Development of accurate predictive models for the assessment of the survival of *Campylobacter jejuni* and *C. coli* under food-relevant conditions

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EXECUTIVE SUMMARY

INTRODUCTION

Campylobacter are major zoonotic pathogens and control in the food chain is a public health necessity. Human infection by *Campylobacter* involves the interaction between environmental and foodborne pathways (Sheppard *et al*., 2009, EFSA 2012, Gras *et al*., 2012). A case-control study undertaken by Tam *et al*. (2009) identified that exposure to poultry related products accounted for 40.6% of cases of campylobacteriosis in humans. Source attribution studies have demonstrated the importance of poultry related products as the primary reservoir of human campylobacteriosis (EFSA 2014). While *Campylobacter* is known to be sensitive to extreme biophysical and biochemical environments, current treatment models used by the food production industry may be deficient insofar as they lack data that describe the biological processes relating to how the organism responds under high intensity stress. Evidence provided by source attribution studies suggests that *Campylobacter* may survive in foods irrespective of pre-treatment strategies and foods may act as vehicles of infection. Existing pre-treatment methods that employ potentailly lethal processes, such as heating and refridgeration, may not fully take into consideration the population biology of *Campylobacter* or how the survival of *Campylobacter* may be enhanced following interactions within a particular substrate. In addition, the survival of different strains may vary according to the type and intensity of the stress encountered (Coroller *et al*., 2006).

AIMS

We aim to assess the behaviour of *Campylobacter* in food products, and determine how survival and growth profiles of *Campylobacter* are influenced by exposure to variation in environmental conditions. In addition, we will assess the ability of *Campylobacter* to survive and recover from exposure to potentially damaging conditions by determining whether sub-lethally damaged cells persist and may therefore pose a continued threat to human health.

Experimental simulations will be evaluated using a semi-mechanistic modelling approach to describe the variation in the underlying response of *Campylobacter* following exposure to combined biophysical and biochemical stress. Predicted response curves generated from these models may then be used by industry partners to inform effective pre-treatment strategies designed to minimise contamination of the food chain by *Campylobacter*.

OBJECTIVES

1. To undertake a systematic review of published literature on the survival of *Campylobacter* in foods and food-related environments.
2. To identify *Campylobacter coli* and *Campylobacter jejuni* (*C. coli* and *C. jejuni*) strains appropriate for use in challenge studies.
3. To use laboratory techniques which take account of the population biology and physiology of *Campylobacter*.
4. Examine the survival of *Campylobacter* at high temperature in laboratory media.
5. Investigate the interaction of *Campylobacter* with food matrices and determine the influence of food substrates on the survival of *Campylobacter* at high temperatures.
6. Investigate the survival and growth of *Campylobacter* in food interiors after non-lethal heat treatments.
7. Generate models to predict the growth and survival of *Campylobacter* in food and food-related environments.
8. Make publically available data and models in a format that is suitable for use by the food industry.
9. Determine the temperature growth limits for a panel of 14 *C. jejuni* and *C. coli* strains.
10. To examine the survival of *Campylobacter* at lower temperatures in laboratory media during sous vide cooking at lower temperatures.
11. Investigate the survival of *Campylobacter* strains when associated with food matrices via external contamination during sous vide cooking at lower temperatures.
12. Investigate the survival of *Campylobacter* strains within food interiors during sous vide cooking at lower temperatures.
13. Develop models, where appropriate, that assesses the survival of *Campylobacter* within food matrices following cooking with sous vide method.

APPROACHES

A full systematic review of the peer reviewed literature on the survival of *Campylobacter* under food relevant conditions was undertaken by two reviewers.

The *C. jejuni* and *C. coli* strains were identified and isolated from different clinical and poultry sources and genetic origins. Strains were subjected to biophysical and biochemcial challenge under laboratory conditions.

Experimental simulations were also undertaken to account for how survival of *Campylobacter* is affected by characteristics of food matrices, namely when bacteria are present on the outside or the inside of poultry meat while including refridgeration as a step to mimic normal processing and storage conditions. For each simulation/experiment there were three biological replicates and some experiments repeated on a number of occasions to determine reproducibility. In addition, we investigated maximum growth temperatures in laboratory media and determined the survival of *Campylobacter* following internal and external contamination of meat under sous vide cooking conditions using commercial equipment. We used highly sensitive recovery techniques to provide data for predictive models.

