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The effect of low-temperature long-time (LTLT) cooking on survival of potentially pathogenic *Clostridium perfringens* in beef

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Abstract

Low-temperature long-time (LTLT) cooking may lead to risk of potential survival of pathogenic bacteria such as *Clostridium perfringens* in cooked meat. In this study, the effect of LTLT cooking on *C. perfringens* was investigated at temperatures commonly used by caterers. Brain heart infusion broth (BHIB) and meat cubes in pouches (vacuumed or non-vacuumed) were inoculated with *C. perfringens* (NCTC 8238) and heated at temperatures of 48°C, 53°C, 55°C, 60°C and 70°C. The viability of *C. perfringens* in BHIB and meat was monitored using plate counting and the D-value of each thermal treatment was determined. The recovery of *C. perfringens* after thermal treatment was assessed using optical density measurements. Flow cytometry analysis was used to assess the physiological status (death/injury) of *C. perfringens* cells in BHIB. The results showed that the required log reduction (6-log) of *C. perfringens* can be achieved at 55°C but not at 48°C or 53°C. The D-values at all temperatures were higher in meat compared to BHIB while the D-value at 55°C was higher in non-vacuum compared to vacuum sealed meat. *C. perfringens* cells were able to recover and grow to pathogenic levels when thermal treatment was unable to achieve the required 6-log reduction. In BHIB, percentage of dead cells

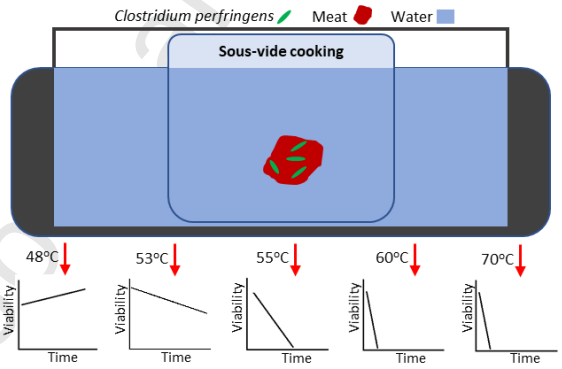
increased gradually at 48°C, 53°C and 55°C while an immediate increase (> 95%) was observed at 60°C and 70°C. These results are important to food safety authorities allowing to set the time-temperature combinations to be used in LTLT cooking to obtain safe meat.

Key words: thermal treatment, sous-vide cooking, vacuum sealed meat, D-values, flow cytometry, sub-lethal injury

Highlights

- Sous-vide at 55°C requires 300 minutes for a 6-log reduction of *C. perfringens*
- Cooking at 48°C or 53°C did not achieve 6-log reduction of *C. perfringens*
- Sub-lethally injured cells can recover after thermal treatment

Graphical Abstract



1. Introduction

Low-temperature long-time (LTLT) cooking is achieved by heating the meat at low temperatures (from 50°C – 65°C) for prolonged time (from minutes to multiple hours) which induces desirable changes in meat characteristics and retains most nutrients (Dominguez-Hernandez et al., 2018). The food industry and caterers have been adopting LTLT cooking methods such as sous-vide and slow cooking of meat as an effective process to address this expectation (James and James, 2014; Ramos et al., 2013). In sous-vide cooking, the meat is vacuum sealed in plastic bags or pouches before heating which reduces oxidation reactions and production of off-flavors as well as prevent cross-contamination after cooking (Baldwin, 2012).

However, cooking at low temperatures increases the potential of pathogenic bacteria surviving in meat that can compromise its safety, particularly at high levels of initial contamination (Silva and Gibbs, 2012). This concern is derived from the ability of adaptive pathogens to survive in a wide range of temperatures and enhance their thermo-resistance. *Clostridium perfringens*, for example, can grow at temperatures up to 52°C and produce highly resistant spores (Novak and Juneja, 2002; Stringer et al., 2012). Moreover, preference to anaerobic environment makes *C. perfringens* a big challenge to food processed under vacuum, such as sous-vide (Juneja et al., 2006). Although, it has been recommended that temperatures of 55°C or above must be used when cooking red meat using sous-vide, temperatures below 55°C have been used by restaurants in many countries and were published in books or on the internet (Stringer et al., 2012). However, there is a gap in knowledge on survival or potential growth of *C. perfringens* at temperatures between 52°C and 55°C (FSA, 2013).

There are no published set time-temperature combinations for cooking at temperatures below 60°C. Therefore, it is critical to validate cooking meat at low temperatures and set the minimum time required to achieve safe meat in order to avoid misinterpreting the application of time-temperature combinations in LTLT cooking. The effectiveness of thermal treatment to achieve safe meat can be assessed by applying an isothermal process to contaminated broth or meat and monitor viability of pathogens at regular intervals (Alnoman et al., 2015; Lahou et al., 2015). Broth-based models inoculated with attenuated strains are widely used to represent contaminated meat in thermal inactivation studies since they guarantee the best control of temperature during cooking which minimize systemic errors and enable data collecting for many observations per treatment (Ross et al., 2008, Wiegand et al., 2012). On meat surfaces the bacteria are immobilized, and they tend to form colonies while their sensibility to environmental stresses is increased (Jeanson et al., 2015). Moreover, the presence of fat in meat may increase resistance of cells to heat (Doyle, 2002). Furthermore, the anoxic environment (reduced oxygen) during sous-vide cooking produced by vacuum sealing of meat in pouches may encourage the growth of facultative anaerobes such as *C. perfringens*.

This study aims to investigate the thermal inactivation of vegetative cells of *C. perfringens* in brain heart infusion broth (BHIB) (nutrient-rich media that simulates meat) and red meat during LTLT cooking and provide recommendations for time-temperature parameters for sous-vide cooking at temperatures below 55°C. BHIB and red meat underwent thermal treatment at temperatures of 60°C, 55°C, 53°C and 48°C to simulate the cooking temperatures used by caterers. The ability of *C. perfringens* to recover after thermal treatment was investigated and the physiological status of *C. perfringens* (death/injury) during LTLT cooking was assessed using flow

cytometry. Also, the effect of limiting oxygen on *C. perfringens* during LTLT cooking in non-vacuum and vacuum sealed pouches (sous-vide cooking) was investigated.

2. Materials and Methods

2.1. Materials

Tryptose sulfite cycloserine (TSC) agar (Oxoid Ltd. CM0587B), egg yolk tellurite emulsion (Oxoid Ltd. SR0054C), Columbia blood agar (Oxoid Ltd. CM0331B), brain heart infusion broth (BHIB) (Oxoid Ltd. CM1135B), TSC Supplement (D-cycloserin) (Oxoid Ltd. SR0088E), phosphate buffer saline (PBS) tablets (Oxoid Ltd. BR0014G), AnaeroGen 2.5L sachet (Oxoid Ltd. AN0025A) were purchased from Fisher Scientific (United Kingdom). A slab of meat (4 kilograms extra-lean top round steak beef) was bought from a local butcher in Birmingham, United Kingdom. The vacuum food sealer rolls (width: 28 cm; length: 6 meters) were purchased from SousVideTools. The two stains propidium iodide (PI) and bis-(1,3-dibutylbarbituric acid) trimethaine oxanol [$DiBAC_4(3)$] or BOX were purchased from Sigma-Aldrich (United Kingdom).

