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Association of Cyclopropane Fatty Acid Synthesis with Thermo-Tolerance of Campylobacter Survival

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Abstract

Aims: To investigate the possession of the cyclopropane fatty acid synthase (cfa) gene by thermo-tolerant and non-thermo-tolerant Campylobacter spp. and to examine the presence of the cfa gene in Campylobacter that survived the scalding stage of poultry processing and to further investigate the cfa gene expression at 37?C in different C. jejuni strains that are able or not able to survive at 52?C. Methods and Results: The presence of the cfa gene in Campylobacter strains was determined by PCR. In order to determine the effect of heating on survival, the thermophilic C. jejuni were grouped by those collected before and those collected after the scalding stage process both groups being exposed to 52?C. Quantitative RT- PCR was performed to verify the gene expression level of cfa at 37?C in C. jejuni strains able or not able to survive at 52?C. Methods and Results:The presence of the cfa gene in Campylobacter strains was determined by PCR. In order to determine the effect of heating on survival, the thermophilic C. jejuni were grouped by those collected before and those collected after the scalding stage process both groups being exposed to 52?C. Quantitative RT-PCR was performed to verify the gene expression level of cfa at 37?C in C. jejuni strains able or not able to survive at 52?C.

Index terms— campylobacter, cfa gene, heat tolerance, survival, poultry processing.

1 I.

Background ampylobacter spp. are the leading cause of bacterial foodborne diarrheal disease worldwide **??**WHO, 2018). Poultry are believed to be the main contributor to human cases of Campylobacter spp. with the bacteria being found in both live and slaughtered chickens (Skirrow and **??**laser, 2000, Wittenbrink, 2002). The majority of Campylobacter spp. associated with chicken carcasses are identified as C. jejuni and C. coli (PHE, 2015, Wieczorek and Osek, 2015) and these two species are most frequently found in human cases in developed countries. Campylobacter spp. are exposed to many stress factors such as survival in the acidity of the host gut, survival on food and in the environment (Oh et al. 2018). The organism is capable of adapting to these stresses by regulating specific gene expression in response to stress (Murphy et al., 2006), such as dps, sodB, trxB, and ahpC, oxidative stress defence genes. Gene expression of these are increased through exposure to acid stress (Birk et al. 2012). During exposure to cold-shock Campylobacter increases the expression of sodB and Cjo358 stress response genes are increased (Stintzi and Whitworth, 2003). Depending on the stress conditions involved Campylobacter has different survival rates (Reid et al.

2 2008).

Many other species of bacteria have the ability to change physiologically during starvation or environmental stress; for example by modifying their membrane lipids in situ by changing the phospholipid unsaturated fatty acid (UFA) to cyclopropane fatty acids (CFA) (Grogan and Cronan, 1997). The presence of cyclopropane ring-containing lipids, especially phospholipids, has been reported for many bacterial species including Escherichia coli and Salmonella typhimurium and there is a strong correlation between acid survival and chlorosome glycolipid

molecules in heat protection (Mizoguchi et al. 2013). Konkel et al. (1998) reported that C. jejuni preferentially synthesises 24 proteins immediately following heat shock. One of the major heat-shock proteins is DnaJ, which enables Campylobacter to colonise chickens while it has been shown that Campylobacter DnaJ mutants cannot colonise chickens. This suggests that DnaJ (HSP 40) plays an essential role in C. jejuni's thermal tolerance at temperatures above 42?C (Konkel et al. 1998). However, DnaK and DnaJ are both found in non-thermophilic Campylobacter and so cannot be a factor in determining thermotolerance (Riedel et al. 2020).

The presence of CFAs in bacterial fatty acid membranes has been shown to provide protection against temperature changes and it is therefore likely that lipid composition of membranes changes when the microbial growth temperatures change **??**Russell et al.1995). The aim of this study was to investigate the possession of the cyclopropane fatty acid synthase gene by thermo-tolerant and non-thermo-tolerant Campylobacter spp. and to examine the presence of the cfa gene in Campylobacter that survived the scalding stage of poultry processing and to further investigate the cfa gene expression at 37?C in different C. jejuni strains that are able or not able to survive at 52?C.

