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Project title: Freezing as an intervention to reduce the numbers of campylobacters isolated from chicken livers

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ABSTRACT

The aims of this study were (1) to determine the prevalence and numbers of campylobacters in 63 samples of raw livers purchased at retail across the UK; and (2) to investigate whether the freezing of chicken livers contaminated with *Campylobacter* was a reliable method for decontamination.

Chicken livers naturally contaminated with campylobacters were subjected to freezing at -15°C and -25°C for one day and seven days. Numbers of campylobacters on the livers were determined immediately before and after a 24 h or 7 d freeze treatment, and daily during three days post-thaw refrigerated storage. Freezing for 24 h at -25°C can reduce numbers of *Campylobacter* by up to 2 $\log_{10}$ cfu g^{-1}. Freezing the livers for 24 h at -25°C, thawing overnight in a fridge set to 4°C and refreezing for another 24 h at -25°C reduced the numbers of campylobacters by up to three log cycles. There were significantly greater reductions in the numbers of campylobacters when the first and second freeze treatments were compared.

Significance and impact of the study: Freezing chicken livers can reduce, but not eliminate, campylobacters. If poultry processors were to freeze livers destined for human consumption as part of routine processing, there is a potential for a reduction in campylobacteriosis associated with the consumption of imperfectly cooked chicken livers and derivatives, such as pâté.

INTRODUCTION

Outbreaks of infectious intestinal disease from foodborne sources have fallen overall in England and Wales over a period of roughly two decades prior to 2008 (Gormley et al. 2011). However, over the same period there was an overall increase in the incidence of human campylobacteriosis largely from poultry-derived foods (Gormley et al. 2011). Recently, there has been growing evidence from source attribution studies (Strachan et al. 2012), investigations of outbreaks of campylobacteriosis (Little et al. 2010; Merritt et al. 2011) and retail surveys (Baumgartner et al. 1995; Fernandez and Pison 1996) which implicates imperfectly cooked chicken livers as a likely source of illness. In particular, chicken livers prepared in catering premises and consumed as 'pan-fried' or in the form of pâté or parfait appear to be making a disproportionately large, and increasing, contribution to human illness (Little et al. 2010).

In Switzerland, *Campylobacter* has often been isolated from chicken livers in numbers up to $5x10^4$ cfu g^{-1} with a prevalence varying between 10% and 100% depending on season (Baumgartner et al. 2011). Although no account was taken of season, an earlier US survey of *Campylobacter* in chicken livers reported an overall prevalence of 48% (Barot et al. 1983) with a more recent US study reporting a 77% prevalence (Noormohamed and Fakhr 2012). A Polish study determined fresh liver *Campylobacter* prevalence to be 31% (Mackiw et al. 2011), whereas in Chile, the prevalence in frozen livers was 93% (Fernandez and Pison 1996). In addition to apparent widespread contamination, there is good evidence that *Campylobacter* contamination is not restricted to the surface of liver, with reports of low numbers of *Campylobacter* inside (Barot et al. 1983) and in 'hotspots' such as the bile ducts (Baumgartner et al. 1995).

Collectively, these studies provide evidence that chicken livers from campylobacter colonised chickens are routinely contaminated with campylobacters. Consequently, the cooking condition required to eliminate *Campylobacter* from chicken livers have been defined as an internal temperature which exceeds 70^{o}C for at least two minutes (Whyte et al. 2006). A key observation made by Whyte and colleagues was that undercooked livers were pink in colour which appeals to consumers. If the livers were cooked for too long, they became an unappealing grey. Both O'Leary and colleagues (2009) and Little et al. (2010) suggested that the desire by caterers to keep livers pink and appealing contributed to UK outbreaks of campylobacteriosis associated with the consumption of chicken liver dishes.

Although adequate cooking kills campylobacters, there are other potential interventions which could help control this bacterium that do not significantly impact on the organoleptic properties of the livers. It has been reported previously that freezing can reduce numbers of viable campylobacters on chicken skin and muscle (Garenaux et al. 2009; Sampers et al. 2010) and that typically, freezing chicken meat or skin for 24 h causes around a one log cycle reduction in the numbers of campylobacters (Oyarzabal et al. 2010; Sampers et al. 2010). However, we were unable to find information describing the effect of freezing chicken livers contaminated with campylobacters. Consequently, this study assesses the effect on numbers of *Campylobacter* if chicken livers are frozen for up to one week at temperatures chosen to mimic domestic and catering freezers before thawing for 24 h in a refrigerator. In addition, the effect of freezing, thawing and then refreezing the livers is reported.

