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Thermal inactivation profiles of *Mycobacterium avium* subsp. *paratuberculosis* in lamb skeletal muscle homogenate fluid

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ABSTRACT

Mycobacterium avium subsp. *paratuberculosis* (*MAP*) causes Johne's disease in livestock and there is a debate about its role in humans in chronic inflammatory bowel disorders such as Crohn's disease, but the relationship remains unproven. Nevertheless livestock health authorities in many countries aim to lower the prevalence of this infection to reduce potential contamination of the human food supply. *MAP* may occur in bovine milk and data on thermal inactivation suggest pasteurisation is an effective process. Recently *MAP* has been identified in skeletal muscle of cattle and sheep but there are no data on its thermal inactivation in these substrates. In this study the inactivation of *MAP* was studied in a fluid homogenate of lamb skeletal muscle at temperatures previously identified as being relevant to cooking processes applied by domestic consumers. A PCR thermocycler was used to ensure accurate temperatures and rapid heat exchange, while radiometric culture was used to ensure sensitive detection of viable *MAP* for determination of *D* and *z* values. Among the two predominant strains of *MAP*, S and C, D_{55} ranged from 56 to 89 min, D_{60} was 8 to 11 min, D_{65} was 26 to 35 s while D_{70} was 1.5 to 1.8 s. Values for *z* were 4.21 C° for the S strain and 4.51 C° for the C strain. At temperatures of 65–70 °C, *MAP* appeared to be less heat tolerant in skeletal muscle fluid than in previous reports using milk as the medium. The total thermal exposure of *MAP* during baking of a sample of 16 leg-of-lamb roasts in domestic ovens was determined to result in more than 20 log reductions in most cases, that is the product was microbiologically safe. Based on the models used in this study, there is a low probability of survival of *MAP* provided that red meat is cooked to recommended standards.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (*MAP*) causes Johne's disease in livestock. Macrophages laden with mycobacteria accumulate in the lamina propria of the intestine of affected animals and *MAP* are progressively shed, sometimes in massive numbers, into the faeces. The organism is an obligate parasite of animals, but can survive for considerable periods in the environment (Whittington et al., 2004). There are two distinct strain groupings, S which predominates in sheep in some countries and C which infects cattle and many other species. The strains are distinguished by substantial genomic insertions and deletions which confer differences in cell wall properties and other phenotypic characteristics (Alexander et al., 2009; Marsh et al., 2006). There is a long-standing debate about the role this organism may play in humans in chronic inflammatory bowel disorders such as Crohn's disease, but the relationship remains unproven. Nevertheless, livestock health authorities in many countries have taken steps to lower the prevalence of this infection and reduce the level of potential contamination of the human food supply.

In addition to infection of the intestine, *MAP* often occurs in extra-intestinal sites in cattle, with dissemination via blood and the lymphatic system (Barrington et al., 2003; Buergelt et al., 2004; Buergelt and Williams, 2004; Koenig et al., 1993). The organism has been found in lymph nodes, liver, spleen, semen, testes, epididymis, seminal vesicle and other parenchymous organs (Ayele et al., 2004; Barrington et al., 2003; Pavlik et al., 2000). There is an extensive literature on the presence of *MAP* in bovine milk [for example see (Ellingson et al., 2005)]. There have been similar findings in tissues, blood and milk of sheep, goats, wild ruminants and primates (Djonne et al., 2003; Eppleston and Whittington, 2001; Gwȼzdz et al., 1997; Gwozdz et al., 2000; Juste et al., 2005; Lambeth et al., 2004; McClure et al., 1987; Morin, 1982; Naser et al., 2000; Reddy et al., 1984; Williams et al., 1983a; Williams et al., 1983b). As Johne's disease clearly has a systemic component, the developing fetus is at high risk of infection and there are reports of congenital infection in many species (Alinovi et al., 2009; Whittington and Windsor, 2009). Most of the evidence points to

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Table 1
D values reported for *MAP* in milk or lactate solution.