3 II.

4 Material and Methods

5 a) Origin of isolates

A total of 60 C. jejuni isolates were collected from commercial poultry processing plants in the UK. Samples were taken from carcasses early during the processing cycle, at the post-bleed, immediately after leaving the scalding tank (post-scald) and at the postchill stages, and 21 isolates (7 from each stage of process) were chosen to be used within the framework of the study. Twenty Campylobacter strains for the environmental group were obtained from the laboratory culture collection and original came from different sources (water, sheep, soil). All Campylobacter isolates had been identified to the species level using conventional multiplex PCR **??**Lund et al. 2004).

6 b) Bacterial strains and culture conditions

The C. jejuni isolates were recovered from frozen storage by direct plating onto blood agar (BA; Oxoid Ltd.). Plates were incubated at 37?C for 48 h in a MACS-MG 1000 anaerobic cabinet (MAC-Cabinet; Don Whitley Scientific, Shipley, UK) with a microaerobic gas mixture consisting of 10% CO 2 , 5% O 2 and 2% H 2 , balanced in N 2 . These cultures were used for Deoxyribonucleic acid (DNA) extraction.

7 c) DNA Extraction

The DNA from the samples was extracted using the QIAamp® DNA extraction Kit (Qiagen, Crawley UK) according to manufacturer's instructions. Eluted DNA was stored at -20?C until used.

8 d) PCR for detection of the cfa gene in Campylobacter

isolates DNA was extracted from C.jejuni isolates in order to examine the presence or absence of the cfa gene, PCR primers specific for this gene were designed based on the gene sequence information in the Campy database which was determined by conventional PCR using custom-designed primers (Table **??**). Twenty-five µl PCR reaction mix were prepared as follows: 12.5 µl GoTag mastermix (Promega, UK), 1 µl forward primer (1:10 dilution), 1 µl reverse primer (1:10 dilution), 9.5 µl nuclease-free water and 1 µl DNA. The cycling conditions were as follows: 95?C for 10 min and then 40 cycles of 95?C for 60 seconds, 50?C for 60 seconds, and 72?C for 90 seconds. Samples were incubated at 72?C for 5 min and held at 4?C until processed. A 10 ?l aliquot was taken from the amplified PCR products and analysed by gel electrophoresis at 100 V for 90 min using 1X TBE (0.89 M Tris borate, 0.02 M EDTA) running buffer on 2% agarose gels (Sigma-Aldrich). Gels were stained with 10 mg/ml ethidium bromide solution (Sigma-Aldrich) and visualised on a UV gel documentation system. A DNA-molecular ladder (50-bp and 100-bp ladder) (Hyper ladder, Bioline, UK) was included in each gel so that the size of products could be determined. Presence of a band of the appropriate length was taken to be a positive result. C. fetus (NCTC 10842) was used as a negative control and C. jejuni (NCTC 11168) was used as a positive control.

9 e) Purification of PCR products for sequencing

The PCR products were purified using the QIAquick® PCR purification Kit (Qiagen) according to the manufacturer's instructions. A 15ul aliquot of purified DNA with a concentration of 1 ng/ µl (150-300 bp) was mixed with 2 µl of 10 pmol/ µl (10 µM) sequencing primer (cfa forward) in a microcentrifuge tube (Eurofins). Purified PCR products were sent to Eurofins Genomics, MWG Operon (Ebersberg, Germany) for sequencing. Sequence data was assembled with Multiple Sequence Alignment (Clustral Omega) and CLC Sequence Viewer 6.7.1 (CLC bio, Aarhus, Denmark) was used to align the sequences.