2.2. Bacterial strain and culture preparation

The enterotoxigenic strain *Clostridium perfringens* NCTC 8238 (Hobbs serotype 2) was obtained from our culture collection and is an established model used in heat treatment studies (Juneja et al., 1994; Novak et al., 2001). This strain was originally isolated from boiled salt beef (Hobbs et al., 1953) and carries the alpha-toxin *cpa* gene and enterotoxin *cpe* gene but not the epsilon-toxin *etx* gene. *C. perfringens* was maintained on a slant of Columbia blood agar at 4°C. A colony from the slant was sub-cultured on TSC agar supplemented with egg yolk emulsion and incubated anaerobically at 37°C for 24 hours. A suspension of *C. perfringens* was prepared by

inoculating a colony from the subculture into 10 mL sterile BHIB incubated in anaerobic Genbox Jars with AnaeroGen sachets at 37°C for 24 hours. The resulting suspensions were used to make overnight cultures of *C. perfringens* in the stationary phase.

2.3. Artificial contamination of BHIB and meat with *C. perfringens*

For the BHIB study, a 0.2 mL inoculum (approximately 10^8 CFU/mL) from the overnight culture of *C. perfringens* was used to inoculate 2 mL of BHIB in 15 mL centrifuge tubes to a final concentration of 10^6-10^7 CFU/mL. For the meat study, the surface of the meat was trimmed aseptically using a sterile knife to reduce bacterial loads on surface of the meat. The whole meat was divided into cubes of 10 grams (thickness: 2 cm) and stored at -18°C. The meat cubes were left at ambient temperature overnight to thaw. A 0.2 mL inoculum (approximately 10^8 CFU/mL) from the overnight culture of *C. perfringens* was used to contaminate the meat in sous-vide vacuum pouches to a final concentration of 10^6-10^7 CFU/g. The sous-vide vacuum pouches (width: 20 cm ; length: 15 cm) were vacuum sealed using the XinBaoLong QH-01 electric vacuum sealer (Foshan Xin Bao Long Packing Machinery Co., Ltd., China). For non-vacuum sealing, the meat was placed in sous-vide vacuum pouches and sealed under atmospheric pressure. Culture concentrations were determined by the established relationship between optical density (OD) and colony forming units (CFU) to calculate the culture volume to obtain the required concentration of contaminated broth or meat (Loske et al., 2014).

2.4. Static thermal inactivation of *C. perfringens* in BHIB and meat

The effect of LTLT cooking was evaluated by heating the *C. perfringens* contaminated BHIB and meat at temperatures of 70°C, 60°C, 55°C, 53°C and 48°C.

The inactivation experiments were carried out by immersing the tubes or sous-vide vacuum pouches containing contaminated BHIB and meat, respectively, in a pre-heated Pasto sous-vide water bath (12 litres) (Grant, United Kingdom). Throughout the LTLT cooking process the temperature of the water bath was monitored using a thermometer (± 0.2°C). The temperature of the broth and meat (at centre) were validated by measuring an uncontaminated sample (tube with BHI and meat cube in a sous-vide pouch) during the process using a thermometer. Although the meat cubes were contaminated only at the surface, LTLT cooking guidelines require measuring the temperature on the inside of the meat to ensure it has reached a safe internal temperature (Stringer et al., 2012). The holding time started as soon as the pouches were placed in the water bath while the internal temperature of the beef cubes reached the cooking temperature within 12-15 minutes depending on the temperature. The time taken for the contaminated BHIB to reach the same temperature of the pre-heated water bath was about 5-7 minutes depending on the cooking temperature. During LTLT cooking, samples were removed at different time points of 0, 60, 120, 180, 240, 300, 360, 1440, 2160 and 2880 minutes and immediately placed in ice-water for 30 seconds to stop the cooking process. A volume of 5 mL of PBS (phosphate buffered saline) buffer solution was transferred to the sous-vide vacuum pouches and using a stomacher the meat was homogenized at 250 rpm for 1 minute to release and disperse the bacteria into PBS. The sous-vide vacuum pouches were massaged thoroughly to obtain an even distribution of bacteria prior to enumeration.

2.5. Enumeration of *C. perfringens* during LTLT cooking

Viable *C. perfringens* cell counts were made using serial dilutions in PBS buffer solution and 1 mL from each dilution was transferred to a Petri dish and mixed with

TSC agar supplemented with egg yolk tellurite emulsion and D-cycloserine (kept at 50°C) by using pour plate technique (Velliou et al., 2013). Once the agar solidified, the plates were incubated in anaerobic Genbox Jars with AnaeroGen sachets at 37°C for 24 hours. The black colonies were counted using a colony counter (CC-1 BOECO Germany) and expressed as colony forming units per milliliter (CFU/mL) in BHIB or per gram (CFU/g) in meat (Byrne et al., 2006; Byrne et al., 2008). Enumeration of viable *C. perfringens* in BHIB or meat at each temperature were carried out in triplicate.

2.6. pH measurements during thermal inactivation process

The pH of the BHIB was measured using a pH meter (Mettler Toledo FE20, Switzerland) by dipping the electrode inside the sample and pH was recorded to the nearest 0.1 pH unit.

2.7. Flow cytometry analysis of *C. perfringens* during thermal inactivation process

During LTLT cooking of BHIB, a 1 mL sample was collected at 0, 60, 120, 180, 240, 300, 360, 1440, 2160 and 2880 minutes and *C. perfringens* were stained with $DiBAC_4$ (3) or BOX (50 ng mL^{-1}) and PI (4 µl mL^{-1}) and incubated in the dark for 5 minutes. BOX is a membrane potential dye that can enter depolarized cells and binds to lipid rich surfaces giving a bright green fluorescence while PI is a membrane integrity dye that can enter cells with a compromised cell membrane and bind to nucleic acids giving a bright red fluorescence (Shapiro, 1995). Flow cytometry analysis was conducted using the Attune NxT flow cytometer (Thermo Fisher Scientific, UK) and samples were excited using a 488 nm solid state laser and particulate noise was eliminated using a Forward scatter height (FSC-H) threshold.

About 10,000 data points were collected, and fluorescence was detected using 530/30 BP (BL1 channel) and 620/15 BP (YL2 channel) filters corresponding to BOX and PI fluorescence respectively. The gate was placed around the unstained cell population on the multiparametric density plot (BL1 *vs* YL2) and percentages of injured and dead cells were determined after staining with BOX and PI which corresponds to an increase in fluorescence intensity at the BL1 and YL2 channels respectively. The data was analysed using Attune NxT software and each experiment was conducted in triplicate.