MATERIALS AND METHODS

Microbiological examination. Each sample of livers was finely chopped into small (0.5cm x 0.5 cm x 0.5 cm) pieces using sterile scissors and a 25g subsample was generated by removing randomly-selected pieces using a sterile spoon to ensure that the meat exudate was also tested. An equal volume of maximum recovery diluent (MRD, Oxoid, Basingstoke, UK) was added to each 25 g liver sample before homogenisation for 1 min using a stomacher-400 (Seward, UK). *Campylobacter* were enumerated using the ISO 10272 part 2 direct-plating method (International Standards Organisation, 2006) with minor modifications. In brief, 2 ml of the initial 1 in 2 (1:1) dilution were spread onto six plates of modified charcoal cefoperazone deoxycholate agar (mCCDA, Oxoid CM0739 plus SR0155). Two ml of the initial suspension was then mixed with 8 ml of MRD to yield a 1 in 10 dilution. All subsequent dilutions were decimal as described by ISO 10272 and made using MRD. One hundred microlitre volumes of the decimal dilutions were plated in duplicate onto mCCDA. Incubation was under microaerobic conditions (CampyGen, Oxoid) at 41.5^{o}C for 48 h. Colonies of *Campylobacter* spp. were confirmed by a positive oxidase reaction, and inability to grow in aerobic atmosphere at 41.5^{o}C on Columbia Blood Agar (Oxoid). In addition, a single colony from each plate was tested using Dryspot *Campylobacter* (Oxoid) according to the manufacturer's instructions.

Sample collection at retail. In total, samples (380 g to 500 g) of fresh (n=33) or frozen (n=30) chicken liver from England, Wales and Scotland were purchased from national and small independent retailers between October 2012 and February 2013. The samples were examined to determine numbers of

campylobacters using the test protocol described above. Before purchase, the surface temperatures of the liver samples were measured with an infrared thermometer (model 59 mini, Fluke, Norwich, UK). Samples were purchased on randomly-selected days (including weekends), labelled, and transported in insulated Styrofoam boxes (Biotherm 45, DGP Intelsius, York, UK) using bubble-wrapped retail packs of frozen peas as refrigerant. Temperature loggers (Tinytag Plus 2; Gemini Data Loggers, Chichester, UK) were placed beside the samples to monitor the transit temperatures. Only samples with temperatures during transit between 0 and 8^{o}C were examined. Samples were stored at 4 °C in the laboratory prior to examination. All microbiological examinations commenced within 24 h of purchase.

Single freezing treatments. Broiler chickens are more likely to be colonised by camplyobacters if they come from flocks that have previously been thinned i.e. taken from rearing sheds where some of the birds had been removed some days previously (Allen et al. 2008). Therefore, in order to maximise the chance of sourcing campylobacter–positive flocks, livers for freezing experiments were collected from a slaughterhouse where thinned flocks could be identified. Additionally, it was only possible at slaughter to collect large quantities of livers from the same flock as those most likely to contain the same strains of *Campylobacter* at constant concentration. Direct collection also meant confidence that the livers had not previously been frozen, and had been processed only to remove gall bladders and bile ducts before cleaning and chilling in slush ice. 7-8 kg of livers were collected into a single bag and transported to the laboratory packed in ice. At the laboratory, four polythene bags, each containing 1.5 kg (roughly 90) randomly-selected livers, were prepared and a temperature logger (Tinytag Plus 2) was placed in the centre of each bag. For each experiment, two bags of livers were placed in a freezer with a set temperature of -15^{o}C, and two were placed in a freezer at -25^{o}C. For each temperature, one bag of livers was removed after 24 h and the other on the 7th day after freezing. After removal from the freezer, the livers were thawed overnight in a fridge set to 4^{o}C. Thirty thawed livers were examined in batches of three pooled samples (n=10) immediately after thawing to determine numbers of campylobacters per g. The remaining sixty livers were returned to the fridge, and 10 samples (each composed of three pooled livers) were examined after 24 h and 48 h. Each freezing trial was undertaken three times over a period of three months and using livers from birds raised on different farms.

Freeze, thaw and re-freezing treatments. Experiments to determine the effect of freezing livers more than once were carried out as described above for single freeze treatments, with minor differences. A polythene bag containing 2.5 kg livers was prepared. The livers were tested to determine pre-freeze numbers of campylobacters, frozen at -25^{o}C for 24 h, thawed for 24h at 4^{o}C,

tested to determine numbers of campylobacters and then immediately refrozen for a second 24 h before a second 24 h thaw at 4°C and re-examination. As before, numbers of campylobacters were determined each day for a further two days of refrigerated storage at 4°C.

Statistical Analyses. Paired and homoscedastic t-tests, and Analysis of Variance (Excel 2010; Microsoft, Redmond, WA, USA) were used to compare the $\log_{10}$ cfu recovered from the various pre- and post-freeze liver samples and the samples collected at retail as appropriate. For those results which were reported as below the limit of detection (<1 cfu/g) a value of half of the limit of detection (0.5 cfu/g) was substituted to allow $\log_{10}$ transformations. For all tests, a *P* value of <0.05 was used to determine any significance between treatments. Pearson product moment correlation coefficients (Excel) were used to presumptively determine any relationships between the retail purchase temperatures and numbers of campylobacters. The least squares algorithm was used to determine the strengths of any presumptive relationships.