10 f) Heat tolerance

In order to determine the effect of heating on survival, 71 C. jejuni strains were divided into two groups: pre and post scald, 36 and 35 isolates were collected from each group respectively. As described by Hughes et al (2009) with slight modification, the isolates were recovered from frozen storage as described above. To determine survival at 52?C, isolates were sub cultured in 50 ml Mueller Hinton broth (Oxoid), in a Bijoux and a 10µl loopful of bacteria was added and mixed thoroughly for each sample. These were incubated microaerobically at 37?C for 48 h using the MAC-Cabinet. Following incubation 50 µl from each sample was pipetted into a 300µl microcentrifuge tube which was placed in a water bath at 52?C for 30 mins. Serial dilutions were made in microtitre plates by pipetting 180 µl of PBS (Oxoid) into the wells of rows two to eight and 40 µl of samples into the wells of row one. Twenty µl of samples from row one was removed and mixed into the next row. This was continued across the plate. To check for the presence of bacteria in the wells 20 µl samples from each well were pipetted onto BA plates and incubated under microaerobic conditions at 37?C for 48 h as described above. The number of bacteria that survived was determined using the following formula. G incubated at 37?C for 48 h in a microaerobic atmosphere using the Campygen gas generating system (Oxoid). A 10µl loopful of culture was inoculated into 7 ml Mueller Hinton broth (Oxoid) in 7 ml Bijoux and incubated under microaerobic conditions at 37?C for 48 hours as described previously. Prior to extraction a 100µl aliquot of culture broth was pipetted into MH broth in a 25 cm 2 tissue culture flask at 37?C for 24 hours. Following incubation 2 ml from each culture was pipetted into 2.0 ml microcentrifuge tubes. The tubes were centrifuged at 8000 g for 10 minutes. To the resulting pellet, 800 µl of tri reagent (Sigma) was added and the tubes left at ambient temperature for 10 minutes. This was followed by the addition of 200 µl chloroform (Sigma) and the tubes were then centrifuged for 10 min at 13000 g. The upper aqueous layer was removed while avoiding the interphase and this was transferred to a clean 2.0 ml microcentrifuge tube (Fisher Scientific). A Qiagen® RNeasy Mini kit was used for RNA extraction following manufacturer's instructions. The extracted RNA was frozen immediately at -80 ?C for further analysis.

11 h) Real Time -Polymerase Chain Reaction

Quantitative RT-PCR was performed to verify the gene expression level of cfa at 37?C in C. jejuni strains able or not able to survive at 52?C. The following primer sequences were used for detection of cfa and 16S rRNA genes in C. jejuni isolates: The forward cfa-RT 5' ACTATGAGCTATTCTTGCGCT 3' (21) reserve cfa-RT 5' AACCCCAGCCACCAACCTATA 3' (20), the forward 16S rRNA CCAGCAGCCGCGGTAAT (17) and the 16S rRNA GCCCTTTACGCCCAGTGAT (19) using the QuantiTect SYBR Green RT-PCR kit (QIAGEN) according to the manufacturer's recommendations. The comparative threshold (Ct) value corresponds to the PCR cycle at which the first detectable increase in fluorescence associated with the exponential growth of PCR products occurs, using comparative threshold cycle (??C ?) (Livak and Schmittgen, 2001). The relative expression of each gene was determined three times in each of three experimental RNA samples, normalised to the 16S rRNA reference gene and expressed as fold difference in quantity of cDNA molecules present in C. jejuni that could survive (+ve) at 52?C to that present in C. jejuni that could not survive (-ve) at 52?C.

12 i) Statistical analysis used in this study

For determination of P-value in heat tolerance experiments.

Fisher's analysis, a 2x2 contingency table and one-tailed P-value was used. P values were considered to be significant <0.05.

P-values for the gene expression work were determined using SPSS, Mann-Whitney U test (P value <0.05 was considered to be significant) using Graph Pad Prism (V.6.0) software package for the graph Mac (Graphpad, San Diego, USA).