2.8. Recovery of *C. perfringens* from BHIB and meat after LTLT cooking

The ability of *C. perfringens* to grow after the thermal inactivation process was assessed at 360, 1440 and 2880 minutes. A 10 mL of fresh BHIB was inoculated with 0.5 mL of the thermally treated samples and incubated in anaerobic Genbox Jars with AnaeroGen sachets at 37°C for 24 hours. The growth of *C. perfringens* was determined by measuring the OD before and after incubation with BHIB.

2.9. Data processing and statistical analysis

The generated results were collected in Excel (Microsoft Corp.) for calculating means, standard deviations, and error bars. The thermal survival curves for *C. perfringens* were fitted by linear regression analysis to determine decimal reduction time (D-value). The D-value is the time required to inactivate 90% of the population and was calculated by linear regression analysis. This value is equivalent to negative inverse of the mean rate of decrease (slope) in the linear portion of the curves (D-value = -1/slope) (Wiegand et al., 2009). One-way analysis of variance (ANOVA) and the Tukey's HSD *post hoc* test was used to determine whether there was statistical

difference among samples using IBM SPSS Statistics software version 23 and differences were considered significant at $P < 0.05$.

3. Results

3.1. Effect of LTLT cooking on viability of *C. perfringens* in BHIB

The viability of *C. perfringens* in BHIB was monitored during LTLT cooking (Figure 1A-E). At 48°C, the viability of *C. perfringens* gradually decreased and was reduced by 3.9-, 3.3-, 3.8- and 4.4-log CFU/mL after 360, 1440, 2160 and 2880 minutes, respectively (Figure 1A). At 53°C, the viability of *C. perfringens* gradually decreased and was reduced by 1.8-, 2.3- and 4.3-log CFU/mL after 60, 120 and 180 minutes, respectively, but inactivation was higher than 6-log after 240 minutes (Figure 1B). At 55°C, the viability of *C. perfringens* was reduced by 4.7-log CFU/mL after 60 minutes but inactivation was higher than 6-log after 120 minutes (Figure 1C). At 60°C and 70°C, *C. perfringens* was inactivated (> 6D) after 60 minutes (Figure 1D and E). These results suggest that LTLT cooking at 48°C was insufficient to achieve a 6-log reduction of *C. perfringens*.

3.2. Effect of LTLT cooking on physiological status of *P. perfringens* in BHIB

Flow cytometry was employed to assess bacterial physiology during LTLT cooking of BHIB using the fluorescent dyes propidium iodide (PI) and $DiBAC_4$ (3) (BOX) to measure viability and membrane depolarization, respectively (Figure 1A-E). At 48°C, the percentage of PI positive cells gradually increased from 41.6% (N = 3, SD = 6.9) after 60 minutes to 85.4% (N = 3, SD = 8.3) after 2880 minutes (Figure 1A). Moreover, the percentage of BOX positive cells gradually decreased from 57.5% (N = 3, SD = 6.6) after 60 minutes to 14.6% (N = 3, SD = 7.8) after 2880 minutes

(Figure 1A). At 53°C and 55°C, the percentage of PI positive cells gradually increased and was > 90% after 300 minutes onwards (Figure 1B) while the percentage of BOX positive cells gradually decreased and was < 10% (Figure 1B) and < 5% (Figure 1C), respectively, after 300 minutes onwards. At 60°C and 70°C, the percentage of PI and BOX positive cells was > 95% and < 5%, respectively, after 60 minutes onwards (Figure 1D and E). These results correlate with viability data showing increased death with loss in viability.

3.3. pH measurements of BHIB during LTLT cooking

The pH was monitored during LTLT cooking of BHIB. At all temperatures the pH fluctuated slightly but remained relatively constant until the end of the cooking process (Figure S1). These results suggest an absence or a very low microbial activity during the duration of experiments, which is in line with the decrease in *C. perfringens* viability.

3.4. Effect of LTLT cooking on viability of *C. perfringens* in meat

The viability of *C. perfringens* in meat was monitored during LTLT cooking (Figure 2A-C). At 48°C, the viability of *C. perfringens* in vacuum and non-vacuum sealed meat remained constant in the first 180 minutes (Figure 2A). The viability of *C. perfringens* significantly increased by ~1-log after 240 minutes to remain constant until 360 minutes before it significantly decreased after 1440 2160 and 2880 minutes. There was no significant difference in viability of *C. perfringens* between both cooking methods up to 1440 minutes. On the contrary, the viability of *C. perfringens* was significantly higher in non-vacuum compared to vacuum sealed meat (2.1 *vs* 4.8-log) after 2880 minutes (Figure 2A). At 53°C, the viability of *C. perfringens* gradually decreased in the first 240 minutes and remained constant until

360 and 1440 minutes in vacuum and non-vacuum sealed meat, respectively (Figure 2B). There was no significant difference in viability of *C. perfringens* between both cooking methods in the first 360 minutes. Also, *C. perfringens* was inactivated (> 6D) after 1440 minutes in vacuum sealed meat while it was inactivated (> 6D) after 2880 minutes in non-vacuum sealed meat (Figure 2B). At 55°C, the viability of *C. perfringens* gradually decreased in the first 240 and 360 minutes in vacuum and non-vacuum sealed meat, respectively (Figure 2C). There was no significant difference in viability of *C. perfringens* between both cooking methods in the first 240 minutes. However, *C. perfringens* were inactivated (> 6D) after 300 minutes in vacuum sealed meat while it was inactivated (> 6D) after 1440 minutes in non-vacuum sealed meat (Figure 2C). At 60°C and 70°C, *C. perfringens* were inactivated (> 6D) after 60 minutes in both cooking methods (Figure S2). These results suggest that LTLT cooking at 48°C was insufficient to inactivate *C. perfringens* in meat and 55°C was more effective in inactivating *C. perfringens* compared to 53°C.

3.5. Decimal reduction time (D-value) of *C. perfringens* during LTLT cooking

The survival curves of *C. perfringens* by which the D-values were determined during cooking during LTLT cooking at 48°C, 53°C and 55°C in the first 6 hours are shown in Figure S3. The D-values of *C. perfringens* in BHIB and meat are shown in Table 1. In BHIB, the D-value at 48°C was the highest (D_{48} = 88.5 minutes) (n = 3, SD = 7.1) while the D-value at 55°C was lower than at 53°C; D_{55} = 18.0 minutes) (n = 3, SD = 0.1) while D_{53} = 38.3 minutes (n = 3, SD = 3.2). In meat, *C. perfringens* was able to grow at 48°C and therefore the D-value could not be calculated. In non-vacuum sealed meat, there was no difference in D-value at 55°C compared to 53°C; D_{55} =

82.7±5.2 minutes while D_{53} = 88.5±14.5 minutes. In vacuum sealed meat the D-value was lower at 55°C compared to 53°C; D_{55} = 50.8 minutes (n = 3, SD = 18.8) while D_{53} = 101.0 minutes (n = 3, SD = 18.5). At 55°C the D-value was lower in vacuum compared to non-vacuum sealed meat (50.8 *vs* 82.7 minutes). These results indicate that the D-value is reduced with increasing temperature and that the D-value is lower in BHIB compared to meat.

