enhanced biofilm formation of most *C. coli* strains and supported growth in favorable conditions. When diluted, retail liver and meat juices enhanced survival of *Campylobacter* strains at low temperatures and increased aerotolerance. In conclusion, beef and chicken liver juices enhanced the survival of *C. jejuni* and *C. coli* strains at low temperatures, which helps explain the high prevalence of *Campylobacter* spp. in retail liver products.

In recent years, campylobacteriosis has been listed as a leading cause of bacterial diarrheal illnesses in the USA. *C. jejuni* and *C. coli* are the primary causal agents for campylobacteriosis, which can lead to immunological disorders like Guillain Barre syndrome and Miller Fisher syndrome. After eradication of poliomyelitis, Guillain Barre syndrome remains the leading cause for flaccid paralysis in multiple countries. *Campylobacter* is found in various reservoirs and also occurs as a commensal organism in poultry. As a foodborne pathogen, *Campylobacter* is usually transmitted via the consumption of contaminated beverages and food, with the latter occurring primarily from retail meat and liver products. Several outbreaks of campylobacteriosis associated with contaminated retail liver products have been reported worldwide.

Retail meat and liver products remain important nutrient sources for humans and provide proteins and micronutrients that are essential for growth and immunity. High folate content and various lipid compositions have been identified in beef and chicken liver. The high choline content in retail liver products is beneficial to human health in terms of normal cell functioning and acetylcholine synthesis. In spite of improvements in the handling process, retail meat and liver products may be contaminated with *Campylobacter* during processing in the slaughterhouse. The high incidence of *Campylobacter* on the surface of retail liver suggests cross-contamination during processing; however, it is important to note that *Campylobacter* spp. may also occur in the internal tissues of retail meats. Other foodborne pathogens such as *Salmonella*, *Staphylococcus*, hepatitis E, *Escherichia* spp. and *Yersinia* spp. are also prevalent in retail liver products worldwide. The higher prevalence of *Campylobacter* and *Staphylococcus* pathogens in retail liver vs. retail meat products has been reported. The high frequency of *Campylobacter* contamination in retail liver products indicates a risk for future outbreaks due to the consumption preference for undercooked liver products. Up to 98% survival of *Campylobacter* has been reported in chicken liver dishes that are undercooked to retain a pinkish coloration.

Although the occurrence of *Campylobacter* in retail meat and liver products largely depends on contamination, bacterial survival during harsh conditions ensures transmission and can result in clinical cases. *Campylobacter* is a fastidious, microaerophilic gram-negative bacterium that grows optimally at 42 °C. It routinely encounters various stressors including temperature fluctuation and oxidative and osmotic stress. *Campylobacter* copes with harsh conditions by deploying multiple survival mechanisms including the viable but nonculturable
Campylobacter was investigated. In this experiment, we incubated eleven C. coli and C. jejuni strains at lower temperatures, although contrasting results have been reported41,48,53,54. In this vs. differential survival rates and stress tolerance mechanisms. A few studies have shown higher survival rates of C. Campylobacter adhesion and attachment of Campylobacter even at low temperatures (0–4 °C), Campylobacter can survive for prolonged periods in retail meat and liver49–51. Hence, the high prevalence of C. jejuni and C. coli in retail liver products is associated with enhanced survival at low temperatures. Furthermore, low numbers of Campylobacter are sufficient to cause infection42. In response to cold shock, genes related to acquisition of cryoprotectants and membrane composition remodeling were abundantly expressed in Campylobacter43. Various components in retail liver might function as cryoprotectant molecules to improve survival at low temperatures.

Biofilm production is another survival mechanism of Campylobacter during aerobic and adverse environmental conditions44. The food matrix influences the formation of Campylobacter biofilms, which occur in retail meat substances such as chicken and pork meat juices45,46. Furthermore, the retail meat environment increases adhesion and attachment of Campylobacter, thus facilitating biofilm formation45. The nutrient and iron-rich environment in retail liver products might contribute to biofilm formation by Campylobacter spp. Although oxidative stress is unfavorable for Campylobacter growth, aerotolerance in aerobic conditions53. Studies on the influence of retail liver environments on aerotolerance and biofilm formation are lacking because the required assays were not readily accessible in food models (e.g. liver slices and homogenates).48,49. Liver juices in retail liver samples represent the actual environment that Campylobacter strains encounter during handling and storage; however, studies including liver juices as a food model for Campylobacter have not been previously undertaken.