13 III.

14 Results

15 a) Presence of Campylobacter cyclopropane fatty acid synthase gene

The presence of the cfa gene in Campylobacter strains grouped by source was determined by PCR (Table **??**). The cfa gene was present in all C. jejuni strains isolated from the poultry abattoir (Table **??**), whereas, in the non-abattoir associated strains, the cfa gene was absent in C. fetus, C. helveticus, C. sputorum and C. fecalis (Table **??**). The strains isolated from abattoirs originally came from chickens and were adapted to 42?C (chicken body temperature). In strains isolated from environmental sources (non-chicken), where the temperature was below 42?C the gene was absent (Table **??**).

16 b) Heat tolerance survival

Thirty-six samples of C. jejuni collected before and 35 collected after the scalding stage process 'both groups were exposed to 52?C' showed to differ significantly in terms of survival $P< 0.0420$ (Table **??**), with the proportion

of strains collected after the scalding process surviving 52?C being roughly twice as high as before the scalding process.

17 c) The cfa gene expression

Two groups of C. jejuni isolated from chicken abattoirs, previously identified to contain a cfa gene using PCR, were exposed to 52?C in a waterbath. The results obtained by QRT-PCR analysis using the cfa as the gene targets showed that expression of cfa mRNA relative to 16S mRNA differed. When cultured at 37?C, C. jejuni strains turned out to have the ability to survive at 52?C and had a significantly higher expression of the cfa gene compared to C. jejuni strains that appeared not to survive at 52 ?C (Figure 1).

18 IV.

19 Discussion

The ability of C. jejuni to tolerate conditions found during processing can be considered an important factor associated with their survival (Oh et al. 2018). To understand the mechanisms involved, Campylobacter spp. isolates from the environment and C. jejuni from various location in the chicken slaughter line were compared for their ability to produce the cfa gene, which has been shown previously to be associated with increased survival at elevated temperature (Grogan and Cronan, 1997, Zhang and Rock, 2008). In the present study, C. jejuni strains isolated from processing plants are more likely to possess the cfa gene, whereas the cfa gene was not present in C. fetus, C. helveticus or C. sputorum isolated from non-abattoir sources (Table **??**). The source was seen to have a great influence on the presence of the G cfa gene and the presence of this gene could explain thermophilic survival temperatures above 37 o C (Table **??**). The upregulation of the cfa gene was seen when the bacterial cell started to enter the stationary phase in E. coli **??**Chang et al. 2000b) and under thermal resistance they modified the profile of their phospholipid fatty membrane **??**Annous et al.1999;Zhang and Rock, 2008). The biosynthesis of CFAs effected stability and integrity of the cell membrane at high temperatures (Guzzo, 2011; **??**ufourcet al. 1984). This change supports the role of CFAs in the stress tolerance which is encoded by the cfa gene and enables cells to physiologically adapt to the condition of heat stress (Guzzo, 2011). Interestingly, all isolates obtained from the processing plants had the cfa gene present (Table **??**), suggesting that Campylobacter strains colonising birds were able to withstand the challenge of the heat during processing plant regardless of cfa gene synthase.

During slaughter, Campylobacter spp. in and on chicken carcasses are subjected to temperatures higher than 50°C (Osiriphun et al. 2012) and this is a form of stress because under normal conditions Campylobacter does not grow at temperatures higher than 42°C (Park, 2002). To mimic circumstances of heat tolerance survival and maintenance of the survival response in C. jejuni strains were examined for survival of 52°C (Table **??**). This experiment demonstrated a higher capacity of post scald C. jejuni strains to adapt to and survive heat (Table **??**). A temperature of 52°C was selected as the whole chicken carcass is subjected to a scald tank with a water temperature of approximately 52 to 55°C to remove the feathers (Lehner et al., 2014).In the present study the differences in survival between isolates collected pre and post scald stage were significant ($P < 0.05$). A large proportion of the C. jejuni strains from the post scald stage can survive higher temperatures compared to those from the pre scald stage (Table **??**). The ability to adapt to higher temperatures correlates with the upregulation of specific genes, including groEL and rpoD that enhance survival of heat-stress **??**Klancniket al. 2008). The heat-stress response mechanisms of C. jejuni resulted in changes in morphology and protein profile when exposed to 48°C and to 55°C for a short time and their culturability and viability correlated with an altered protein profile and decreased virulence properties (Klancnik et al. 2014).