3.6. Detection of surviving *C. perfringens* after enrichment

The ability of *C. perfringens* to recover and grow after LTLT cooking was assessed. Regardless of the cooking method, *C. perfringens* was able to recover at 48°C after 360, 1440 and 2880 minutes while no recovery occurred at 60°C and 70°C. *C. perfringens* was unable to recover in BHIB at 53°C and 55°C. However, *C. perfringens* was able to recover at 53°C from non-vacuum sealed meat after 360 and 1440 minutes while it was able to recover from vacuum sealed meat after 360 minutes. Also, *C. perfringens* was able to recover at 55°C from non-vacuum sealed meat after 360 minutes while no recovery occurred from vacuum sealed meat.

4. Discussion

Time-temperature relationships during cooking is an important factor that can determine the growth and survival of pathogens in meat. This study was done to characterize growth and survival of *C. perfringens* in meat during LTLT cooking methods. BHIB or meat was contaminated with *C. perfringens* and their viability and physiological status (death/injury) were monitored over time during LTLT cooking at different temperatures. *C. perfringens* inocula were made of vegetative cells in the stationary phase, described as having the highest heat resistance (Byrne et al., 2002). Furthermore, the inoculum consisted mainly of vegetative cells observed by

microscopy. *C. perfringens* can exist as a vegetative cell or in the dormant spore form in food. Survival of *C. perfringens* spores during meat cooking is a big challenge to meat safety due to their high thermal resistance and ability to germinate in a wide range of temperatures (Akhtar et al., 2009). In this study spores may have formed during LTLT cooking which can germinate into new cells.

In BHIB, LTLT cooking at 48°C did not achieve the minimum destruction level, as required by the Food Standard Agency which states that a thermal treatment should cause ≥ 6-log reduction of vegetative pathogens (Stringer et al., 2012). The critical temperature range for growth of *C. perfringens* is approximately 10°C - 52°C (Doyle, 2002; Stringer et al., 2012). The lethal effect of an excessive cooking process even at 48°C on the survival ability of this bacteria was observed, but it was not sufficient to achieve the required 6-log reduction. However, the viability was below 4-log CFU/mL after 240 minutes onwards. The Food Safety and Inspection Service (FSIS) have stated that the worst reported case of meat contamination with *C. perfringens* was 10^4 CFU/g (Kalinowski et al., 2003; FSIS, 1999). Therefore, ≤ 4-log CFU/g of *C. perfringens* in raw meat and meat products is considered safe and acceptable.

Although it has been reported that *C. perfringens* can grow at temperatures up to 52^{o}C (Huang, 2016; Stringer et al., 2012) our results showed an inactivation at 48^{o}C in BHIB. This discrepancy may be attributed to various reasons including strain differences, growth media and growth conditions. In a study investigating the growth of vegetative cells of three different strains of *C. perfringens* in meat and meat products at constant temperatures of 49-51.6^{o}C it was found that the strain NCTC 8239 was inactivated at 49^{o}C while the strains 6867 and A91 were able to grow at that temperature (Hall and Angelotti, 1965). Roy et al. (1981) investigated the growth

of vegetative cells of two different strains of *C. perfringens* in autoclaved ground beef after growth at constant temperatures of 37^{o}C, 41^{o}C, 45^{o}C, or 49^{o}C and found that the strain NCTC 8798 was more thermal resistant than NCTC 8238. Moreover, the maximal growth temperature of *C. perfringens* type A isolates carrying a chromosomal enterotoxin gene (*cpe*) ranged from 52 to 54^{o}C while *C. perfringens* type A isolates carrying a plasmid *cpe* gene ranged from 48 to 51^{o}C (Li and McClane, 2006). The thermal resistance of *C. perfringens* also depends on the growth medium. *C. perfringens* showed an increase in heat resistance with reduction of the lag phase and generation time when grown in autoclaved ground beef (Willardsen et al.,1978) and beef roasts (Smith et al., 1981) compared to Fluid Thioglycollate medium, a synthetic medium commonly used to evaluate growth of anaerobic microorganisms. Moreover, *C. perfringens* growth during thermal treatment is enhanced in meat in comparison to tryptic soy broth (Smith et al., 2004). *C. perfringens* is a strictly anaerobic bacterium, unable to survive in the presence of oxygen for too long (Briolat and Reysset, 2002). In this study LTLT cooking in BHIB was conducted under aerobic conditions, therefore, the growth conditions may not be optimal for *C. perfringens* as it can cause oxygen stress which impedes its growth (Huang, 2016). Willardsen et al. (1979) reported that the survival and growth of *C. perfringens* was enhanced in autoclaved ground beef in comparison to raw ground beef since autoclaving before conducting thermal treatments results in expulsion of the oxygen present and generation of reducing substances.

At 53°C the required 6-log reduction was achieved after 240 minutes and the viability of *C. perfringens* was below 4-log CFU/mL after 180 minutes. There is a gap in scientific knowledge on growth of *C. perfringens* at temperatures between 40°C - 55°C (Stringer et al, 2012). The United States Department of Agriculture recommend

that the lowest cooking temperature is at 54.4°C for 112 minutes in its Appendix (A) (USDA, 1999). Stringer et al. (2012) has reported that *C. perfringens* are able to grow at 52°C and using ComBase database they estimate that the maximum growth rate of *C. perfringens* at 52°C is greater than 1 log unit per hour. They suggest that meat should not be heated at less than 52°C for more than 60 minutes since minimally processed food is at risk of its surviving vegetative cells, such as LTLT cooking of meat (Stringer et al., 2012). Moreover, there is no scientific data on potential growth of *C. perfringens* at temperatures between 52°C and 55°C and even though ComBase database has thermal survival curves of *C. perfringens* in culture medium, there is no data on thermal treatment at 53°C (FSA, 2013). Furthermore, inactivation data for thermal treatments at 55°C were for short time (< 60 minutes) (Stringer et al., 2012). In this study, *C. perfringens* showed higher survival during LTLT cooking at 53°C compared to 55°C. At 55°C the required 6-log reduction was achieved after 120 minutes and the viability of *C. perfringens* was below 4-log CFU/mL after 60 minutes. In a study by Vajda (2016), cooking at 55°C reduced the viability of *C. perfringens* by > 7-log CFU/mL in RCM (Reinforced Clostridial Medium) broth after 80 minutes. Cooking at a temperature of 55°C is accepted by the UK Food Standards Agency which states that safe meat can be achieved by cooking at low temperatures of 55°C - 69°C and the treatment should be equivalent to the thermal process at 70°C for 2 minutes or 60°C for 45 minutes at the coldest point of meat (FSA, 2013).

As expected, *C. perfringens* was rapidly inactivated (> 6D) at 60°C and 70°C. Temperatures above 60°C will generally inactivate vegetative cells (Doyle, 2012). It was shown that thermal treatment at 60°C and 65°C results in > 7-log reduction of *C. perfringens* in RCM broth after 45 minutes and 4 minutes, respectively (Vajda, 2016).