In previous studies from our laboratory, a high prevalence of C. jejuni and C. coli strains were reported in retail meat and liver products52. Interestingly, C. coli was recovered from retail beef liver, although beef cuts were not contaminated53. Retail liver might contain Campylobacter in internal tissues or may get contaminated during processing. In both conditions, higher survival rate of C. coli strains in the retail liver environment compared to other food matrices might have contributed to the higher prevalence; however, a precise explanation remains unclear. Most studies on Campylobacter survival and gene expression have been conducted with C. jejuni54,37,43,51 however, it is important to note that genomic differences between Campylobacter spp52 might be associated with differential survival rates and stress tolerance mechanisms. A few studies have shown higher survival rates of C. jejuni vs. C. coli strains at a lower temperatures, although contrasting results have been reported41,48,53,54. In this study, we investigate the influence of retail meat and liver juices (chicken and beef) on different strains of C. jejuni and C. coli during biofilm formation, survival or growth at variable temperatures, and oxidative stress (aerotolerance). This study provides further insights regarding the influence of retail meat and liver environments on Campylobacter survival.

Results

Survival at 4 °C. The influence of retail liver and meat juices on the survival of Campylobacter strains at 4 °C was investigated. In this experiment, we incubated eleven Campylobacter strains including reference strain C. jejuni NCTC11168 (Table 1) in retail meat and liver juices at 4 °C for five weeks. Retail meat and liver juices significantly enhanced survival (P < 0.0001) in ten of the eleven strains; an exception was C. coli YV1-223, which showed higher survival in MHB than BJ. Strains showed higher survival in BJ and BJ than BJ, CJ and MHB (P < 0.0001) (Fig. 1). Statistical analyses (MANOVA) showed a significant influence of retail meat and liver juices (P < 0.0001) but not the origin of juices (chicken vs. beef) on the survival of Campylobacter strains. A rapid reduction in bacterial counts (up to 5.76 log reduction) was observed for Campylobacter NCTC11168 at 28 days of incubation in BJ (Fig. 1a). A 1.57-, 1.61- and 2.41-log reduction in bacterial numbers was observed for NCTC11168 incubated in BJ, CJ, and BJ, respectively, at 35 days of incubation. BJ resulted in higher survival of C. jejuni strains T1-21, OD2-67, WP2-202, and NCTC11168 throughout the experiments as compared to MHB and BJ (P < 0.0001) (Fig. 1a); however, only C. coli strains HC2-48 and YV1-223 survived for 35 days in BJ (Fig. 1b). C. coli strains WA3-33, CF2-75, CO2-160, and ZV1-224 did not survive the 35-day incubation period in BJ, BJ or MHB (Fig. 1b). The overall viability of Campylobacter strains was lowest in BJ (P < 0.0001). None of the strains inoculated in MHB produced visible colonies on Mueller Hinton Agar (MHA) plates after a five-day incubation period.

| Campylobacter strains | Source | Accession number (chromosome, plasmids) |
|-----------------------|--------|----------------------------------------|
| C. jejuni NCTC11168   | Clinical (reference) | AL111168.1 |
| C. jejuni T1-21       | Chicken meat | CP013116.1, CP013117.1 |
| C. jejuni OD2-67      | Chicken liver | CP014744.1, CP014745.1, CP014746.1 |
| C. jejuni WP2-202     | Chicken gizzard | CP014742.1, CP014743.1 |
| C. jejuni CG1-109     | Beef liver | NA |
| C. coli WA3-33        | Chicken liver | CP017875.1, CP017874.1 |
| C. coli HC2-48        | Beef liver | CP013034.1, CP013035.1 |
| C. coli CF2-75        | Beef liver | CP013035.1, CP013036.1, CP013037.1 |
| C. coli CO2-160       | Beef liver | CP013032.1, CP013033.1 |
| C. coli YV1-223       | Pork meat | NA |
| C. coli ZV1-224       | Pork meat | CP017875.1, CP017876.1, CP017877.1 |

Table 1. Campylobacter strains used in this study. All Campylobacter strains (except C. jejuni NCTC11168) were isolated and whole genome sequenced in our laboratory30,32,50,56–83.
The survival of *Campylobacter* strains in diluted retail meat and liver juices (5% v/v in MHB) was investigated at 4 °C. With the exception of MHCJ, all diluted retail meat and liver juices significantly enhanced *Campylobacter* survival relative to MHB ($P < 0.0001$) (Fig. 2). *Campylobacter* strains failed to grow in MHCJ after 5 days. The addition of 5% laked horse blood and defibrinated horse blood to MHB (MHBB and MHFB, respectively) also enhanced *Campylobacter* survival, with most strains showing higher survival in MHBB vs. MHFB (Fig. 2). For *C. jejuni* strains, survival was highest in MHBLJ, MHBB and MHCLJ than unamended MHB ($P < 0.0001$). Survival rates in MHBB, MHBLJ, and MHCLJ were comparable to 100% BJ for several *C. jejuni* strains (Figs 1a, 2a).