RESULTS AND DISCUSSION

In total, 33 unfrozen and 30 frozen samples of livers were purchased from 51 different retail outlets throughout Great Britain. From the identification codes it was concluded that the 63 samples originated from 14 different EU-registered-slaughterhouses. Samples were collected from supermarket chains (n=48), independent butchers (n=10), delicatessens (n=1), commercial catering suppliers (n=2) and convenience stores (n=2). The sample test results are summarised in Figure 1. Of the 63 samples, 55 contained countable numbers of *Campylobacter* and eight contained fewer than limit of detection of the test method (<1 cfu/g liver). It was observed that there was a tendency for livers purchased unfrozen to have higher counts than livers purchased frozen, and statistical comparison revealed a significant difference (t-test, P<0.02).

Further analyses revealed that there was no relationship between the sample temperature at purchase and the numbers of campylobacters detected, either when the entire dataset was compared, or when only the test results above the limit of detection were compared (i.e. the positive samples). Furthermore, when the entire dataset was compared, there was no correlation between numbers of campylobacters detected and the number of days of shelf life remaining, or when the numbers on fresh or frozen samples were individually compared (results not shown).

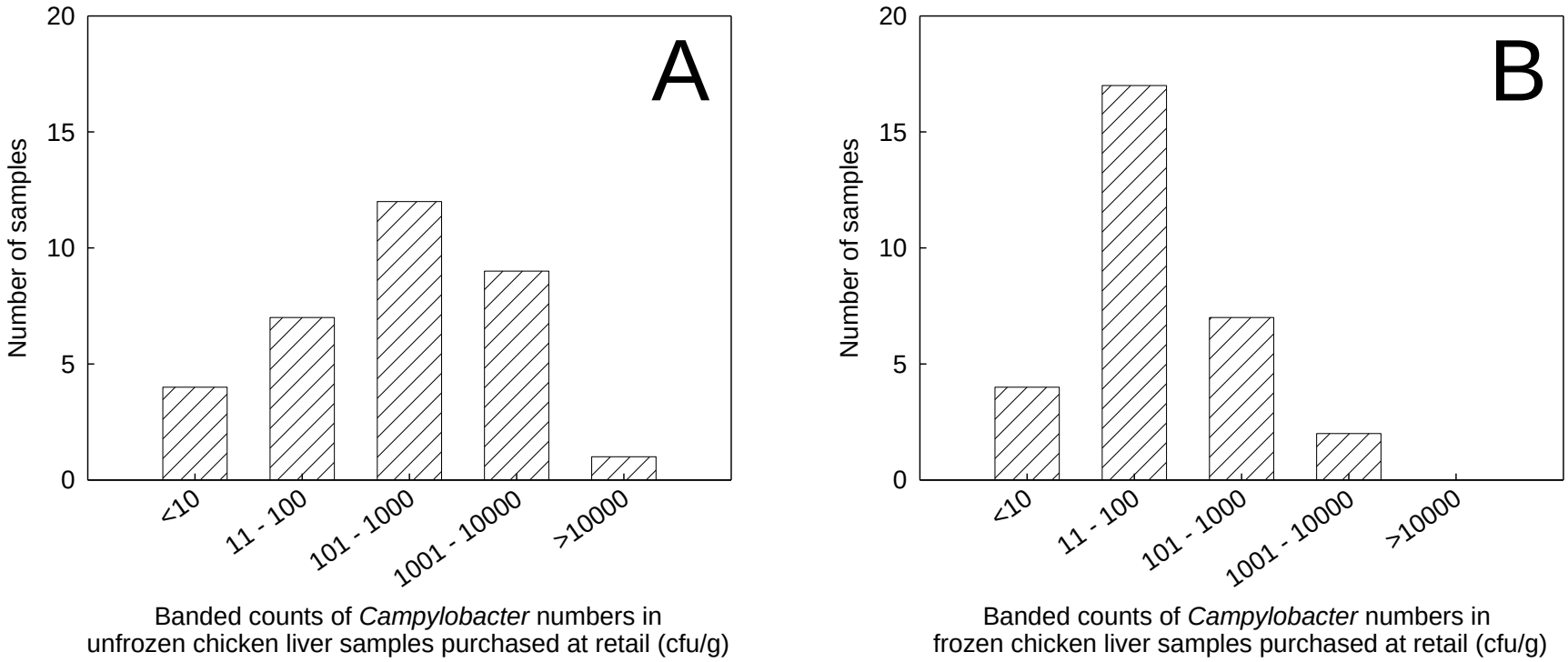


Figure 1: The distributions of the concentrations of campylobacters isolated from unfrozen (A, n=33) and frozen (B, n=30) chicken liver samples purchased at retail in GB between October 2012 and February 2013