We used two modelling approaches during the course of this study. In our initial appraoch we used the R statistical computing platform to generate generalised non-linear least squares models to determine the underlying mechanism responsible for producing the observed response (Pinheiro and Bates 2000). Five non-linear functions were used to generate predictive models. A sixth model, the mixed Weibull distribution model was adapted to the R framework from literature (Coroller *et al*., 2006). Models developed in R were overly complex for use by industry and it became necessary to find an alternative solution that was both easy to use and informative. In our second approach, we used GlnaFiT (Geeared *et al*., 2005), a freely available predictive modelling program implemented in Microsoft Excel. The program was originally designed to provide access to predictive models for end-users within industry unfamiliar with advanced non-linear regression techniques (Geeared *et al*., 2005). There are 10 non-linear functions that allow models to be fit to data. Each model is designed to describe a particular type and shape of response curve.

Key Findings

Objective 1 - To undertake a systematic review of published literature on the survival of Campylobacter in foods and food-related environments:

A formal meta-analysis of data generated by the literature review was not possible due to the multitude and often unique experimental approaches adopted by different researchers. Furthermore, there was little conformity with regard to the species and strains used during experimental studies. A refinement of data sources was necessary in order to identify those studies that were most relevant to the survival of *Campylobacter* in laboratory media, poultry meat and/or other relevant meat products. Thirty two data sources were retained for indirect comparison of published studies. A total of 25 different types of enumeration media have been used to explore aspects of *Campylobacter* survival under differing bio-physical challenges. Heterogeneity was also found in different temperatures used for recovering *Campylobacter* following challenges where seven different temperature profiles were noted (30^0C, 37^0C, 39^0C, 41.5^0C, 42^0C and 43^0C). Similarly, recovery incubation times following heat challenges also varied between studies (minimum 24 – 96 hours and maximum 48 – 96 hours). Moreover, not all studies explored the effects of selective and non-selective media on the recovery of sub-lethally damaged *Campylobacter* cells. For example, only 17 of the 32 studies explored this potentially important aspect of recovery of *Campylobacter*. Hierarchical cluster analysis was used to investigate patterns and groupings among studies according to their experimental design and the effects of each challenge on the survival of *Campylobacter*. Findings illustrated a high degree of heterogeneity amongst studies, due to non-standardisation in experimental design and this therefore precludes comparing the resistance of different strains to different treatments from the literature. In addition, we also used canonical correspondence analysis (CCA) to explore the trends in the use of recovery and enumeration techniques and to identify major trends of variation in the experimental design of studies. Heterogeneity in experimental design with regard to combinations of enumeration media used and the variability in temperature and temporal profiles are such that a synthesis of methods and findings for the purposes of formal meta-analyses is not possible. Therefore, it is not currently possible to generate such models from the published data without addressing underlying heterogeneity in the design and execution of experiments.

Objective 2 - To identify Campylobacter coli and C. jejuni strains to be used in challenge studies:

Table 1. shows the entire panel of *C. jejuni* and *C. coli* isolates selected for use in heat challenge studies in laboratory media (56^oC, 60^oC and 64^oC), which represent a range of different sequence types and clonal complexes common in human clinical cases and poultry meat. Isolates were derived

from poultry meat or human clinical cases (strains kindly provided by Professor Andrew Fox, Public Health England). All isolates were subject to minimal passage on receipt at Liverpool University and kept in stock cultures prior to resuscitation for each individual experiment. Isolates marked with an asterisk indicate the smaller panel used in further challenge studies, as it was not possible to perform all experiments on all members of the panel. The sub-set of isolates for further study was selected from initial results based upon their apparent differences in response to initial heat challenge experiments, with one *C. coli* isolate used for further experiments, as all four isolates of this species appeared to have a similar response in initial heat challenge experiments.

Table 1. The full panel of 14 *C. jejuni* and *C. coli* isolates used in this project, their sequence type and source.