Among *C. coli* strains, MHBLJ promoted survival throughout the 35-day experiment. HC2-48 was the only *C. coli* strain producing visible colonies on MHA plates at 11 days after inoculation in MHCLJ, MHBJ, MHBB, and MHFB.

**Survival of *C. jejuni* and *C. coli* in various food matrices at 4 °C.** A comparative analysis of viability among *Campylobacter* strains was conducted using log bacterial cell count reduction rates. A higher reduction in bacterial counts (log N/No) was generally observed for *C. coli* strains as compared to *C. jejuni* in variable food matrices. Survival patterns were prolonged for all *Campylobacter* strains incubated in CLJ, BLJ, and MHLBJ (Fig. 3). Interestingly, the survival rate of *C. coli* HC2-48 was significantly higher than other *C. coli* strains in CJ.
BJ, MHB, MHCLJ, MHBB, and MHFB but comparatively lower than most of the *C. jejuni* strains. No strains survived more than five days in MHB and MHCJ. Two tailed ANOVA for species level differences in individual juices showed significant species level differences of survival rate in retail meat juices (CJ and BJ) and all diluted juices (except MHB and MHCJ) (P < 0.0001). However, MANOVA for interaction including all experimental results for the interaction of *Campylobacter* species, media and time showed significant strains level differences (P < 0.0001) but not species-level differences (P = 0.38) (see Supplementary Table 1).

**Survival at −20 °C.** Reference strain *C. jejuni* NCTC11168 was used to investigate the influence of retail meat juices on the survival of *Campylobacter* at freezing temperature (−20 °C). We included *C. jejuni* NCTC11168

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**Figure 2.** Survival of (a) *C. jejuni* and (b) *C. coli* strains incubated in diluted retail meat and liver juices (5% v/v in MHB) at 4 °C. *Campylobacter* strains were incubated in diluted beef liver juice (MHB LJ), chicken liver juice (MHCLJ), beef juice (MHB), chicken juice (MHCJ), laked horse blood (MHBB), defibrinated fresh horse blood (MHFB) and MHB at 4 °C. Survival curves for *C. jejuni* T1-21 in MHBJ and MHFB are not available. Standard errors (vertical bars) were calculated from mean values of triplicate viable cell counts (LogCFU/ml).
to compare results with a previous report of survival assay in CJ at freezing temperature (−18 °C)38. A rapid decrease in viable cell counts was observed within the first week of incubation (~3.7 logs for MHB, ~2.76 logs for CJ, ~2.17 logs for BLJ, ~1.7 logs for CLJ, and ~1.67 logs for BJ); cell counts then remained relatively constant until the end of experiment in most juice matrices (Fig. 4). Diluted retail juices (MHBLJ, MHCLJ, MHBJ, and MHCJ) and horse blood (MHBB, MHFB) increased survival of *C. jejuni* NCTC11168 relative to the unamended MHB media at −20 °C; however, no significant differences were observed in NCTC11168 survival rates among different retail meat and liver juices.

**Growth and survival at 37 °C.** The influence of retail meat and liver juices on growth and survival of *Campylobacter* strains (Table 1) at favorable growth temperatures was evaluated at 37 °C in microaerobic conditions. As expected, MHB supported higher growth and survival of all strains up to the end of the experiment (Fig. 5). CJ was the best matrix for growth and survival among juices. Chicken liver juice (CLJ) was more
favorable for growth at 37 °C than BLJ and BJ for all C. coli strains and C. jejuni 11168 and CG1-109. None of the strains survived after four days in BLJ. C. jejuni NCTC11168 and CG1-109 and all C. coli strains failed to survive more than two days in BJ; however, C. jejuni T1-21, OD2-67, and WP2-202 survived until the end of the experiment. Interestingly, cell counts of C. jejuni T1-21 and WP2-202 decreased at 48 h and then increased, which might be caused by metabolic adaptation to the available nutrients after the initial incubation period.