These results corroborate with a study by Byrne et al. (2006) showing that *C. perfringens* is inactivated in pork meat at 60°C (D_{60} = 8.5 minutes) and 65°C (D_{65} = 0.8 minutes).

The inoculum of *C. perfringens* before LTLT cooking showed a high percentage of BOX positive cells ranging from 70% to 80%. During LTLT cooking of BHIB the percentage of dead cells increased as the viability decreased. Also, there was an inverse correlation between the percentage of PI and BOX positive cells which can be attributed to the physiological changes that occur in *C. perfringens* as it transitions from injury to death during LTLT cooking. During LTLT cooking of BHIB at 48°C, the percentage of dead cells was highest after 1440 and 2880 minutes and the percentage of sub-lethally injured cells were 14% and 15% after 1440 and 2880 minutes, respectively. Sub-lethally injured cells have the ability return to their normal state during a resuscitation period (Busta, 1976). When no colonies of *C. perfringens* were observed on agar plate the percentage of dead and injured cells at 53°C was 81.2% and 17.5%, respectively, while the percentage of dead and injured cells at 55°C was 81.2% and 17.1%, respectively. Moreover, it may be possible that viable but non-culturable (VBNC) cells may have formed and exist within the injured cell population. Stress can induce the VBNC state in bacteria to become highly resistant to a variety of stresses (e.g. thermal treatment) and can resuscitate back to the actively metabolising state (Ayrapetyan and Oliver, 2016). In contrast, when no colonies of *C. perfringens* were observed on agar plate the percentage of dead cells at 60°C and 70°C was > 95% after 60 minutes confirming the immediate lethal effects of such temperatures on *C. perfringens*.

In meat, the viability of *C. perfringens* followed a similar trend as in BHIB. At 48°C, *C. perfringens* were able to grow during LTLT cooking in the first 360 minutes. Survival of *C. perfringens* was higher in non-vacuum compared to vacuum sealed meat showing that packaging quality and adherence of cooking bags may affect heat transfer, and therefore slow down the inactivation kinetics of micro-organisms. Moreover, LTLT cooking at 48°C was not sufficient to achieve the required 6-log reduction. *C. perfringens* can grow in different meats at temperatures ranging from 15°C to 51°C, with reported generation times as short as 8.5 minutes (Doyle, 2002). These results corroborate with modelling data showing that *C. perfringens* cells are able to grow and proliferate in medium cooked at 48°C (ComBase, 2015). According to ComBase model estimation created by Stringer and Metris (2018), sous-vide cooking at 49.5°C for 228 minutes causes an increase in cells of *C. perfringens* by 2.9-log.

Regardless of the cooking method, it took longer time to achieve the required 6-log reduction at 53°C compared to 55°C. Although at 53°C the required 6-log reduction was achieved in vacuum sealed meat after 1440 minutes, this thermal treatment may not be widely acceptable since the maximum cooking time at temperatures considered in the “danger zone” is 240 minutes (NSWFA, 2015). Duan et al. (2016) found that LTLT cooking at 53°C inactivated thermally adapted *C. perfringens* in pork and chicken meat and reduced the counts below detection level within 600-1800 minutes depending on the length of the adaptation period. In contrast, Stringer and Metris (2018) reported that LTLT cooking at a temperature of 53°C allows incubation and/or growth rather than inactivation of *C. perfringens*. However, this discrepancy may be attributed to strain differences of *C. perfringens* that cause them to respond differently to thermal treatment (Sarker et al., 2000).

It is shown here that safe meat can be obtained through sous-vide cooking at 55°C for 300 minutes. The cooking time established by Baldwin (2012) at 55°C for meat in order to achieve a 6-log destruction of vegetative pathogens varies from 213 to 460 minutes according to the thickness of meat. In this study, *C. perfringens* showed > 6-log reduction in a cooking time as short as 60 minutes at 60°C and 70°C. In a study by Smith et al. (1981), LTLT cooking at 60°C resulted in a 3-log and 12-log reduction of *C. perfringens* in beef after 12 minutes and 150 minutes, respectively. At these temperatures, vacuumed and non-vacuumed pouches showed no difference in terms of microbiological safety of the meat.

As expected, D-values decreased with increasing temperatures. In BHIB, the D-value at 55°C was 18.0 minutes (n = 3, SD = 0.1) which is comparable to D-values obtained in literature for RCM broth (D_{55}= 11.7 minutes) and pork meat (D_{55}= 16.3 minutes) (Byrne et al., 2006, Vajda, 2016). In vacuum sealed meat, the D-value was 50.8 minutes (n = 3, SD = 18.8) which is comparative to D-value found for sous-vide cooked marinated chicken (D_{55} = 47.4) (Juneja, 2006). Regardless of cooking temperature, the D-value in meat was higher compared to BHIB. Pathogenic bacteria express more thermal resistance on meat surface than broth which may increase the survival chances of bacteria at temperatures above 55°C (Velliou et al., 2013). Also, the presence of fat in meat can increase resistance of cells to heat. It has been reported that pathogens express more thermotolerance in media with high fat content, and pathogenic cells attached to the surface of meat are more resistant to cooking than dispersed cells (Lahou et al., 2015). Moreover, it is unlikely that fat resulted in heat resistance in this study since the meat used was an extra-lean top round steak beef.

In this study, *C. perfringens* was able to recover after LTLT cooking of meat or BHIB at 48°C. At 53°C, no recovery occurred after LTLT cooking in BHIB. *C. perfringens* was able to recover after 360 and 1440 minutes from vacuumed and non-vacuumed pouches, respectively. One of the most important characteristic of *C. perfringens* is its ability to rapidly reach pathogenic level during prolonged cooling of contaminated food. Juneja et al. (1994) observed a 4-5-log CFU/g increase in *C. perfringens* during the 1080 minutes cooling of meat after cooking. In this study, the ability to recover correlates with viability results suggesting that some of the sub-lethally injured cells may recover. According to Doyle (2002), LTLT cooking of meat is not sufficient to destroy *C. perfringens* spores. Akhtar et al. (2009) demonstrated that *C. perfringens* spores are able to germinate at slow cooking temperatures of 55°C, 60°C and 65°C which are lethal for vegetative cells. However, spore germination is a complex process and activation temperature of spores depends on the strain of *C. perfringens* and can range from 65 – 100°C while only 0.13 – 3.6% of spores from *C. perfringens* strains associated with food poisoning germinate without heat activation (Doyle 2002).