Isolate ID	Sequence Type and Clonal Complex	Species	Source
13126	ST-21, CC-21*	*C. jejuni*	Poultry
13121	ST-45, CC-45*	*C. jejuni*	Poultry
13136	ST-45, CC-45	*C. jejuni*	Poultry
13163	ST-21, CC-21	*C. jejuni*	Poultry
11253	ST-825, CC-828	*C. coli*	Human
11762	ST-829, CC-828	*C. coli*	Human
12628	CC-828*	*C. coli*	Poultry
12610	CC-828	*C. coli*	Poultry
11368	ST-574, CC-574	*C. jejuni*	Human
12645	ST-51, CC-443	*C. jejuni*	Poultry
12662	ST-257, CC-257*	*C. jejuni*	Poultry
12720	ST-51, CC-443	*C. jejuni*	Poultry
12745	ST-257, CC-257	*C. jejuni*	Poultry
12783	ST-574, CC-574	*C. jejuni*	Poultry

Subsets of isolates used in all experimental challenge simulations are indicated *

Objective 3 - Use laboratory techniques, which take account of the biology and physiology of Campylobacter:

Standard operating procedures were developed for all experiments and used both selective media, namely modified charcoal cefoperazone deoxycholate agar (mCCDA) containing the selective

supplements cefoperazone and amphotericin and the non-selective Columbia blood agar containing 5 % defibrinated horse blood (CAB) with *Campylobacter* growth supplement (FBP), which contains oxygen quenching agents, to allow a comparison between the recovery capabilities of the two media, and to assess the recovery of sub-lethally damaged cells on the latter media.

A comparative analysis was undertaken where the numbers of sub-lethally damaged cells were determined for strains 13121 (ST-45, CC-45) and 11168 (ST-43, CC-21) (note this strain was not part of the main panel, but used as a comparator for this objective alone, as it has been used as a reference strain within the literature) by comparing recovery on either CAB-FBP or mCCDA when isolates were challenged at 56^{o}C. This temperature was used for this assessment as it allowed the response of the strains and their recovery on the two types of media to be modelled, as the response was more repeatable within strains. The impact of varying initial inoculum on the numbers of cells recovered was also assessed comparatively using strains 13121 (ST-45, CC-45) and 13136 (ST-45, CC-45). Findings for the comparison of enumeration media suggest that media type influenced the numbers of cells recovered for strain 11168 (ST-43, CC-21), where greater numbers of sub-lethally damaged cells were recovered from media type CAB-FBP in comparison to mCCDA. Greater numbers of cells were recovered when using CAB-FBP (log 1.74 CFU/ml^{-1}) *P-value* = 0.0001. However, there was no statistical differences in the numbers of cells recovered between media types at 16.0 minutes (asymptote) (*P-value* = 0.206) (Figure 62). This may be due to the fact that the numbers of bacteria at the asymptote also represents the limits of detection for recovery methods used in these experiments and with such low numbers it was not possible to show significant differences between CAB-FBP and mCCDA. ***Non-selective media (CAB-FBP) will allow greater recovery of cells when challenged with heat at sub-level temperatures compared to selective media (mCCDA with antibiotics), therefore we recommend that this media is used for such studies and not selective media.***

Objective 4 - To examine survival of Campylobacter at high temperature in laboratory media:

Experiments were undertaken to examine differences in the underlying survival rates of *Campylobacter* strains in pre-heated laboratory media at different temperatures and pH values. In each instance, *Campylobacter* strains were exposed to three different temperatures (56 ^{o}C, 60 ^{o}C and 64^{o}C) in different experiments. These challenge studies were then repeated using media with different pH values (4.5, 5.5, 6.5, 7.0 and 8.5). Survival curves produced from the challenge of isolates at 56 ^{o}C or 60^{o}C were found to be highly reproducible on repeat experiments. Challenges at higher temperatures resulted in poorer repeatability between experiments and greater heterogeneity in strain death rates. Differences were observed between different strains of

Campylobacter in terms of their survival during the heat treatments and the levels of sub-lethal injury seen. Variation was noted in survival curve profiles between isolates of the same clonal complex as well as between clonal complexes and this precluded generating a single model able to predict survival even at a single temperature. In terms of survival at low pH (4.5) survival was poor across all strains, with mild alkaline pH (8.5) the reduction in the response was less apparent, but survival poorer than between pH 5.5 and 7.0 for all temperatures tested.