The lower bacterial counts in CLJ, BLJ, and BJ indicated that these matrices were less suitable food sources for growth than CJ in favorable environmental conditions (e.g. 37 °C). MANOVA showed significant species-level differences (P = 0.0035). Differences in growth on the basis of strain, juice, and origin of juice were also significant (P < 0.0001) (see Supplementary Table 2).

**Influence on biofilm formation.** On polystyrene surfaces, incubation in CLJ significantly enhanced biofilm formation all C. coli strains except YV1-223 (Fig. 6b). Biofilm formation was higher for C. jejuni T1-21 and C. coli HC2-48, CF2-75, YV1-223 and ZV1-224 in CJ, whereas other strains did not produce significant biofilms in CJ (Fig. 6a,b). All the juices failed to promote biofilm formation in C. jejuni OD2-67, CG1-109, and WP2-202. Biofilm formation on borosilicate glass was highest when C. jejuni NCTC11168 was incubated in CJ, whereas the other juices did not promote biofilm formation (Fig. 6c). Statistical model analysis showed significant effects of strain-media (P < 0.0001) and species-media interactions on biofilm formation (P < 0.0001) (see Supplementary Table 3).
Influence on aerotolerance. In comparison to non-amended MHB, *C. jejuni* NCTC11168 and OD2-67 and *C. coli* WA3-33 and CF2-75 showed enhanced aerotolerance in MHB amended with 10% beef and chicken liver juice (MHBLJ, MHCLJ) at 24 h of aerobic incubation (Fig. 7). The addition of laked horse blood to MHB (MHBB) enhanced survival in all strains exposed to aerobic conditions but bacterial counts were lower than MHBLJ, MHCLJ and MHBJ; this indicates the presence of an additional factor in retail liver and meat juices that enhances aerotolerance. In general, the addition of BJ to MHB also enhanced aerotolerance, but CJ and non-amended MHB did not.
Figure 7. The influence of retail meat and liver juices on aerotolerance of C. jejuni NCTC11168 and OD2-67 and C. coli WA3-33 and CF2-75. Dilutions (10% v/v) of retail meat and liver juices in MHB were prepared and included MHBLJ (beef liver juice), MHCJL (chicken liver juice), MHBJ (beef juice), MHCJ (chicken juice). MHBB (10% laked horse blood) and MHB (reference media) were also included in this experiment.

Discussion

Campylobacter strains routinely encounter low temperatures, and a few studies have speculated that cold tolerance and the acquisition of cryoprotective molecules are survival mechanisms.24,25. The prolonged survival of Campylobacter in cold storage conditions has been reported in media and food models,38–41,48, which are influenced by the food matrix composition. Retail meat juices from chicken,38,39,45, pork,46 and beef56 have been used as models to represent the surface of retail meat products. Analogous to meat juice models, retail liver juice models (BLJ and CLJ) represent the retail liver environment that foodborne pathogens would encounter after contamination. Retail liver possesses choline and other nutrients which are beneficial for human health18–20. Heme and nonheme proteins and other nutrients and minerals are also highly abundant in retail meat and liver products.27,28. The elevated survival of Campylobacter in retail liver juices in this study indicates that liver juices provide a nutritious and favorable environment for acquisition of cryoprotective molecules and nutrients for metabolism29 during survival at low temperatures.

During harsh environmental conditions, Campylobacter spp. often enter the VBNC state8 and undergo a morphological change to coccoid forms29. The reported high prevalence in retail liver products20–32 and high survival rate at lower temperatures (this study) indicates that the food matrix provided by retail liver products helps retain culturable conditions of Campylobacter cells for prolonged periods. Our experimental design represents culturable cell counts of Campylobacter on MHA plates after incubation in various media at reduced temperatures. One previous report showed a rise in the C. jejuni population in inoculated chicken livers at 4 °C for a 24-h incubation, but a reduction in bacterial numbers was observed in chicken skin medallions and chicken meat.29. Higher viability (culturability) of C. coli strains in retail liver juices vs. retail meat juices (Fig. 1b) correlated with the higher prevalence of reported C. coli in retail liver products as compared to retail meat products.30. Similarly, the high viability of C. jejuni in retail liver and meat juices with comparable survival rates (Fig. 1a) might explain the reported high prevalence of C. jejuni in retail meats and liver products.30,32,50. The decline in bacterial counts in our study when using retail liver juices is higher than previous studies, which found no significant reduction in bacterial counts in artificially-inoculated liver samples (liver slices and liver homogenate food models) at 4 °C.38,39,45. Discrepancies in experimental design (food models used) and differences in the Campylobacter strains used in our study might explain the reduced survivability in our study as compared to previous reports.