The results from the retail survey were used to inform the laboratory-based freezing experiments, and they also verified that the numbers on the samples from slaughterhouses were broadly equivalent to samples purchased at retail by consumers. Two freeze temperatures were selected to represent best and worst cases in terms of freezing effectiveness. The worst-case freezer, set to -15°C, was unable to freeze the liver masses to the target temperature within 24 h (Figure 2A) achieving only -11°C. After two days, the minimum temperature achieved was -14°C (Figure 2B). In contrast, the freezer set to -25°C lowered the temperature in the centre of the liver masses to -26.2°C within 6 h (Figure 2C) with a further 1°C temperature reduction by over the course of several days (Figure 2D). It was observed that in both freezers the rate of freezing diminished at around -1°C, as the water contained within the livers turned to ice. The reduced rate of freezing at the phase transition temperature was 6 h longer in the freezer set to -15°C compared with the freezer set to -25°C, and was considered to be the primary reason that the less effective freezer failed to lower the temperature of the livers to -15°C within 24h.

Livers frozen for 24 h in the freezer set to -15°C had significantly reduced (t-test; $P<0.01$) numbers of campylobacters compared with the unfrozen controls (Figure 3A). A similar observation was made for livers frozen for 24 h in the freezer set to -25°C (Figure 3B). The colder freezer reduced numbers of cfu by approximately 1.5 log cycles, which was significantly greater (t-test; $P<0.01$) than the 0.8 log reduction observed for the livers in the freezer at -15°C.

There were significantly fewer campylobacters after freezing at -15°C for 7 d, compared with the numbers after a 24h at -15°C. Overall, freezing at -15°C for 7 days caused a 1.5 log reduction in numbers of campylobacters which was broadly comparable to the reduction observed at -25°C for 24h. There was no significant difference between in the reductions obtained after 24 h or 7 d in the -25°C freezer (t-test; $P>0.05$). Comparison of numbers of cfu g^{-1}, for individual samples, immediately after thawing and after chilled storage for three more days using ANOVA found no significant differences. Thus, we found no evidence to support a hypothesis that freezing causes sub-lethal injury to *Campylobacter* with a potential for recovery during subsequent thawing and extended chilled storage. Equally, there was no evidence of continued die-off if livers were stored chilled after freezing and thawing.