Examining the impact of both pH and temperature has demonstrated that strains are able to survive well at lower pH ranges, with optimal survival for one strain (12662, CC-257) at pH 5.5 even when challenged at 64^{o}C. Thus, Campylobacter may not be as sensitive to acid pH as previously thought. However, this could have, perhaps, been expected because in chickens it has to survive passage through the gizzard, proventriculus and crop which all have low pH values. Such serial passage of strains may impact on their ability to subsequently tolerate low pH values.

Objective 5 - To investigate the interaction of Campylobacter with food matrices and its influence on survival at high temperatures:

In general, attachment to chicken meat pieces (0.1g) enhanced *Campylobacter* survival at sub-level temperatures, such as 56^{o}C when direct heating was applied. However, at higher temperatures (60, 64, 68 and 70^{o}C) attachment had no impact on *Campylobacter* survival. Given that meat will be pre-chilled before cooking, chicken inoculated with *Campylobacter* was subject to overnight chilling in a fridge (4^{o}C) and then subjected to direct heating at 60, 64, 68 and 70^{o}C. The impact of pre-chilling varied between strains but, due to insufficient reproducibility at higher temperatures, it could not be determined whether such differences were truly significant. However, at sub-lethal temperature challenge, pre-chilling in general appeared to enhance survival. In addition to this work extended time sampling was undertaken at 64°C (12 minutes) and 68°C (8 minutes) for two of the most heat resistant strains, 12628 (*C. coli,* CC-828) and 12662 (*C. jejuni*, ST-257), as a residual population was noted at the endpoint of previous experiments. The extended time sampling further confirmed the survival of such residual populations of *Campylobacter* after an initial Log_{10} 3.5-4.0 reduction, therefore demonstrating that a very small population was able to survive for these longer heating times.

As whole portions of meat are subject to gradual heating, small pieces of chicken meat were inoculated on their surface with *Campylobacter* as above and then subjected to gradual heating in a water-bath. The water-bath had a starting temperature of 25°C when the inoculated meat pieces were added and was set at 70^{o}C and it took approximately 16 minutes for the water-bath to reach this temperature. Once the water-bath reached 70^{o}C it was then held for 6 minutes (22 minutes

from the start of the experiment) at this temperature, within this time period there was a large reduction in counts (Log_{10} 2.7-4.2) for all tested strains, however *Campylobacter* could still be detected intermittently during this time, suggesting very low levels at the limits of microbiological detection/recovery. ***A high inoculum level (10^7 CFU/g) was used in these experiments to allow modelling of the death curve, with up to 10^5 CFU/g (FSA retail surveys) reported to be found on naturally contaminated poultry meat. However, given the small size of these fragments, such survival is of concern when extrapolating to larger meat portions and also internal contamination reported in poultry meat, these results may suggest the low level persistence of viable cells. These resistant sub-populations which remain after heating at such high temperatures are of concern and may suggest that 2 minutes at 70^{o}C is insufficient at eliminating all viable cells when the broiler meat is highly contaminated.***

Objective 6 -To investigate the survival and growth of Campylobacter in food interiors after non-lethal heat treatments:

In order to investigate the survival of internally contaminated meat with *Campylobacter,* meat pieces (2g) were internally inoculated with ~10^6 CFU and subject to direct or instant heating (added to pre-heated media), or gradual heating as in objective 5. The direct heating of chicken meat samples at 64^{o}C following the internal inoculation with *Campylobacter* (Log_{10} 6.5) resulted in a Log_{10} 3.5- 4.0 reduction for all isolates tested, this reduction was largely within first 5 minutes whilst the internal temperature of the meat reached 64^{o}C, however the residual population of *Campylobacter* persisted after 22 minutes at this relatively high temperature. Furthermore, after 14 minutes at an internal temperature of 68^{o}C (with the water-bath set at 70^{o}C), all *Campylobacter* strains could still be recovered with approximately Log_{10} 2.5 detected, again demonstrating a sub-population persisting at such high temperatures. It was not possible to reach an internal temperature of 70^{o}C using the water-bath, but these and earlier results from other objectives suggest that 70^{o}C for 2 minutes may be inadequate for eliminating all viable cells when chicken meat is highly contaminated. In further experiments, which involved heating to high temperatures (68°C), followed by gradual cooling for up to 40 minutes demonstrated that *Campylobacter* could be recovered, with approximately a Log_{10} 4.0 reduction. However, there was no evidence of a change in viable counts of *Campylobacter* during this gradual cooling process. In addition there was no further reduction in numbers of *Campylobacter* following refrigeration for 24 hours (cook and chill) after such experiments. ***Direct heating of chicken meat internally contaminated with high levels of Campylobacter (~10^6 CFU) at high temperature (68^{o}C internally) showed a rapid decline in Campylobacter numbers, however a residual population was present up to 14 minutes after being***

kept at this temperature. Whilst high numbers were inoculated into meat, these results have implications in terms of the FSA recommendations concerning 70^{o}C at 2 minutes being adequate for removing all viable Campylobacter.

Objectives 7 & 8 -To use the data generated to model and predict the growth (where relevant) and survival of Campylobacter in food and food-related environments and to make the data and models publically available in R in a format that is suitable for use by the food industry:

Selecting an appropraite non-linear function to generate predicted response curves that best describes the response of *Campylobacter* is a complex task. The manner in which *Campylobacter* responds to challenge may be a combination of strain characterisitcs and also the magnitude and type of challenge used during experimental simulations. Our findings indicated that no single model is adequate under all circumstances and that the selection of an appropriate model should be based on visualising the type and shape of survival curve generated by experimental data. Nevertheless, we conducted an intensive literature search in an attempt to identify a generic non-linear modelling approach capable of describing the survival response curves of *Campylobacter* following exposure to specific biochemical and biophysical challenge. The general model prospoed by Coroller *et al*. (2006) was identified as a possible solution to this problem and the potential of this modelling approach was presented to the steering group. The steering group decided collectively to assess the suitability of this model alongside other models incorporated in to a free software package provided by the University of Leuven.The GlnaFiT (1.6) package was developed by Geeraerd *et al*. (2006) and provides a user friendly means of fitting and evaluating ten different models capable of generating predicted response curves, including the general Weibull model proposed by Coroller *et al.* (2006). This software is freely available and maintained by University of Leuven: http://cit.kuleuven.be/biotec/downloads.php.

A user manual has been produced to assist with users in selecting appropriate models.

Objective 9 -To determine the temperature growth limit for a panel of 14 C. jejuni and C. coli strains:

Data for the upper temperature growth limit for *Campylobacter* species is lacking, especially for strains currently associated with poultry and causing human clinical disease, which may differ in their heat resistance and be of different genetic backgrounds. The growth-limit of *Campylobacter* strains (Table 1) was analysed using a linear mixed-effects model, assessing differences in the population of cells post incubation (18 hours) between strains at four temperatures (37, 41, 44, 45 and 46^{0}C). Further increases in temperature greater than 41^{o}C resulted in significant decreases in

the numbers of cells grown, however there was evidence that most isolates could still grow at 45^{o}C albeit at a reduced rate.

Mixed-effects models were used whereby individual strains were ranked according to their predicted growth limits. This is illustrated in the caterpillar-plot (Figure 380) where the average growth limit for all strains has been rescaled to zero, and the rank order of each strain is then determined by whether the individual estimate is greater than, or less than average of all strains. The rank-order of each strain as determined by BLUP was calculated and indicated that for *C. coli* strains (CC-828) a greater than average number of viable cells were observed and that a trend was apparent for higher upper growth limits for *C. coli* when compared to *C. jejuni* strains used in this study. ***C. coli strains appeared to have greater adaption for growing at higher temperatures, but with both C. coli and C. jejuni strains being able to grow albeit at a reduced rate at 45^{o}C.***