During the processing and handling of retail meat and liver products, dilutions of substances naturally occur in the working environment and on the surface of retail meat and liver products. In previous studies, media supplemented with blood was compared with full-strength retail meat juices in survival assays however, the effect of dilutions on survival of Campylobacter at lower temperatures has not been previously studied. In this study, 5% dilutions of juices, laked blood and fresh horse blood in MHB were compared to detect the influence of the blood component and additional factor(s) in juices on the survival of Campylobacter strains at low temperatures. Enhanced survival of Campylobacter in MHB supplemented with 5% laked or fresh horse blood (MHBB or MHFB) (Fig. 2) demonstrates the influence of blood components on survival. The higher survival of selected strains in MHBB vs. MHFB indicates that the lysed blood environment improves survival. The prolonged storage of retail meat and liver products at lower temperatures promotes lysis due to the freeze-thaw process, which creates a more favorable environment for contaminant Campylobacter cells. In our study, the higher survival in MHBLJ than MHBB or MHFB for most Campylobacter strains (Fig. 2) indicates that BLJ contains other essential nutrients in addition to blood components. For C. jejuni strains, all diluents except MHCJ enhanced survival (Fig. 2a); however, only MHBLJ improved the survival of all C. coli strains (Fig. 2b). MHB enhanced the higher survival of some C. jejuni strains more than 100% BJ, which might be due to a more balanced...
nutritional composition in MHBJ vs. full-strength BJ. None of the Campylobacter strains survived well in MHCJ at low temperatures (Fig. 2), which shows the potential loss of the protective or favorable environment observed with full-strength CJ (Fig. 1). These results indicate different nutritional or environmental requirements among Campylobacter spp. for survival at low temperatures.

Significantly higher survival of C. jejuni than C. coli in CJ, BJ, and diluted juices (p < 0.0001) (Fig. 3) supports our contention that the two Campylobacter spp. have different nutritional requirements. Our results suggest that C. coli might require more nutritional support than C. jejuni strains. Hence, highly nutrient rich conditions (like BJL and CLJ) might have supported similar survival rate for both species which differed for other juices with different nutritional contents. A similar inference was made in a previous report showing the higher survival of C. jejuni than C. coli in water samples maintained at 4 °C and 20 °C. MANOVA for interaction of species, strains, media, and time by including all results for survival at 4 °C, also showed significant strain level differences (P < 0.0001). C. jejuni strains were previously shown to be more acid tolerant than C. coli. Among C. jejuni strains, used clinical strains survived better than poultry strains at low temperatures (4 °C and 10 °C) in a previous report. Likewise, a waterborne C. jejuni strain showed better survival in defined fresh water media at 4 °C than a foodborne strain. In contrast, a few reports have shown similar survival rates for both species and higher survival rate of C. coli at low temperatures in different food models.

At freezing temperatures (−20 °C), all retail liver and meat juices and diluents enhanced survival of C. jejuni NCTC11168 relative to MHB (Fig. 4). Hence, retail liver juices likely function as a protective food matrix composition for Campylobacter spp. at subzero temperatures. Similar inference for the presence of protective materials in CJ for Campylobacter at freezing temperature had been proposed in a previous report. The rapid decrease of bacterial numbers after inoculation into juices maintained at −20 °C (Fig. 4) is similar to previous studies where a rapid decline in bacterial numbers was observed as early as 0.5 h after inoculation.

At a favorable growth temperature (37 °C), CJ promoted growth at 37 °C for Campylobacter strains (Fig. 5a,b), which agrees with previous reports documenting the favorable nutrient composition of CJ for enhanced growth. Chicken liver juice also supported growth and higher survival of all Campylobacter strains in comparison to BLJ and BJ (Fig. 5a,b). Although BLJ enhanced survival of Campylobacter strains at low temperatures (Figs 1, 3, 4), it did not support growth at 37 °C. Hence, BJL might provide cryoprotectant molecules and required nutrients for survival at low temperatures, but these factors are not conducive for growth or potentially toxic at favorable temperatures.