5. Conclusion

Currently, there are insufficient data in the literature on microbiological safety of LTLT cooked meat at temperatures below 55°C. The survival and growth of *C. perfringens* during LTLT cooking was investigated and the time-temperature combinations to reduce the level of *C. perfringens* in meat to safety levels have been determined. The findings have revealed that a temperature of 48°C was insufficient to achieve the required 6-log reduction of *C. perfringens*. Although a temperature of 53°C was able to achieve the required 6-log reduction it may not be acceptable since the time that was needed exceeds the maximum cooking time acceptable in the

"danger zone". At a temperature of 55°C the required log reduction of *C. perfringens* was prolonged in non-vacuumed pouches which suggests that improper or insufficient vacuum sealing may lead to a compromise in meat safety. Moroever, in real life situations, bacteria may be exposed to variuos stresses (e.g. heat shock caused by the gradual increase of temperature in the water bath) that can increase their thermal resistance. Also, strain difference in *C. perfringens* exist, and each may possess different thermal resistance. Therefore, other strains will be studied in the future to compare the heat resistance amongst the different strains of *C. perfringens.* It is also important to note that the results of this study were based only on surface contamination of meat only while contamination inside meat is not uncommon (Gill, 1980).

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Captions

Figure 1. The viability in log CFU/mL and physiological status (death/injury) results from flow cytometry analysis of *C. perfringens* during slow cooking of broth at (A) 48°C, (B) 53°C and (C) 55°C, (D) 60°C and (E) 70°C. The left-side Y-axis shows the percentage of cells and the data is represented as lines; the right-side Y-axis shows the viability in log CFU/mL and the data is represented as bars; the X-axis represents the slow cooking time in minutes. The viability and physiological status were monitored at 0, 60, 120, 180, 240, 300, 360, 1440, 2160 and 2880 minutes. Flow cytometry analysis reveals the percentage of PI (red) and BOX (green) positive cells, indicative of cell death and injury respectively. Bars represent mean ± SD

taken from a minimum of 3 independent experiments. The data were analysed with one-way ANOVA.

Figure 2. The viability in log CFU/g of *C. perfringens* during sous vide and slow cooking of raw meat at (A) 48°C, (B) 53°C and (C) 55°C. The viability was monitored at 0, 60, 120, 180, 240, 300, 360, 1440, 2160 and 2880 minutes. In sous-vide cooking the pouches were vacuum sealed. Bars represent mean ± SD taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P < 0.05$). The data were analysed with one-way ANOVA.

Table 1. Decimal reduction times (D-values) of survival curves for *C. perfringens* in BHIB, non-vacuum and vacuum sealed meat during LTLT cooking at 48°C, 53°C and 55°C. Results represent mean ± SD taken from a minimum of 3 independent experiments.

Figure S1. The pH values of broth during slow cooking at 48°C, 53°C, 55°C, 60°C and 70°C. The pH was monitored at 0, 60, 120, 180, 240, 300, 360, 1440, 2160 and 2880 minutes. Bars represent mean ± SD taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P < 0.05$). The data were analysed with one-way ANOVA.

Figure S2. The viability in log CFU/g of *C. perfringens* in vacuum sealed meat and non-vacuum sealed meat during LTLT cooking at (A) 60°C and (B) 70°C. The viability was monitored at 0, 60, 120, 180, 240, 300, 360, 1440, 2160 and 2880 minutes. Bars represent mean ± SD taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P < 0.05$). The data were analysed with one-way ANOVA.

Figure S3. Thermal survival curves for *C. perfringens* during slow cooking of (A) broth, (B) non-vacuum sealed meat and (C) vacuum sealed meat at 48°C, 53°C and 55°C.

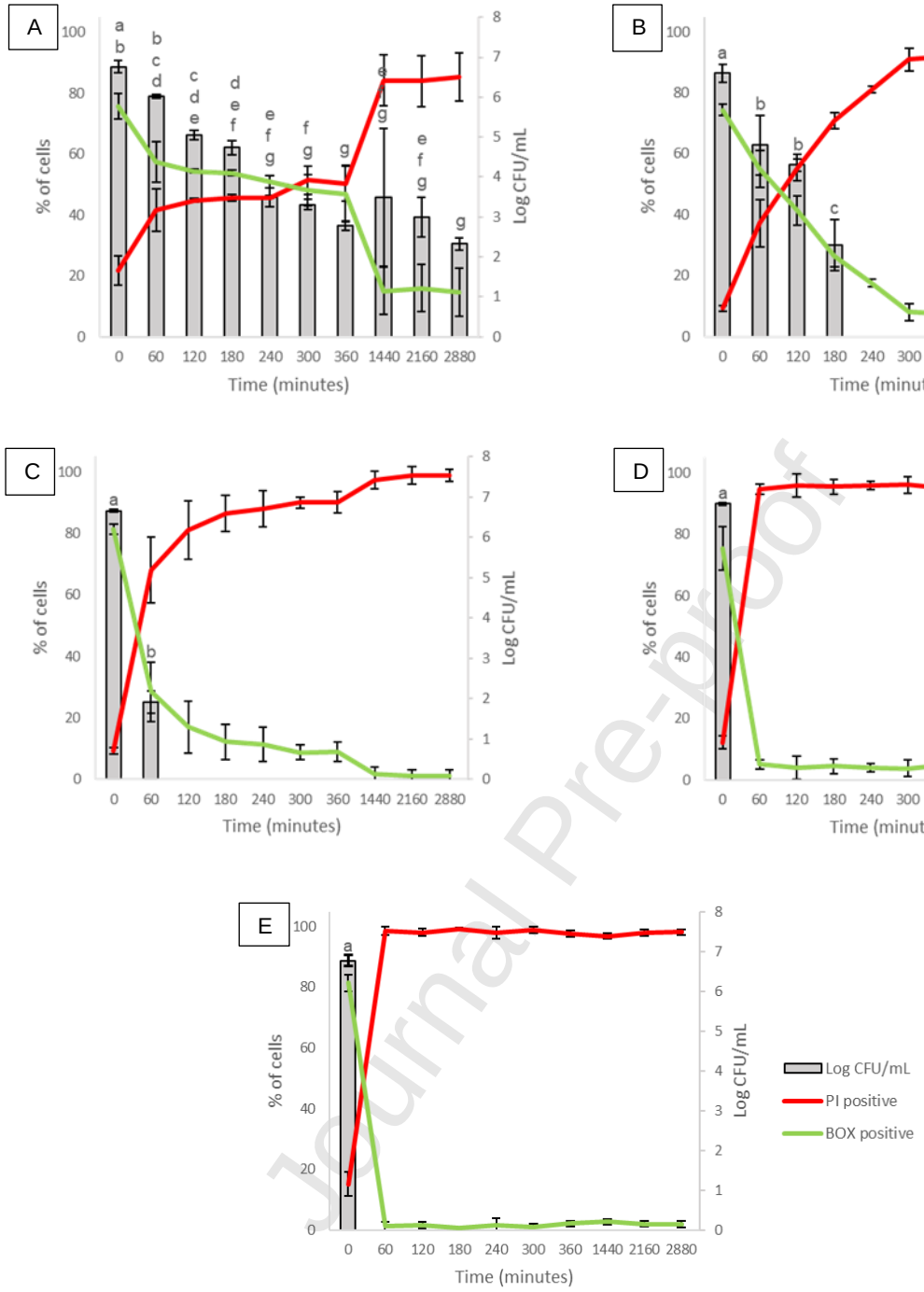

Figure 1. The viability in log CFU/mL and physiological status (death/injury) results from flow cytometry analysis of *C. perfringens* during slow cooking of broth at (A) 48°C, (B) 53°C and (C) 55°C, (D) 60°C and (E) 70°C. The left-side Y-axis shows the percentage of cells and the data is represented as lines; the right-side Y-axis shows the viability in log CFU/mL and the data is represented as bars; the X-axis represents the slow cooking time in minutes. The viability and physiological status were monitored at 0, 60, 120, 180, 240, 300, 360, 1440, 2160 and 2880 minutes. Flow cytometry analysis reveals the percentage of PI (red) and BOX (green) positive cells, indicative of cell death and injury respectively. Bars represent mean ± SD taken from a minimum of 3 independent experiments. Mean

values with different letters are significantly different ($P < 0.05$). The data were analysed with one-way ANOVA.