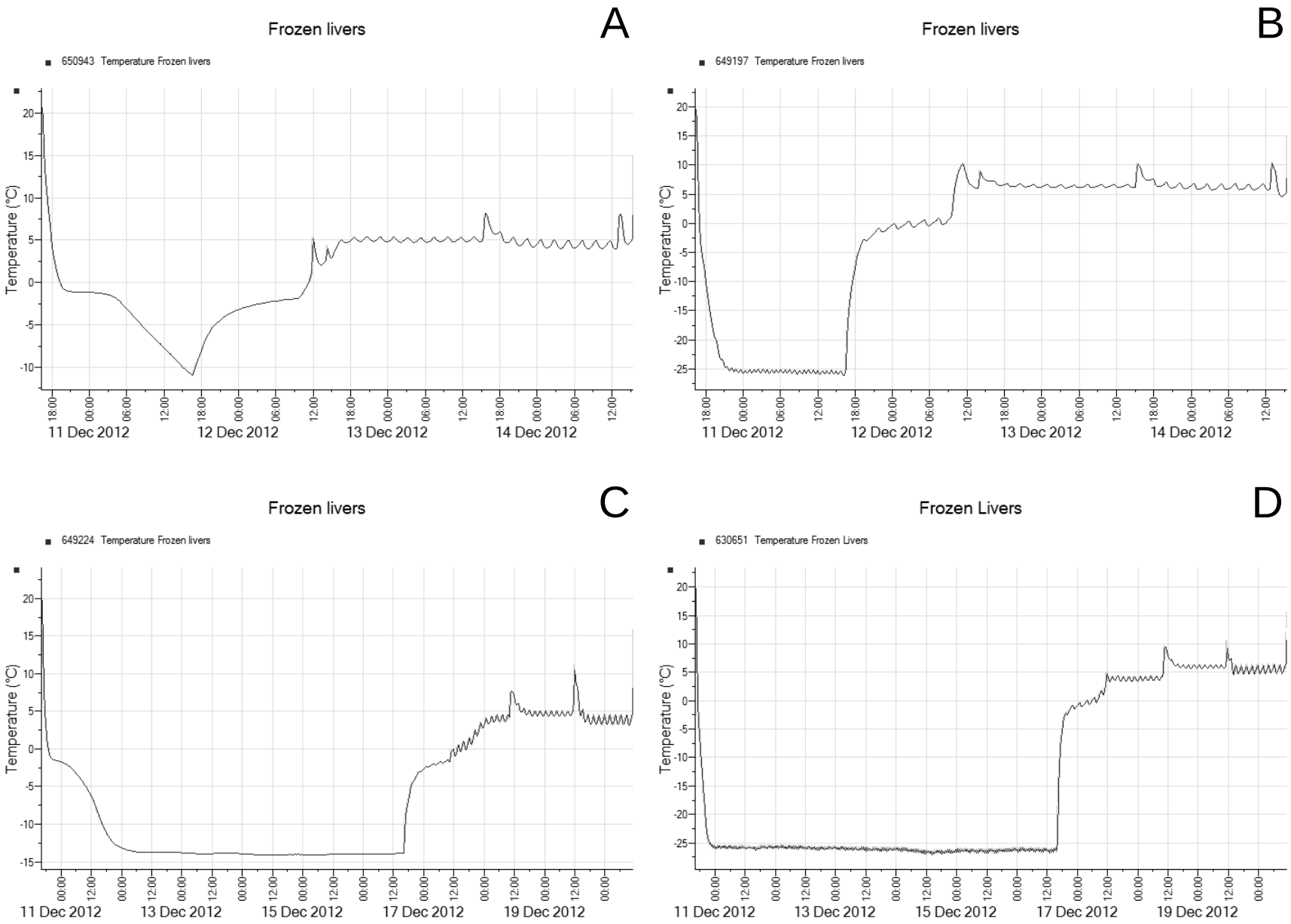
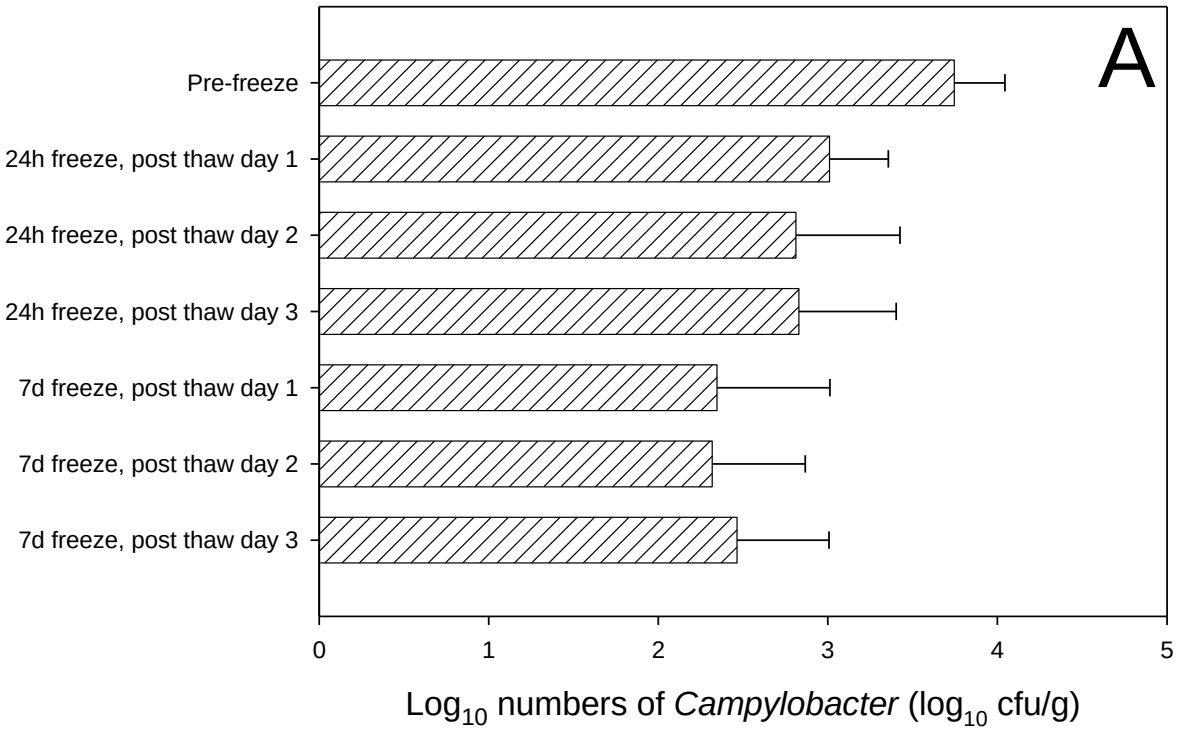


Figure 2: Typical temperatures achieved inside a 1.5 kg bag of chicken livers placed in freezers set to -15°C (A and C) and -25°C (B and D) for 1 day (A and B) or seven days (C and D) followed by three days refrigerated storage