Strain	Method[a]	Media	Inoculum level/ml	Temp (°C)	*D* value[b]	Reference
ATCC19698	ST	Milk	10^6	62	119.9 s	Sung and Collins (1998)
ATCC19698	ST	Milk	10^6	65	70.6 s	Sung and Collins (1998)
ATCC19698	ST	Milk, lactate	10^6	68	22.8–23.8 s	Sung and Collins (1998)
ATCC19698	ST	Milk	10^6	71	16.5 s	Sung and Collins (1998)
ATCC19698	ST	Lactate	10^6	62	324.6 s	Sung and Collins (1998)
ATCC19698	ST	Lactate	10^6	65	55.7 s	Sung and Collins (1998)
ATCC19698	ST	Lactate	10^6	71	13.0 s	Sung and Collins, (1998)
ATCC 43015 (c)[c]	CT	Milk	10^6 to 10^7	60–63	2.6–11.0 min	Keswani and Frank (1998)
ATCC 43015(d)	CT	Milk	10^6 to 10^7	60–63	1.6–11.5 min	Keswani and Frank (1998)
Bovine isolate 62 (c)	CT	Milk	10^6 to 10^7	60–63	2.1–8.6 min	Keswani and Frank (1998)
Bovine isolate 62 (d)	CT	Milk	10^6 to 10^7	60–63	1.8–8.2 min	Keswani and Frank (1998)
Bovine isolate 785991(c)	CT	Milk	10^6 to 10^7	60	2.9–8.6 min	Keswani and Frank (1998)
Bovine isolate 785991(d)	CT	Milk	10^6 to 10^7	60	2.5–14.1 min	Keswani and Frank (1998)
ATCC 19698, human and bovine field isolates	P	Milk	10^2 to 10^3	63	10.8–26.2 s	Pearce et al. (2001)
ATCC 19698, human and bovine field isolates	P	Milk	10^2 to 10^3	66	4.4–7.6 s	Pearce et al. (2001)

[a] ST, sealed tube in water bath; CT, capillary tube in water bath; P, pilot-scale pasteurizer.
[b] min: minutes; s: seconds.
[c] c: clumped; d: declumped.

extra-intestinal spread occurring more often in advanced sub-clinically or clinically affected animals compared to animals with early stage infection.

Various species of non-tuberculosis mycobacteria may be detected in meat (Shitaye et al., 2009) but there have been few surveys for *MAP*. Negative results were obtained in the USA from 200 ground beef samples that were tested using PCR (Jaravata et al., 2007). The organism was recovered from prescapular and popliteal lymph nodes, but not from two skeletal muscles from several infected cows in the USA (Antognoli et al., 2008). In 2009, isolation of *MAP* was reported for the first time from skeletal muscle; diaphragm samples from 13% of 47 cattle from Spain were culture positive; the six affected animals all had the severe multibacillary pathological form of the disease and four had clinical signs of paratuberculosis (Alonso-Hearn et al., 2009). In a concurrent study from Australia, *MAP* was found in meat of sheep at a concentration of $10^{0.7}$ to $10^{1.4}$ viable cells/g and in peripheral lymph node at $10^{1.4}$ to $10^{1.8}$ viable cells/gram; there were similar findings in a small number of cattle (Reddacliff, unpublished data). In experiments in which *MAP* was introduced into the blood stream of normal sheep, the organisms were distributed to most tissues including skeletal muscle in 48 h (unpublished data). Levels of *MAP* of 10^6 to10^8 per gram faeces are commonly seen in advanced cases of Johne's disease (Jorgensen, 1982; Whittington et al., 2000). Therefore faecal contamination of the surface of a carcase during processing may lead to contamination of the cut surface of red meat. Consequently, *MAP* DNA was detected using PCR in swabs taken from the anal region of some bovine carcases (Meadus et al., 2008) and the organism was grown from hides and carcasses in another study (Wells et al., 2009).

In a recent critical review (Eltholth et al., 2009), no primary studies were found on the efficacy of food processing on the viability of *MAP* in meat products. However, considerable research has been conducted to determine the thermal inactivation parameters for the C strain of *MAP* that are relevant to pasteurisation of milk (Chiodini and Hermon-Taylor, 1993; Grant et al., 1996; Hammer et al., 2002; Stabel et al., 1997; Sung and Collins, 1998). *D* values have been reported less often than the time required for complete inactivation of a defined starting amount of *MAP* (Keswani and Frank, 1998; Sung and Collins, 1998). *D* value is very useful because it enables an estimate to be made of the time required for inactivation of 1 log of an organism but can be extended to any starting amount of microorganism at a given temperature. In milk, 1 log of the C strain of *MAP* can be inactivated in about 15 s at 70 °C (Table 1). Recently, using a PCR thermocycler to ensure accurate temperatures, and thin-walled plastic tubes to ensure rapid heat transfer, Gumber found that 10^6 *MAP* were inactivated in less than 1 min in phosphate buffered saline at 70 °C (Gumber and Whittington, 2009).