To our knowledge, our study is the first to use retail liver juices to investigate the influence of retail liver environments on biofilm formation and aerotolerance, which was not feasible with previously used food models (liver slices and liver homogenates). Chicken juice induced high levels of biofilm formation for C. jejuni strains NCTC11168 (borosilicate glass surface) and T1-21 (polystyrene) and C. coli strains HC2-48, CF2-75, YV1-223 and ZV1-224 (polystyrene) (Fig. 6a–c). In a previous report, full strength as well as 5% dilution of CJ was shown to enhance the attachment of Campylobacter strains to abiotic surfaces and biofilm formation. It has been found that CJ environment enhances biofilm formation of both motile and non-motile variants of Campylobacter. Among tested juices, remarkably high biofilm formation was seen for most C. coli strains in CJL (Fig. 6b), but C. jejuni biofilms were not significantly different in CLJ vs. MHB (Fig. 6a,c). In general, we observed higher biofilm formation among C. coli than C. jejuni strains in retail juices, which agrees with a previous study. Significant strain-dependent differences in biofilm formation were observed in our study for the various retail liver and meat juices (P < 0.0001). Extracellular DNA (eDNA) has been shown to be a major component in biofilm formation of Campylobacter, where DNase and eDNase (from Campylobacter strains) treatment could rapidly remove or inhibit Campylobacter biofilms. DNA components available in retail meat and liver juices after lysis of blood, meat and liver cells might enhance biofilm formation of foodborne pathogens like Campylobacter. Feng et al. previously reported higher biofilm formation of Campylobacter spp. in polymicrobial environments than in monomicrobial conditions. Other bacterial contaminants found in retail liver products could contribute to biofilm formation by Campylobacter in the retail liver environment.

Although Campylobacter spp. are microaerophilic, aerotolerant strains show enhanced survival in aerobic conditions. The oxidative stress response is also associated with the mechanistic basis of iron acquisition. Heme-containing proteins in retail meat and liver products function as cofactors for important enzymes in the oxidative stress response, including catalase and superoxide dismutase. Iron content in media influences the aerotolerance mechanism of Campylobacter regulated by regulatory proteins (PerR and Fur) and genes like ferroxoxin (fdxA) and alkyl hydroperoxide reductase (ahpC). Hence, the aerotolerance observed for the four Campylobacter strains in our study (Fig. 7) might be related to the iron and nutrient content found in diluted (10%) retail meat and liver juices. Although diluted CJ did not enhance aerotolerance; other diluted retail meat and liver juices enhanced survival in aerobic conditions, possibly because of their higher nutrient composition or iron content. It is also important to note that overly high iron levels can promote the formation of toxic superoxide radicals that may be detrimental to Campylobacter metabolism. We mention this because it could explain the survival data shown in Fig. 5, where full-strength juices resulted in reduced bacterial counts relative to MHB at 37 °C. The iron content of retail meat and liver juices might also play a significant role in survival at lower temperatures, since the oxidative stress response is activated during cold shock.

Functional metabolic activities and genomic expression data have been reported in Campylobacter at lower temperatures (4 or 5 °C) in various growth conditions. Similarly, differential expression of genes related to quorum sensing and glycosylation of flagellin have been reported in CJ when compared to artificial Brain heart infusion media. A variety of genes were essential for survival at low temperatures in nutrient-rich or nutrient-poor media. All transcriptomic and genome fit analyses of Campylobacter at low temperatures have been conducted with C. jejuni. Genomic differences between C. jejuni and C. coli strains might contribute to differences in survival. Further investigations are needed to validate the effect of genomic differences on survival at low temperatures.
In conclusion, our results show that retail liver juices enhanced the survival of all Campylobacter strains at low temperatures, whereas other retail meat juices and dilutions had differential effects on survival. This is a highly relevant finding with respect to food safety since retail liver juices represent an environment encountered by food-borne Campylobacter after contamination. Overall, C. jejuni strains showed greater survival at 4°C in chicken juice, beef juice, and diluted retail meat and liver juices as compared to C. coli. Chicken liver juice enhanced biofilm formation of most C. coli strains and supported growth in favorable growth conditions. Further investigations are needed to explore the mechanisms by which the retail liver environment is enhancing the survival of Campylobacter at 4°C.