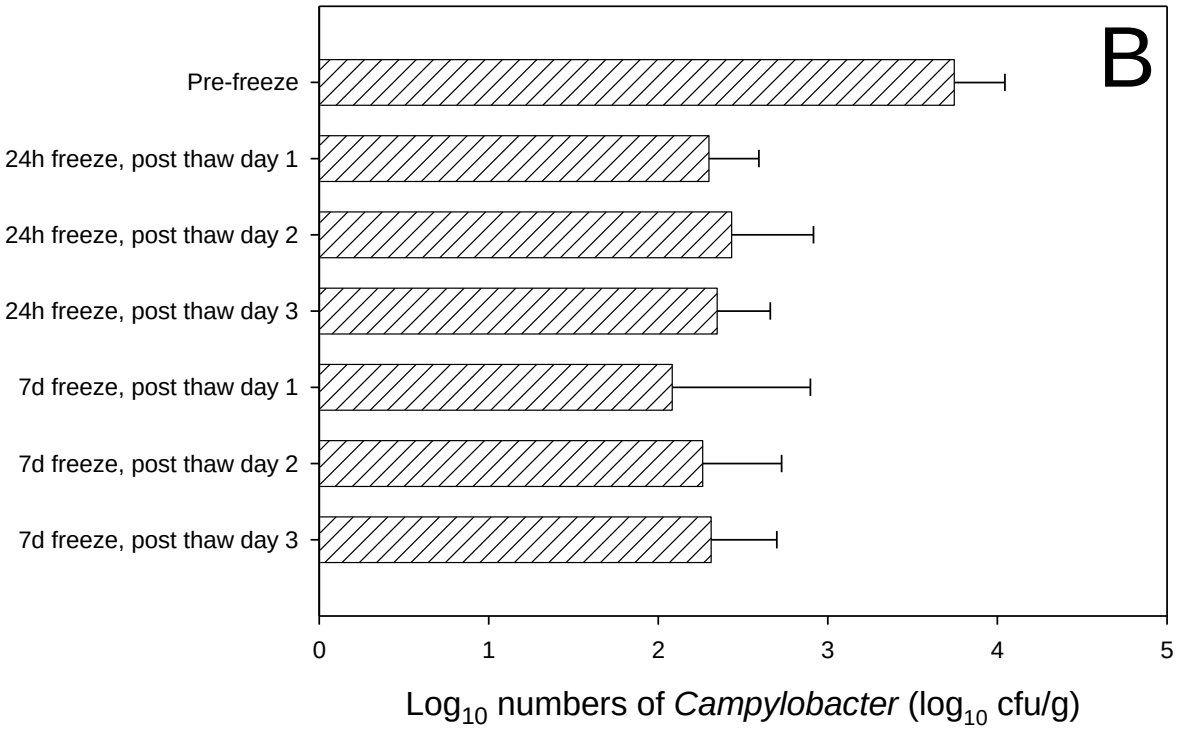


Figure 3: The effect of freezing to target temperatures of -15°C (A) and -25°C (B) on numbers of campylobacters in naturally-contaminated chicken livers. Data are the result of three independent trials with 10 replicates per trial (n=30).

When contaminated livers were frozen twice to -25°C for 24 h with thawing for 24 h in-between (Figure 4), reductions to numbers of campylobacters were observed after each freeze/thaw treatment (Figure 5). In broad agreement with the results of the single treatments reported above, the first treatment caused a significant reduction of about two log-cycles (t-test; $P<0.01$). The second freeze/thaw treatment caused a further decrease of around 0.75 log-cycles (Figure 5). The increased effect of two versus one freeze/thaw treatment was significant (t-test; $P<0.01$). Overall, two freeze/thaw treatments reduced the numbers of campylobacters by about three log cycles. As before, comparison of numbers of cfu g^{-1}, for individual samples, immediately after thawing and after chilled-storage for three more days using ANOVA found no significant differences.