As the thermotolerance of microorganisms is generally related to the medium and as there are no data on the survival of *MAP* in skeletal muscle during heating, the aim of this study was to determine the thermal conditions required to inactivate *MAP* in a substrate derived from red meat. Temperature profiles were previously determined in 16 leg-of-lamb roasts during baking in domestic ovens (unpublished). Maximum core temperatures ranged from 59.6 to >70 °C. This informed the design of the present study, in which the inactivation of *MAP* in a matrix of muscle homogenate fluid was studied over a range 55 to 75 °C, which also encompassed the United States Department of Agriculture (USDA) Food Safety Inspection Service (FSIS) guideline target internal temperature of 71.1 °C for microbiological safety (http://www.fsis.usda.gov/Frame/FrameRedirect.asp?main=http://www.fsis.usda.gov/OPPDE/rdad/FRPubs/95-033F/95-033F_Appendix_A.htm). Both the C strain and the S strain of *MAP* were included in this study.

2. Materials and methods

2.1. Preparation of lamb skeletal muscle homogenate fluid

Two aliquots of 100 g of lean fresh lamb meat, each mixed with 400 ml of sterile saline, were homogenised in a food blender, filtered through a sterile cotton cloth and a 100 to 150 μm mesh filter. The filtrate was allowed to settle for 30 min and was clarified by centrifugation at 2800×*g* for 20 min. The supernatant was passed through an 8 μm filter then sterilised by passing through a 0.2 μm filter. The lamb skeletal muscle homogenate fluid was stored at 4 °C until required for use the next day.

2.2. Isolates and cultivation of MAP

The cultivation of *MAP* in modified BACTEC medium and identification of the organism using IS*900* PCR was conducted as described previously (Whittington et al., 1999). *In vitro* grown *MAP* S strain Telford 9.2, a pure culture defined by RFLP analysis and IS*1311* PCR-REA was used. A lyophilized seed stock was reconstituted in water, inoculated into modified BACTEC 12B medium, incubated for 3 weeks, then subcultured to modified Middlebrook 7H10 agar and incubated for 4 weeks. Colonies from 40 slopes were harvested in 1 ml of phosphate buffered saline (PBS) with 0.1%v/v Tween 20 (PBST), vortexed for 1 min to remove clumps, diluted 1:10 in PBST, passed through a 26 g needle, filtered through an 8 µm filter and examined microscopically at ×400 magnification to confirm that the suspension consisted predominantly of single cells; the suspension was dispensed in 1 ml aliquots and frozen at −80 °C. Prior data indicated that the viable cell count is maintained when the frozen suspension is thawed. A parallel culture of *MAP* strain CM00/016, which is a field isolate of a C strain, non-clonal, was also prepared using the same method, except that slopes were incubated for 6 weeks. The approximate concentration of these suspensions was 10^7 cells per ml, estimated using microscopy.

2.3. Enumeration of viable MAP

The viable cell count was determined by end point titration in BACTEC 12B medium using two methods. The first (Method 1) was a standard most probable number method (Australian-Standard, 1991). Briefly, a single ten fold dilution series of the parent suspension was prepared from 10^0 to 10^{-10}. An aliquot of 50 µl of each dilution was added to each of three BACTEC 12B vials. The viable count per 50 µl was calculated as the MPN index read from standard three tube MPN tables multiplied by the dilution factor. In Method 2, three separate 10 fold dilution series were prepared as above, and 50 µl of each was inoculated into a single BACTEC 12B vial. MPN was determined from 3 tube tables as above. For both methods, the dilution series were prepared using both PBS and lamb skeletal muscle homogenate fluid as diluent media. All BACTEC vials were incubated at 37 °C and were read weekly to twice weekly for up to 12 weeks to determine growth index (GI).

In addition, for method 2, after growth was first detected, daily growth readings were taken for up to 59 days. GI was summed across days and the time in days required for the cumulative GI (CGI) to reach 1000 (CGI1000) was estimated. The number of days to CGI1000 is correlated with inoculum size (Reddacliff et al., 2003). A standard curve with 95% confidence limits was generated using simple least squares linear regression of log10 viable count and days to CGI1000 using Genstat (GenStat Release 10.1 2007, Lawes Agricultural Trust, Rothamsted Experimental Station). Viable counts of *MAP* in suspensions following heat treatment were predicted from the appropriate strain-specific standard curve (Figs. 1 and 2).