Methodology

Bacterial strain and growth conditions. Campylobacter isolates (four C. jejuni and six C. coli strains) were used in this study (Table 1) and were previously isolated from retail meat and liver products. C. jejuni NCTC11168 (clinical isolate) was used as a reference strain. The eleven strains were subcultured from −70°C stock cultures and grown on Mueller Hinton Agar (MHA) supplemented with 5% laked horse blood at 42°C for 48 h in microaerobic conditions (6% O2, 13% CO2, 81% N2, Thermo Forma incubator, model 3130). Prior to harvesting bacterial cells, strains were further subcultured for 18 h on a fresh plate of MHA with 5% laked horse blood for survival, biofilm and aerotolerance assays. Bacterial cells were harvested in phosphate buffered saline (PBS) (pH 7.4), and cell suspensions were adjusted to OD600 = 0.1. In general, bacterial inoculum was prepared similarly for each assay, including survival and growth at variable temperatures, biofilm formation, and aerotolerance.

Preparation of retail meat and liver juices. For food models, retail meat and liver juices were prepared. Chicken juice (CJ) was prepared as described previously. Briefly, frozen retail whole chickens without giblets were purchased from various retail meat shops and thawed overnight at room temperature. A similar procedure was used to obtain beef liver juice (BLJ) and chicken liver juice (CLJ) from frozen beef liver slices and chicken livers, respectively. Beef juice (BJ) was collected from retail meat shops after opening packets containing big chunks of beef cuts. Juices were collected aseptically in sterile containers and stored at −20°C prior to further processing. After thawing overnight at 4°C, CJ was centrifuged at 10,000 rpm for 15 min, whereas other meat and liver juices (CLJ, BLJ, and BJ) were centrifuged at 15,000 rpm for 30 min to exclude larger particles. Juices were filter-sterilized with a 0.45 µm membrane filter (Nalgene Rapid-Flow) and stored at −20°C. The absence of any microbial contaminants in filtered retail meat and liver juices was confirmed by culturing (in aerobic, microaerobic and anaerobic conditions at 25°C, 37°C and 42°C) in MHA supplemented with 5% laked horse blood. Microaerobic and anaerobic incubation at variable temperatures were done in gas jars containing microaerobic gas generating kits and anaerobic gas generating kits (Mitsubishi Gas Chemical, New York, NY, USA) respectively. Dilutions of meat and liver juices (5% or 10% v/v) were also prepared with MHB for survival studies at low temperature and aerotolerance assays. Similarly, dilutions of laked horse blood and fresh horse blood (5%) in MHB were also included to study whether the blood in retail meat and liver products influenced the survival of Campylobacter at low temperatures.

Survival at 4°C. Appropriate volumes of cell suspensions were added to pre-incubated juices and dilutions to create bacterial concentrations of approximately 7 logs CFU/ml. Strains and log CFU/ml were as follows: C. jejuni NCTC11168, 6.63; T1-21, 6.6; OD2-67, 6.98; CG1-109, 6.73; WP2-202, 6.17; and C. coli WA3-33, 7.02; HC2-48, 7.28; CF2-75, 7.17; CO2-160, 7.47; YV1-223, 7.14; and ZV1-224, 6.85. MHB was used as a reference medium. An equal volume of inoculated media and juices were filled to the rim of 5 ml disposable polystyrene test tubes with caps and incubated at 4°C to ensure microaerobic conditions. At specified time intervals, 10 µl samples were taken and serially diluted with 0.1% peptone in saline solution. Two spots of the 10 µl sample from each dilution were spotted onto MHA and incubated at 42°C for at least 48 h. Viable cell counts were taken, and data analysis was performed with mean values of triplicate experiments.

Survival at −20°C. C. jejuni NCTC11168 (7.6 log CFU/ml) was used as inoculum in this study for investigating the influence of retail meat and liver juices at freezing temperature. 500 µl of inoculated meat and liver juices (100%), a 5% dilution of meat and liver juices, and MHB were dispensed into 2 ml Eppendorf tubes and maintained at −20°C. At allocated times, triplicate samples were thawed at room temperature for 10 min and serial dilutions in 0.1% peptone saline were plated. Viable cell counts were taken as described previously.

Survival at 37°C. Inoculated juices and MHB (1.5 ml) were dispensed into a 96-well storage plate, (Square Well, 2.2 ml) and incubated at 37°C in microaerobic conditions. 40 µl samples were removed at specified time intervals and serial dilutions were prepared as described previously. MHA plates spotted with serial dilutions were incubated at 37°C in microaerobic conditions for at least 48 h before viable cell counts were determined.