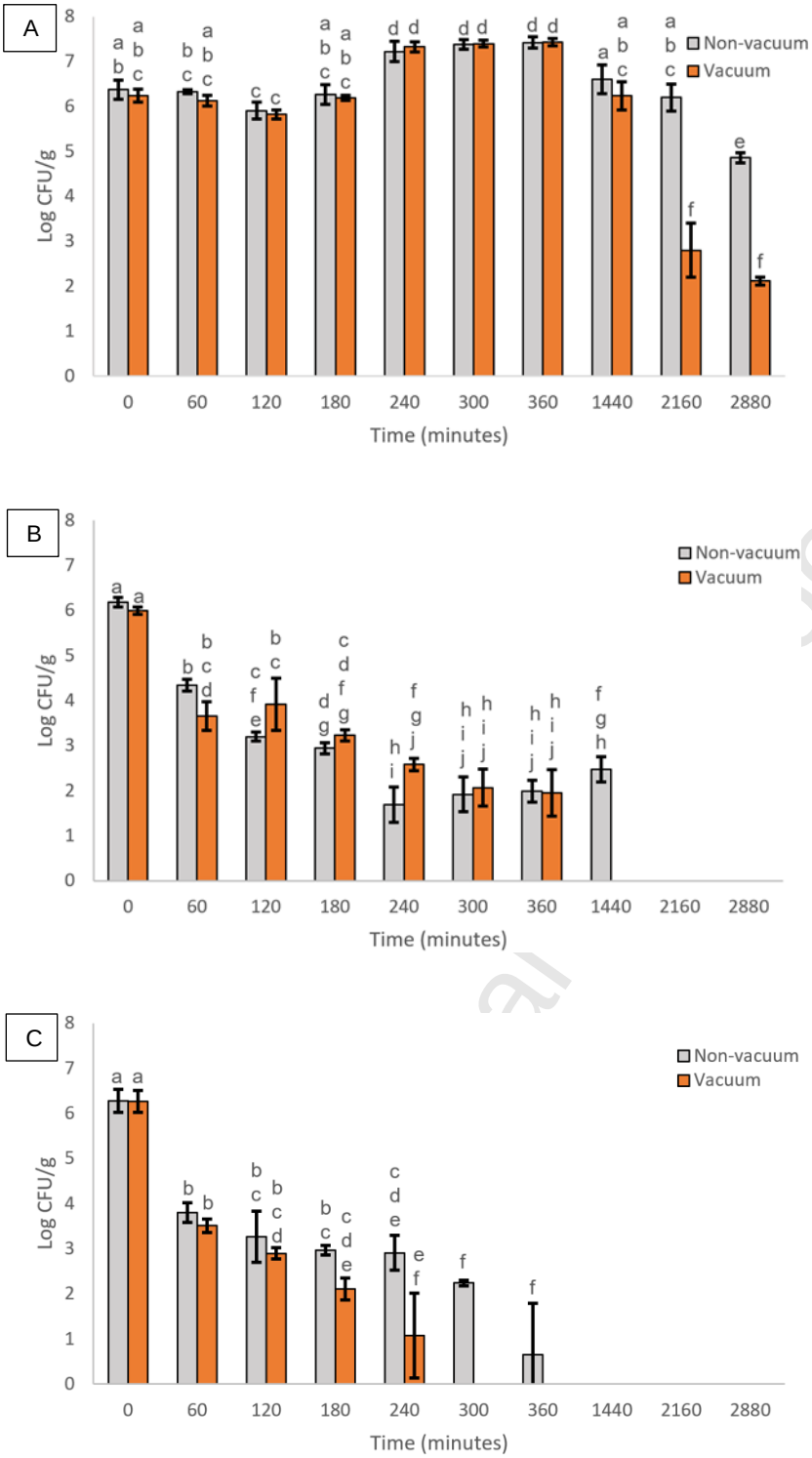

Figure 2. The viability in log CFU/g of *C. perfringens* during sous-vide and slow cooking of raw meat at (A) 48°C, (B) 53°C and (C) 55°C. The viability was monitored at 0, 60, 120, 180, 240, 300, 360, 1440, 2160 and 2880 minutes. In sous-vide cooking the pouches were vacuum sealed. Bars represent mean ± SD taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P < 0.05$). The data were analysed with one-way ANOVA.

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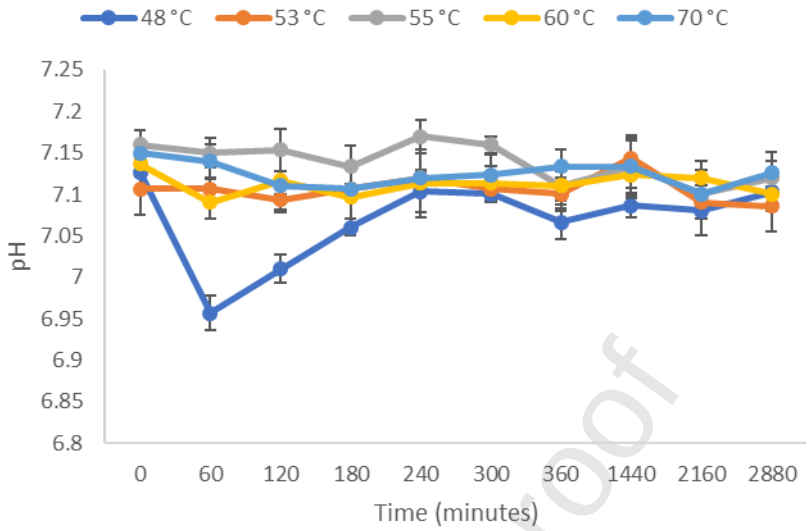
Temperature (^{o}C)	D-values (minutes)		
	BHIB	Non-vacuum sealed meat	Vacuum sealed meat
48	88.5 ± 7.1	n/a	n/a
53	38.3 ± 3.2	88.5 ± 14.5	101.0 ± 18.5
55	18.0 ± 0.1	82.7 ± 5.2	50.8 ± 18.8