2.4. Complete inactivation of 5 logs of MAP

To determine conditions that are required for the complete inactivation of 5 logs of *MAP*, suspensions of *MAP* were diluted in lamb skeletal muscle homogenate fluid to final viable concentrations of 1.5×10^5 and 5.8×10^5 *MAP* per 100 µl for the S strain and the C strain, respectively. Triplicate 100 µl aliquots of suspensions were placed in thin-walled 200 µl capped plastic tubes in a PCR thermocycler (Corbett Research, Sydney) at 22 °C. The machine was programmed to provide temperatures (in separate runs) from 55 °C to 75 °C in increments of 5 °C, and to hold at these temperatures for set times (Table 3). This temperature range was wider than that recorded during cooking of whole lamb roasts (unpublished) and included the FSIS recommended temperature of 71 °C. The come up times from 22 °C to 55, 60, 65, 70 and 75 °C were 38, 42, 46, 47, and 50 s, respectively. After holding at the desired temperature for the appropriate time, the tubes were cooled to 22 °C; the come down times ranged from about 45 to 75 s and were inversely related to the holding temperature. An aliquot of 50 µl from each tube was inoculated immediately into a BACTEC vial and incubated at 37 °C for up to 12 weeks. The survival duration observed among the three replicates at each temperature was recorded; the maximum duration of survival was defined as the longest observed for any replicate.

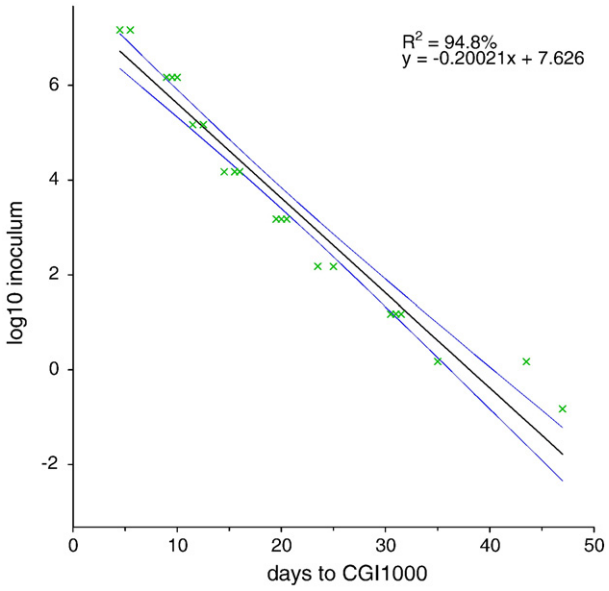

Fig. 1. For enumeration of *MAP* following heat treatments, the linear regression of $\log_{10}$ inoculum (*y* axis) and days to cumulative growth index 1000 (*x* axis) was used. Data for the S strain.

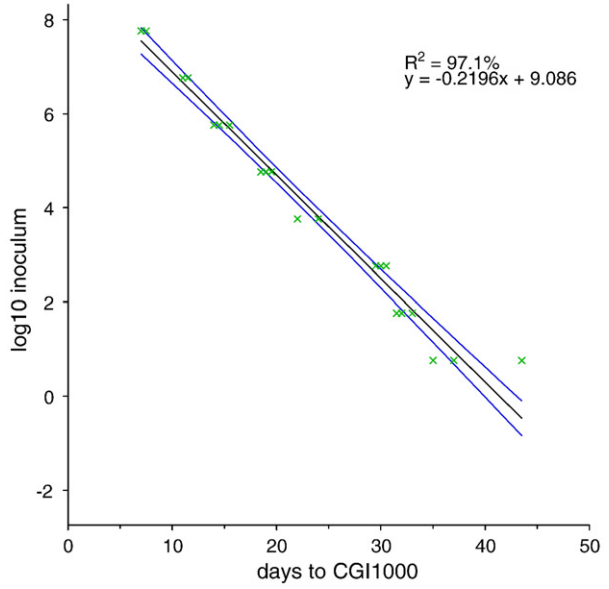

Fig. 2. For enumeration of *MAP* following heat treatments, the linear regression of $\log_{10}$ inoculum (*y* axis) and days to cumulative growth index 1000 (*x* axis). Data for the C strain of *MAP*.