Heat stress is a key feature of poultry processing and only the bacteria that survive these abattoir stresses can reach human hosts. Since high temperature causes a physical change in the composition of the bacterial membrane lipids (Zhang and Rock, 2008), it has been demonstrated that the cfa gene has evolved responsively to this change (Chang et al. 2000). In the present study the thermophilic C. jejuni strains that survived at 52 0 C and cultured at 37 0 C were significantly more likely to increase the level of the relative cfa/rRNAgene expression compared to the control strain of C. fetus (Figure 1). This suggests that the cfa gene alters the fatty acid composition significantly in order to stabilise the membrane **??**Dufourc et al. 1984, Chang andCronan, 1999). C. fetus does not have the cfa gene (Table **??**) so is not able to grow at temperatures higher than 37 0 C. This confirms the involvement of cfa in cyclopropane fatty acids biosynthesis. The findings presented here show that there is significant variation in the relative cfa/rRNA levels expressed between strains surviving at 52 0 C and strains unable to survive at this temperature when both groups are cultured at 37 0 C (Figure 1). C. jejuni strains surviving at 52 0 C expressed the cfa gene and are adapted to survive when cultured at 37 0C as evidenced by its increased response characteristics of altered CFA during temperature growth. Similar studies observed that the cfa gene was regulated under stress conditions in other pathogens. For example, E. coli expressed the cfa gene under acid adaptation (Grandvalet et al. 2008) and S. typhimurium induced the expression of the cfa gene under acid stress (Kim et al. 2005).

The proportion of cfa mRNA transcripts increases with the increasing amount of the CFA in the bacterial membrane (Chang and Cronan, 1999). In the present study different expression was seen with C. jejuni strains that were able to survive at 52 0 C, where some strains had a low level of expression of mRNA in the cfa gene

(less than 0.2 relative cfa/ 16S rRNA levels of gene expression) (Figure 1). This suggests that not all strains express cfa to the same extent. In previous studies the low level of CFA synthesis was linked to the level of conversion of UFAs to CFAs and to the substrate specificity of the CFA synthase (Grandvalet et al. 2008).

Although the two major shock proteins DnaK and DnaJ are found in non-thermophilic Campylobacter, their role is small and limited. Some non-thermophilic Campylobacter are unable to colonise the chicken gut (Kempf et al. 2006) as the chicken's body temperature is 41.7 ?C. Results suggest that the cfa gene may in some way play a role in the ability of C. jejuni to colonise chickens and/or to persist in the chicken gut. This to suggest that the lack of the cfa gene could be a factor in determining thermo-tolerance. Thermo-tolerant bacteria are able to colonise chickens, as the presence of the gene will allow cells to adapt to survive above 37 ?C. Campylobacter isolates that can colonise birds were able to withstand the challenge of the processing plant regardless of cfa gene synthase (Table **??**). Absence of the cfa gene was associated with the inability of the organism to colonise birds **??**Hermans et al. 2011).

This study also found that the source of Campylobacter species and the presence of the cfa gene could explain the difference in ability of strains to tolerate high temperatures. These data support the role of the cfa gene in the thermophilic pathogen for promoting its survival as reported in other studies **??**Chen et

20 G

Temperatures above 42 0 C may not be optimal for Campylobacter spp. to grow (Stintzi, 2003), but C. jejuni strains are adapting their survival at 52 0 C by expressing the cfa gene. The expression level of cfa synthase involved in levels of different heat stress response mechanisms was described by Klancnik et al. (2006) with C. jejuni having elevated level of gene products in response to heat stresses. The change of the fatty acid membrane composition depends on the biosynthetic reactions that use modified lipid acyl components (Russell, 2002). The expression of the cfa synthase gene is reported to be important in the survival of S. typhimurium during the stationary phase, as this bacterium expresses high levels of this gene when entering this stage and the expression of cfa mutants was absent (Kim et al. 2005).