Supplementary material



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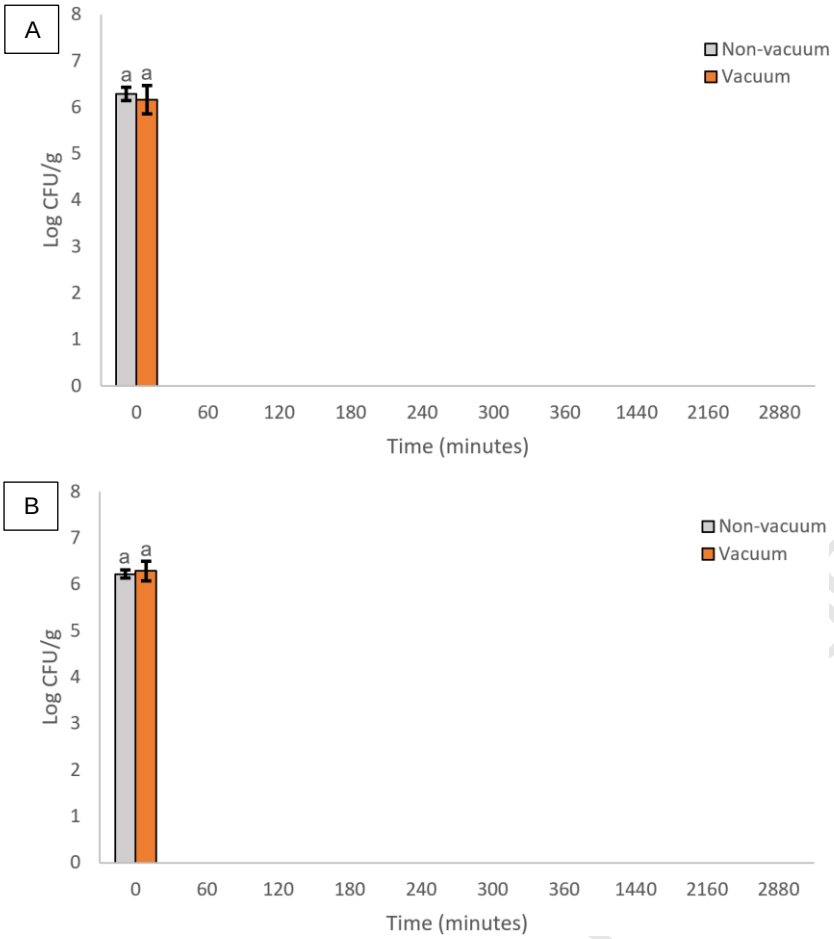

Figure S2. The viability in log CFU/g of *C. perfringens* in vacuum sealed meat and non-vacuum sealed meat during LTLT cooking at (A) 60°C and (B) 70°C. The viability was monitored at 0, 60, 120, 180, 240, 300, 360, 1440, 2160 and 2880 minutes. Bars represent mean ± SD taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P < 0.05$). The data were analysed with one-way ANOVA.

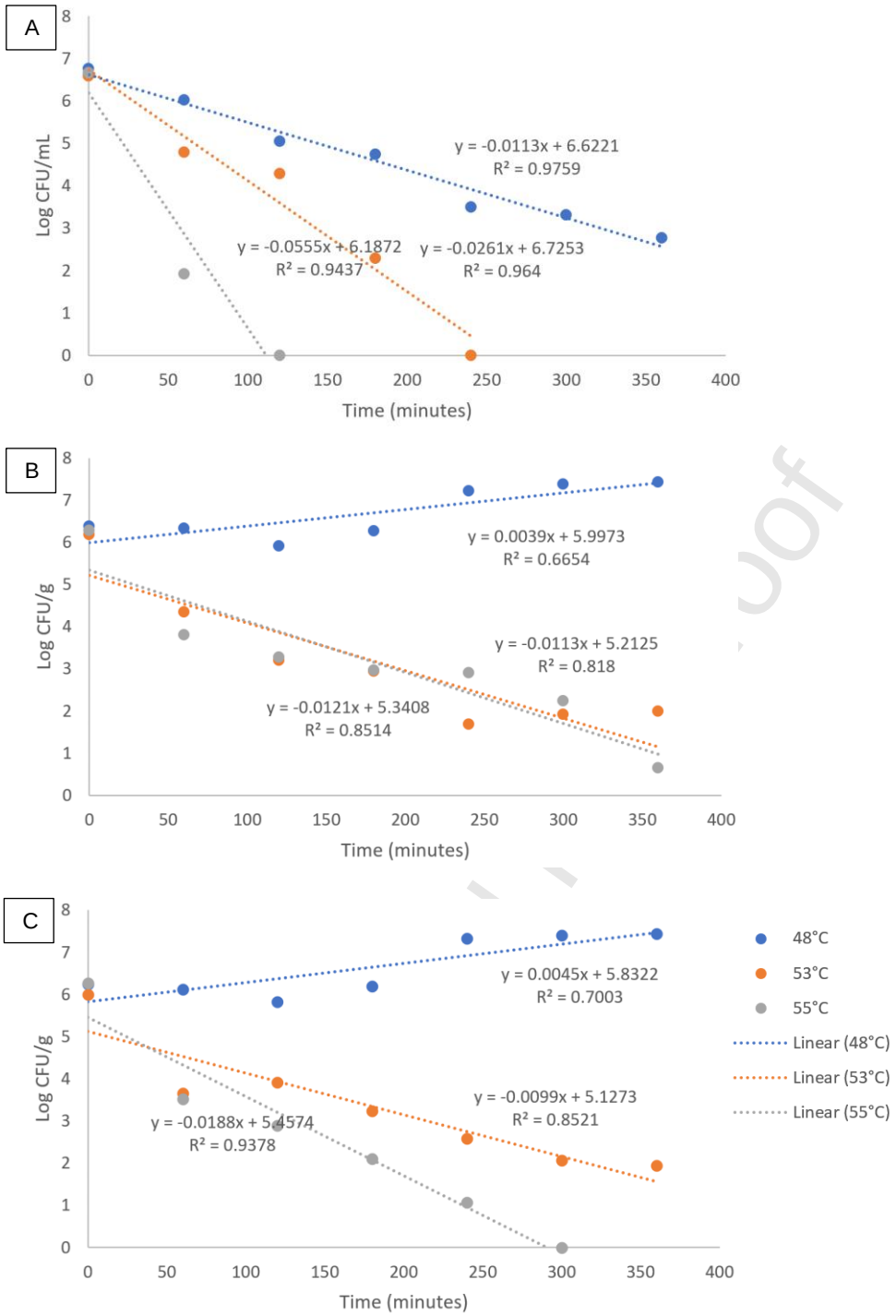

Figure S3. Thermal survival curves for *C. perfringens* during slow cooking of (A) broth, (B) non-vacuum sealed meat and (C) vacuum sealed meat at 48°C, 53°C and 55°C.