2.5. D value

D value is the time required at a constant temperature for a 1 log (or 90%) reduction in viable count, determined by linear regression of experimental data, where $D = -1/\text{regression coefficient}$. Data were obtained above at 55, 60, 65, 70 and 75 °C. After heating for specified periods, an aliquot of 50 µl from each tube was inoculated into a BACTEC vial and the inoculum size was estimated using days to CGI1000; the control was the average MPN count for the unheated suspension (Table 2).

2.6. z value

The *z* value is the temperature change that results in a ten-fold change in *D* value. The *z* value was calculated by least squares linear regression of *D* values, where $z = -1/\text{regression coefficient}$ expressed in Celsius degrees (C°). The *z* value was calculated using D_{55}, D_{60}, D_{65} and D_{70}.

2.7. F_p value and estimation of the decimal reductions in MAP counts achieved during cooking

The F_p value of a thermal process quantifies the total heating effect and expresses it in equivalent minutes at a reference temperature for microorganisms with known *z* values. Using F_p, the severity of thermal processes can be compared with that achieved by the FSIS guideline of 71.1 °C at which temperature sufficient lethality of target pathogens is considered to be achieved instantaneously. Raw data were utilised from a previous study in which 16 legs of lamb were baked; oven temperature was monitored and core temperatures were measured at two locations in each sample using thermocouple probes (unpublished). Following their conversion into excel format the data from each of the 16 cooking trials were imported into proprietary software (DWC FoodTech Thermal Analyser, DWC FoodTech Pty. Ltd., Melbourne) for calculation of the so-called F_p values via the General Method as described (Gaze, 1992; Hersom and Hulland, 1980; Stumbo, 1973; Warne et al., 2009). The reference temperature was 70 °C and the *z* values of 4.21 and 4.51 C°, for *MAP* strains S and C, respectively were used (see results). The average oven temperature was the average of the actual temperatures recorded by the thermocouple in the oven after it had reached cooking temperature; the cooking time was the elapsed time that the sample was held in the oven, as indicated by the time–temperature data gathered by the thermocouple recording oven temperature; the final F_p value was the accumulated F_p value after the sample had cooled. The number of decimal reductions of *MAP* that would be achieved by cooking was calculated from the total F_p value for the entire baking process (including heating, holding and cooling) divided by the *D* value of the specific strain of *MAP*.

3. Results

3.1. Linear regression of MPN counts and days to CGI1000

The viable counts of *MAP* in the parent suspensions were first calculated by end point titration using two most probable number techniques. Two diluents were used, PBS and lamb skeletal muscle homogenate fluid. The latter dilution medium resulted in viable counts of *MAP* that were 1 log higher than those for suspensions of the same strains prepared in PBS (Table 2). An average of the results obtained from both methods for the suspensions that were prepared in lamb skeletal muscle homogenate fluid was used in subsequent analyses (Table 2).

The linear relationship between log10 inoculum size, which was determined from the MPN data in Table 2 and the dilution steps of the dilution series, and days to CGI1000 was determined for each strain of *MAP*. These relationships, accounted for 95–97% of the variation in the data and were used to predict viable counts in suspensions that had been exposed to the various heat treatments (see below) (Figs. 1 and 2).

Table 2
Most probable number counts for the S and the C strain of *MAP* in two suspension media, and inoculum sizes used in heat inactivation experiments.

Strain	Medium	MPN/50 µl Method 1	MPN/50 µl Method 2	Average MPN/50 µl in parent suspension[a]	Total number of viable *MAP* in each 100 µl aliquot used in heat inactivation experiments
S	PBS	4.3×10^5	4.3×10^6		
	Lamb skeletal muscle homogenate fluid	1.5×10^7	1.5×10^7	1.5×10^7	1.5×10^5
C	PBS	9.3×10^6	2.3×10^6		
	Lamb skeletal muscle homogenate fluid	9.3×10^7	2.3×10^7	5.8×10^7	5.8×10^5

[a] 50 µl is the standard inoculum size for each BACTEC vial; these data were used for the control and in regression analysis.

3.2. Temperature and time required for inactivation of 5 logs of MAP

A reduction of at least 5 logs in viable count of the C strain of *MAP* in a substrate of lamb tissue homogenate fluid required heating at 65 °C for more than 7 min, at 70 °C for 15–20 s or at 75 °C for less than 5 s (Table 3). The suspension of S strain required slightly less time to inactivate: 4–5 min, 10–15 s and <5 s at 65 °C, 70 °C and 75 °C, respectively.