Biofilm formation. Suspensions of all Campylobacter strains were prepared and adjusted to OD600 = 0.1 in PBS from an 18-h culture. Biofilm assays were prepared as described previously. Briefly, the cell suspension was diluted to 1:10 in meat and liver juice, and MHB was used as a reference media. Biofilm formation on glass surfaces was investigated by incubating 1 ml of C. jejuni NTC11168-inoculated media and juices at 37°C in 10 ml borosilicate glass tubes. After a 72 h-incubation, bacterial cell suspensions were removed, and wells were washed twice with 1.2 ml of sterile PBS (pH 7.4); plates were agitated gently to disagglomerate cells. MHB (1.2 ml) supplemented with Triphenyl Tetrazolium Chloride (TTC) (0.05% w/v) was added to each well and incubated for 72 h at 37°C. The other Campylobacter strains (excluding C. jejuni NCTC11168) were used to evaluate biofilm formation on polystyrene surfaces. Samples (150 µl) were incubated in sterile polystyrene 96-well microtiter plates at 37°C in microaerobic conditions. After a 72-h incubation, cell suspensions were removed, and wells were
washed twice with 180 µl sterile PBS (pH 7.4) and agitated gently to remove unbound cells. MHB (180µl) supplemented with TTC was added to each well and incubated for 72 h at 37 °C. The remaining MHB/TTC solution was then removed, and wells were air-dried. Bound TTC dye was dissolved using a solution containing acetone (20%) and ethanol (80%); absorbance was measured at 492 nm with an Appliskan Multimode Microplate Reader (Thermo Scientific). All experiments were conducted in triplicate and repeated two or more times.

**Influence of liver and meat juices on aerotolerance.** Aerotolerance assays were conducted using four *Campylobacter* strains (*C. jejuni* NCTC11168, *C. jejuni* OD2-67, *C. coli* WA3-33, and *C. coli* CF2-75) as described previously 47 with minor modifications. Similar approach has been used for aerotolerance assay in previous reports with incubation temperature at 37 °C or 42 °C.7,24,25,27. Sterilized retail meat and liver juices were mixed with MHB to prepare 10% meat and liver juices. We used 10% dilutions because full-strength (100%) retail meat and liver juices coagulated more quickly, thus hindering assays of viable cell counts. Bacteria were removed from 18-h subcultures on MHA supplemented with 5% laked horse blood. Bacterial suspensions were then diluted to OD 0.018 0.2 in PBS (pH 7.4). Bacterial suspensions (1 ml) were added to 9 ml of preincubated, diluted juices and then incubated aerobically at 42 °C, with agitation at 200 rpm (New Brunswick I2400 Incubator Shaker). 50 µl samples were removed at 0, 12, and 24 h, and viable cell counts were evaluated on MHA as described previously. All experiments were performed in triplicate.

**Statistical analysis.** For survival assays, statistical analysis was conducted with log CFU/ml values of triplicate experiments. Statistical test used with survival data was a repeated measures MANOVA with Strain effect (both *C. jejuni* and *C. coli* strains), Growth-Media effect, Time effect (cultures repeatedly sampled over time), Strain × Growth-Media interaction, Strain × Time interaction, Growth-Media × Time interaction, and Strain × Growth-Media × Time interaction. The standard methods for approximating the F statistic were used (Wilks’ Lambda test, Pillar’s Trace test, Hotelling–Lawley test and Roy’s Max Root) when needed, which all gave the same statistical conclusion throughout our statistical analysis. For analysis of species level effect on survival, similar statistical test with the survival data was used by a repeated measures MANOVA with species replacing the strain effect (i.e. Species effect, Growth-Media effect, Time effect, Species × Growth-Media interaction, Species × Time interaction, Growth-Media × Time interaction, and Species × Growth-Media × Time interaction).

The initial data analysis was performed with respect to the origin of the *Campylobacter* strains (beef, chicken and pork) and the type of retail liver juice added to the growth media (BLJ, CLJ or none). Repeated measures MANOVA was also done with Origin effect, Juice effect, Time effect, Origin × Juice interaction, Origin × Time interaction, Juice × Time interaction, and Origin × Juice × Time interaction. We then examined whether liver juice specifically was significant (retail liver juice [regardless of origin or none]) by replacing juice origin (beef and chicken) in the analysis. For survival and growth at 37 °C, statistical analysis mirrored that of the survival experiment at 4 °C.

For biofilm assays, the study was conducted with mean values of absorbance (OD 0.04). An ANOVA with Strain effect, Growth-Media effect, and Strain × Growth-Media interaction was performed. Student’s t-test and two-tailed ANOVA were conducted for each assay of survival and biofilm as needed.

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