The observation of a decrease in *Campylobacter* counts of up to 2.5 logs when the livers were frozen for only 24h is consistent with those of a number of authors for fresh broiler meat generally (Georgsson et al. 2006; Maziero and de Oliveira 2010). It has been previously reported that the length of time taken to freeze is important for bacterial survival in meat, with slow freezing tending to kill more bacteria than rapid freezing (Gill 2002, Archer 2004). When water takes a long time to freeze, motile microorganisms can to move into currently unfrozen pockets of water within foods. However, any solutes present in the unfrozen water become increasingly concentrated as freezing progresses, exposing both migrant and bacteria indigenous to the niche to increased osmotic stress and consequent enhanced damage (Archer 2004). There is also evidence that superoxide radicals form during freezing which are similarly concentrated in unfrozen pockets of water and consequently contribute towards the death of campylobacters (Stead and Park 2000). In contrast to previously published studies, our results showed that the freezer set at -15°C caused less bacterial death after 24 h compared with the freezer set at -25°C. There are a number of potential explanations, including that the livers placed in the freezer set to -15°C were soft-frozen with a slushy non-rigid consistency. In contrast the livers placed in the freezer set to -25°C were hard-frozen. Figure 2 shows that it took around 12 h for freezing in the least effective freezer compared with only 4 h in the freezer set to -25°C. Furthermore, associated with each bag of livers were 10-20 ml of viscous exudate which had leaked from the livers and which took longer to freeze than the livers themselves. It is possible that sugars or other components in the liquid were acting as a cryoprotectants for campylobacters in the freezer set to -15°C. In addition, it has been previously reported that chicken-meat exudate can upregulate stress response genes in campylobacters and help protect them from low temperature damage (Ligowska et al. 2011). It is also possible that the longer time taken to freeze the livers placed in the -15°C freezer enabled the campylobacters to upregulate any cold temperature defences.

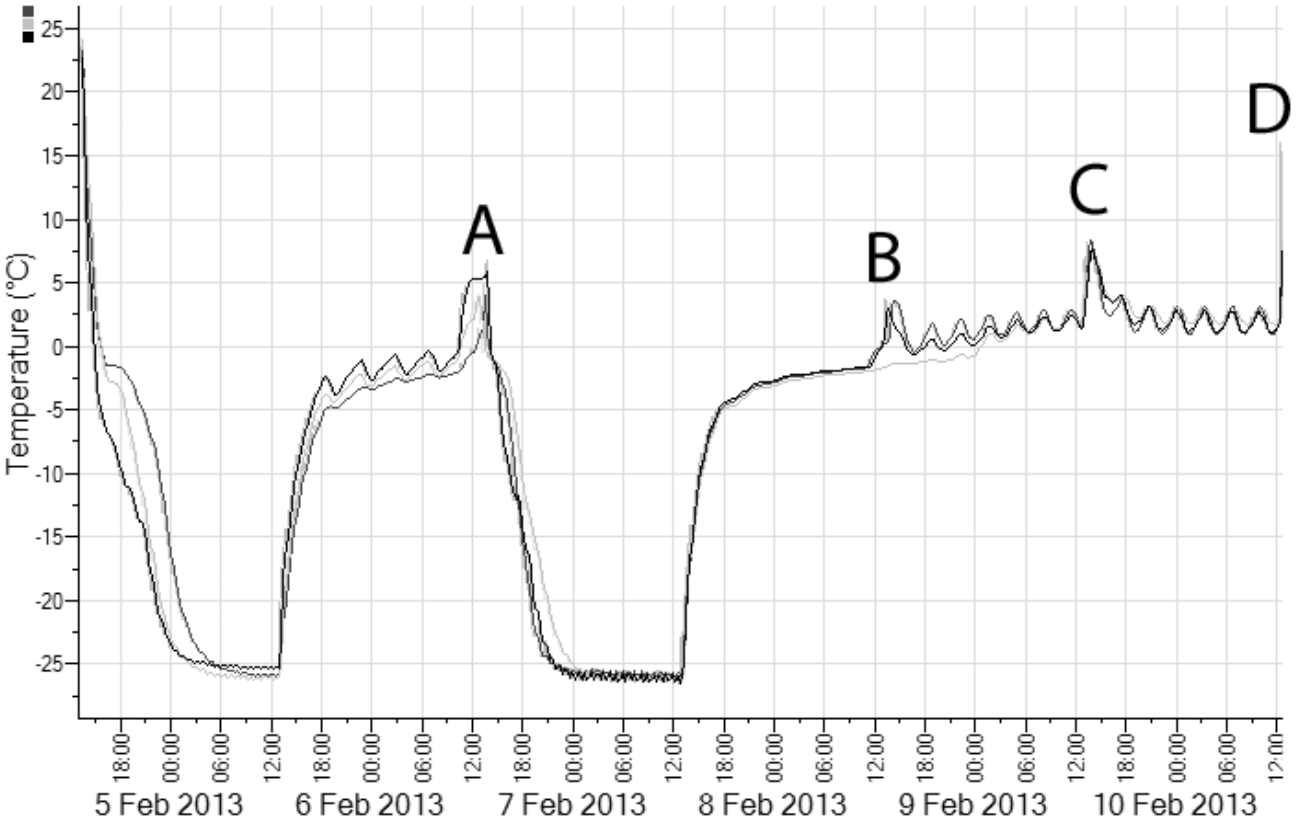


Figure 4: The temperatures at the centre of three separate 2.5-kg bags of chicken livers. The numbers of campylobacters were determined before freezing (not shown on figure) and after each bag of livers had been frozen and thawed (A), refrozen and re-thawed (B) and during refrigerated storage post thaw for two days (C and D).