3.3. D value

D value is the time required at a constant temperature for a 1 log (or 90%) reduction in viable count. *D* values were determined for each temperature and are specific for the medium used, lamb skeletal muscle homogenate fluid (Table 4). For the S strain of *MAP*, *D* value ranged from almost 90 min at 55 °C to 1.5 s at 70 °C. *D* values for the C strain at the higher temperatures were longer than for the S strain, but paradoxically D_{55} was lower than for the S strain (Table 4). There were too few observations of growth after heating at 75 °C to determine a D_{75}.

Table 3
Temperature and time combinations used to study the inactivation of 10^5 *MAP* suspended in 100 µl lamb skeletal muscle homogenate fluid.

Temperature °C	Sampling times[a]	Maximum observed duration of survival[b]	
		S strain	C strain
55	0, 15, 30, 45, 60, 75, 90 min	≥90 min	≥90 min
60	0, 5, 10, 15, 20, 25, 30, 35 min	≥35 min	≥35 min
65	0, 1, 2, 3, 4, 5, 6, 7 min	≥4, <5 min (1 of 3 reps)	≥7 min (1 of 3 replicates)
70	0, 5, 10, 15, 20, 25, 30, 35 s	≥10, <15 s	≥15, <20 s
75	0, 5, 10, 15, 20, 25, 30, 35 s	<5 s	<5 s

[a] min: minutes; s: seconds.
[b] Results are for all 3 replicates unless otherwise shown.

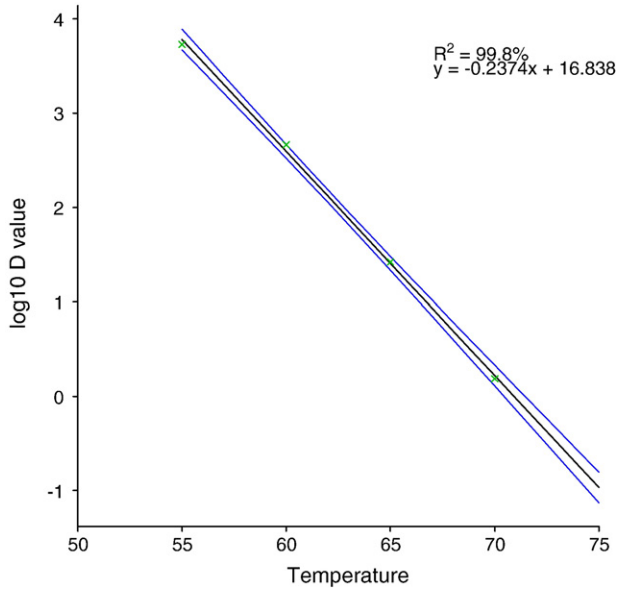

Fig. 3. To estimate *z* value in lamb skeletal muscle homogenate fluid, the linear regression of $\log_{10}$ *D* value (*y* axis) on temperature (*x* axis) was used. Data for the S strain of *MAP*.

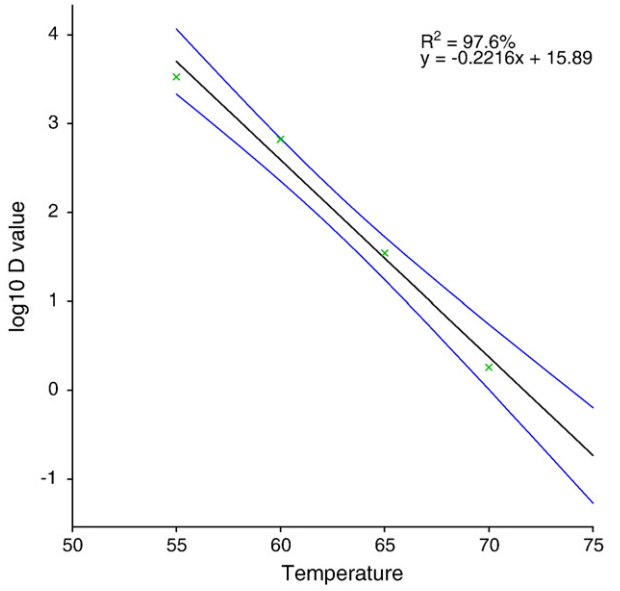

Fig. 4. To estimate the *z* value in lamb skeletal muscle homogenate fluid, the linear regression of $\log_{10}$ *D* value (*y* axis) on temperature (*x* axis) was used. Data for the C strain of *MAP*.