The present study shows that there is a degree of variation seen in the relative cfa/rRNA levels expressed in the strains surviving at 52 0 C (Figure 1). Some C. jejuni strains have a higher cfa gene expression than other strains also surviving at 52 0 C and this indicates that these strains may alter their fatty acid composition in a different way during survival to stabilise their membrane and to cope with higher temperatures. This finding is supported by Hughes et al. (2009) who found that C. jejuni altered different fatty acids due to temperature challenge. The difference in the cfa gene expression levels has also been observed in several strains of E. coli when the bacteria is induced to express the cfa gene and the subsequent synthesis of CFA in cell membrane phospholipids that increases to protect the cells from death (Chang and Cronan, 1999). As shown in Figure 1 some C. jejuni strains surviving at 52 0 C show lower levels of mRNA expression at 37 0 C and this may account for a lack of the major change in fatty acid composition, but these strains are still able to express the cfa gene which is required in order to alter their fatty acid composition to tolerate high temperatures. The findings indicate that the cfa synthesis gene was present in abattoir strains but not in the non-poultry abattoir group, suggesting that cfa contributes to the ability of Campylobacter to grow at temperatures above 37 0 C. Strains of C. jejuni that survived passage through the abattoir scald tank were more likely to be able to survive at elevated temperatures than pre-scald strains.

This study shows that the change in gene expression induced by C. jejuni survival at 52 0C is evidence to suggest that the expression of the cfa gene is an important factor for survival at elevated temperatures and allows the consequential survival of Campylobacter spp. throughout the poultry processing chain which ultimately leads to infection in humans (Skarp et al. 2016).

In conclusion, these results provide evidence that thermophilic C. jejuni cells induced the cfa gene that is important for acquisition of heat resistance. A link of this thermo-tolerance to the cfa gene expression associated synthesis of high levels of cyclic fatty acids impacts the survival during the scalding stage of poultry processing. Understanding the mechanism of Campylobacter during poultry abattoir processing may help in improving control measures to reduce the burden of Campylobacter and implementing strategies to prevent disease.

21 Declaration of conflicting interests:

The authors declared no potential conflicts of interests with respect to publication of this article.

Authors' contributions: H. M. designed and carried out the experiment and wrote the manuscript. L K. W., E. VK and T. C. helped in analysis and interpretation of the data andprovided critical feedback and helped shape the research.

*Abattoir samples: thermophilic C. jejuni; Non-abattoir: other Campylobacter species Table **??**: Heat survival of C. jejuni strains at 52 ?C from abattoirs isolated before and after exposure to the scald tank. Fisher's exact test was used to determine the differences between the heat tolerance groups. P-values less than 0.05 were considered significant. [1]

[1]© 2022 Global JournalsAssociation of Cyclopropane Fatty Acid Synthesis with Thermo-Tolerance of Campylobacter Survival