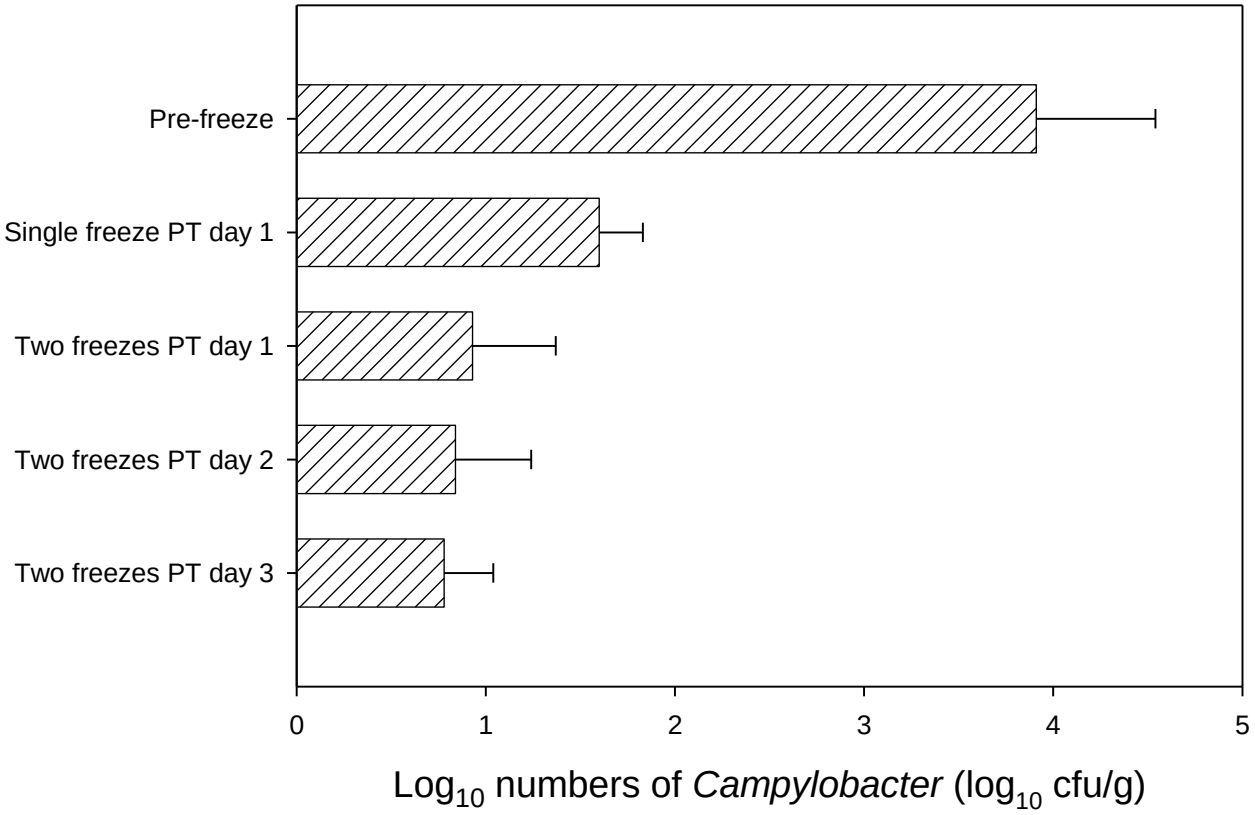


Figure 5: The effect of freezing chicken livers on two occasions on the numbers of cfu g^{-1} *Campylobacters*. The numbers of campylobacters were determined prior to freezing; post thawing (PT) after freezing to -25°C for 24h, PT from a second freeze to -25°C for 24h and after refrigerated storage for one and two days. Data are the result of three trials with 10 replicates per trial (n=30).

Test results for fresh livers at retail (Figure 1) indicated that it is rare for numbers of campylobacters to exceed 10,000 cfu/g fresh liver, a finding in keeping with previous reports (Baumgartner and Felleisen 2011). Thus it seems likely there would be a significant public health benefit were poultry processors to routinely freeze livers destined for human consumption. A literature search did not reveal any risk assessments specific for the consumption of chicken livers, although risk assessment models for *Campylobacter* in broiler meat generally were identified. A comparison of six independently-generated models concluded that the most effective intervention measures for human illness were those that reduced the *Campylobacter* concentrations on chicken meat (Nauta et al. 2009).

In summary, our findings show significant reductions to the numbers of campylobacters when contaminated livers were frozen. There are some chicken processors in the UK which freeze all of the livers collected in their plants and thus it seems plausible to conclude that livers could be frozen as part of routine processing from all plants without significant economic impact. Advice could also be issued to consumers that undertaking a second freeze would be beneficial in terms of a reduced likelihood of illness were the livers to be cooked inadequately.

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