Objectives 10, 11, 12, & 13 - To examine the survival of Campylobacter strains at lower temperatures in laboratory media, when associated with food matrices and in food interiors during sous vide cooking at lower temperatures and modelling of the data:

Three strains *C. coli* ST-1773 [CC-828], *C. jejuni* ST-257 [CC-257] and *C. jejuni* ST45 [CC-45] were used to explore the survival following cooking under vacuum conditions at low temperatures using a commercially available sous vide waterbath. Due to the nature of vacuum packing of samples prior to low temperature heat challenge, this precluded being able to practically perform experiments in laboratory media only, or with external inoculation of small meat pieces as used in our earlier work. Due to the flattening of smaller chicken meat pieces under vacuum, whole chicken fillets were used instead and these are also more realistic in terms of what is likely to be cooked using sous vide, but subject to greater variability in terms of size and thickness (100-120g). Furthermore, the ST-21 isolate 13126, was also chosen from the panel of 4 (from all earlier experiments) for these experiments, however problems were encountered with growing this isolate up in broth with a number of experimental replicates failing, therefore the work with this isolate could not be continued within the timescale of the project.

Each fillet was inoculated with *Campylobacter* (10^{7} CFU) into the mid-point of the fillet and was placed individually in to a vacuum sealed bag and the fillets stored overnight at 4^{o}C. This was done for both logistic reasons in the laboratory and also to mirror the fact that naturally contaminated meat products would also be kept at this temperature prior to cooking.

Experimental simulations were undertaken covering a range of potentially inadequate heating temperatures (50-56^{o}C) in a sous vide water-bath. At 56°C, *C. jejuni* was eliminated after 1 hour at this temperature, however 1/3 replicates of *C. coli* (12628) had ~ 1 log CFU detected after an

hour. Additionally, simulations were extended and samples taken following 2 hours at 52°C resulting in a Log_{10} 4.6 reduction and 3 hours at 50°C resulting in a Log_{10} 2.5 reduction, indicating that a population of *Campylobacter* was able to survive at these lower temperatures for long periods of time and that long sous vide cooking times would not be sufficient to kill all *Campylobacter* present. ***Campylobacter could not be eliminated from whole artificially inoculated chicken fillets cooked at low temperature (50-52^{o}C) even after 3 hours of cooking at 50^{o}C, however it is worth noting that the chicken meat appeared under-cooked (raw) after such cooking times. At 56^{o}C the meat did not appear under-cooked after cooking for one hour and for one replicate of three, a C. coli strain could still be detected at 1 log CFU, indicating for some strains that this temperature for one hour was inadequate to kill all Campylobacter present in the fillet, although again fillets were inoculated with an artificially high inoculum level.***

CONCLUSIONS

1. Data generated in this project show that *Campylobacter* can survive at lower pH ranges (pH 5.5) when exposed to high temperatures.
2. *Campylobacter* associated with chicken meat enhanced its survival only at sub-lethal temperatures (eg 56^{o}C), with pre-chilling having limited impact on sensitivity to heat treatment.

3. Gradual heating of chicken meat pieces inoculated externally and internally to 70^{o}C has demonstrated survival of low numbers of *Campylobacter*, which is of concern.

4. Given that retail surveys have found levels of up to 10^{5} CFU/g contamination of *Campylobacter* on carcasses, these results may suggest that the current recommendation of 70^{o}C for 2 minutes may be inadequate in inactivating all *Campylobacter* present in meat when contaminated at such levels. However, more work is required to investigate if such sub-populations of viable cells are still able to cause infection and therefore represent a significant public health threat. Further work should also be undertaken to elucidate the mechanisms involved in the survival of these residual populations and their association with meat surfaces.
5. Cooking chicken at low temperature (50 and 52^{o}C) for three and two hours respectively was inadequate in eliminating *Campylobacter*; *Campylobacter* was still isolated from whole chicken fillets following these heat treatments, with *C. coli* also recovered at 56^{o}C also after one hour of cooking.

6. Our findings indicate that no one single model describes the survival of *Campylobacter* under all circumstances, and that the selection of an appropriate model should be based on individual strain and response to type and magnitude of challenge used during experimental simulation.