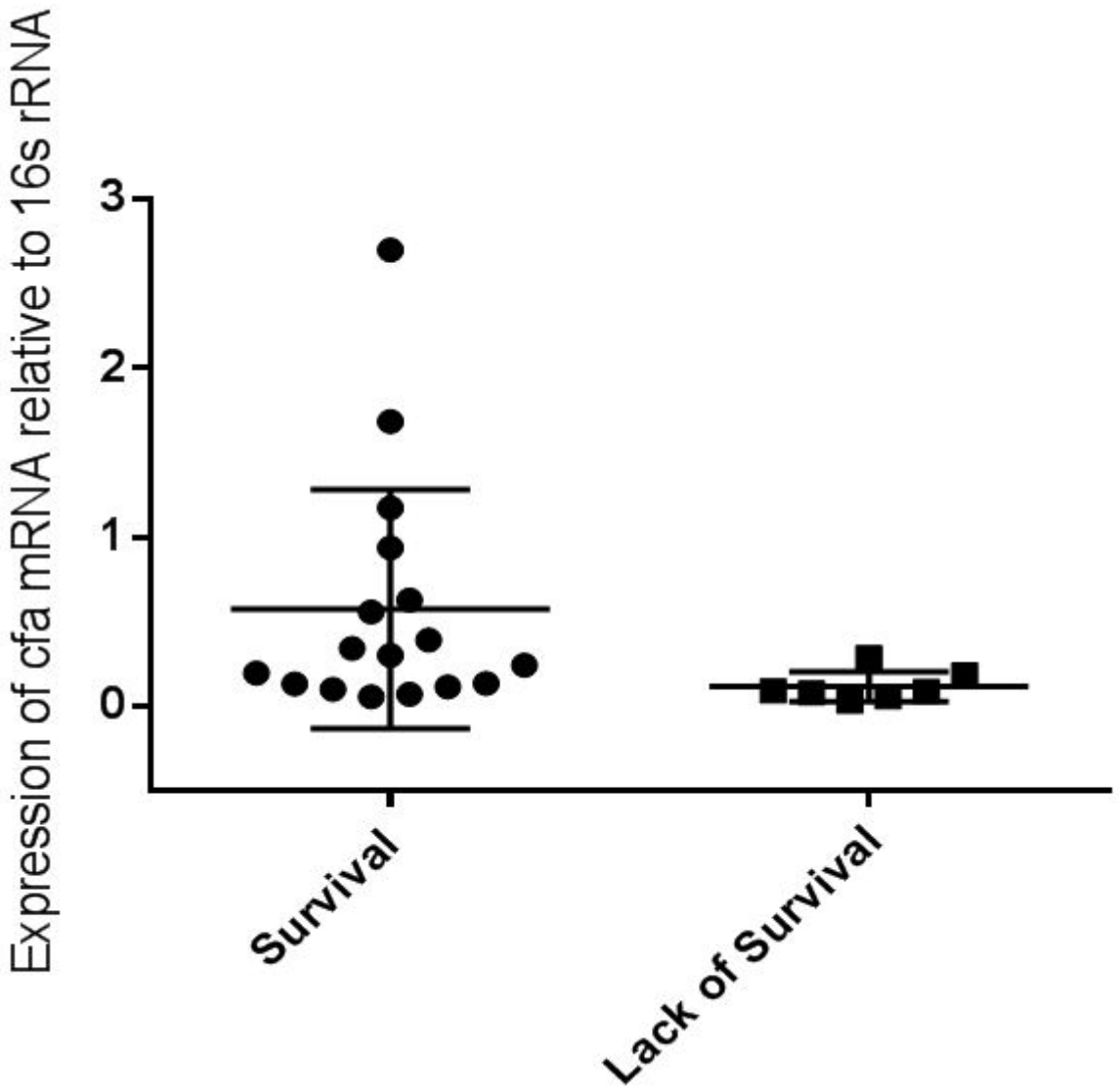

Figure 1:

Figure 2:

	C. jejuni1A2/3		Pre	C4	Abattoir
	C. jejuni1A3/3		Pre	C12	Abattoir
	C. jejuni3A4/2		Pre	C5	Abattoir
	C. jejuni1B5/1		Post	C23	Abattoir
	C. jejuni3B2/3		Post	C5	Abattoir
	C. jejuni3B3/2		Post	C7	Abattoir
	C.sputorumss.sputorum NCTC 11528		-	-	Non-abatt
	C. sputorum ss. fecalis NCTC 11367		-	-	Non-abatt
	C. jejuni ss doylei NCTC 11951		-	-	Non-abatt
	C. sputorum ss. fecalis NCTC 11415		-	-	Non-abatt
Year 2022	C. helveticus NCTC 12470 C. fetus ss fetus NCTC 10842		–	–	Non-abatt
					Non-abatt
Volume XXII Issue II Version I	Pre-scald Post-scald	N. of sam-ple 36 35	+ve survive 52 ?C 7 15		-ve survive 29 20
D D D D) (Medical Research Global Journal of					

[Note: G 16 © 2022 Global Journals]

Figure 3:

21 DECLARATION OF CONFLICTING INTERESTS:

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