3.4. z value

The *z* value is the temperature change that results in a ten-fold change in *D* value and was estimated using the data for four temperatures: 55, 60, 65 and 70 °C. The linear regression explained 98 to almost 100% of the variation in the data (Figs. 3 and 4). The *z* value for S strain was 4.21 C° and for C strain was slightly higher at 4.51 C°.

3.5. F_p values achieved during cooking

The results of the calculation of F_p values achieved during each of the leg-of-lamb cooking trials are shown in Table 5. With the exception of sample 7, for which minimum F_p values recorded by Probe 1 were zero for both strains, and for Probe 2 were 0.6 and 0.7 min for S and C strains respectively, all other samples had cooking processes which delivered F_p values $\geq$2.4 and 2.7 min, for S and C strains, respectively. This means that with the exception of one sample, all processes were at least equivalent to 144 s (2.4 min) and 162 s (2.7 min) at 70 °C for S and C strains, respectively. When the severity of these heat treatments was converted to equivalent time at 71.1 °C to enable comparison with the USDA standard, the least severe process (excluding sample 7) was equivalent to 78.9 and 92.4 s for the S and C strains, respectively. Both values are well in excess of the recommended USDA standard which requires that the product reach 71.1 °C rather than be maintained at that temperature for a specified time.

The data for sample 7 for Probe 2 indicate that the heat treatment was equivalent to 36 s and 42 s at 70 °C, or 19.7 and 24.0 s at 71.1 °C for S and C strains, respectively. Whilst these treatments exceed the recommended USDA standard they are close to the values of 30 and 35 s at 70 °C recorded for the maximum duration of survival of 4.3×10^6 viable cells of S and C strains, respectively, of *MAP*/mL in PBS (Gumber and Whittington, 2009). The sole example for which the severity of the heat treatment was marginal and less than the USDA standard, and also less than the necessary exposure determined by Gumber and Whittington (2009), was that calculated for Probe 1 with sample 7. In this instance F_p values of zero were recorded for both strains of *MAP*. The maximum temperature recorded by Probe 1 was 59.6 °C at which temperature the rate of destruction for the S and C strains, respectively, of *MAP* would be <0.003 and <0.005 times that achieved at 70 °C. While the heat treatment for Probe 1 with sample 7 was marginal, the severity of the heat process received by the other 15 legs of lamb were more than sufficient to have eliminated all S and C strain *MAP*.

Table 4
D values for *MAP* in lamb skeletal muscle homogenate fluid at selected temperatures.

Temperature °C	Units[a]	Co-efficient	Constant	*R*-squared	*D* value[a]
S strain					
55	min	−0.011202	6.071	90.1	5356.2 s
60	min	−0.12966	5.9828	98.4	462.7 s
65	min	−2.271	6.486	95.9	26.4 s
70	s	−0.6507	6.264	98.2	1.5 s
75	s	not valid			
C strain					
55	min	−0.017864	7.7143	95	3358.7 s
60	min	−0.09028	7.042	84.2	664.6 s
65	min	−1.721	6.918	84.7	34.9 s
70	s	−0.5497	7.892	87.9	1.8 s
75	s	Not valid			

[a] min: minutes; s: seconds.

3.6. Decimal reductions in MAP counts that would be achieved during cooking

The results of the calculation of decimal reductions of *MAP* that would likely have been achieved during the cooking trials are shown in Table 6. As a corollary of the wide range in F_p values delivered by the cooking processes, there was a correspondingly wide range in the number of log reductions that would have been achieved for both strains of *MAP*. Cooking of 15 of 16 samples would have delivered at least 24.0 and 23.3 log reductions for strain S and C *MAP*, respectively, and therefore rendered the product microbiologically safe. The remaining sample was the least severe of all processes (Probe 1 with sample 7); the maximum core temperature was 59.6 °C. This would have failed to bring about a single log reduction in the population of the S strain *MAP*.

Table 5
Summary of average oven temperatures, cooking times and final F_p values achieved in domestic trials by volunteers who baked leg-of-lamb roasts.

Leg of lamb ID	Weight of leg (kg)	Average[a] oven temp (°C)	Cook[b] time (min)	Final F_p value[c] for S strain		Final F_p value[d] for C strain	
				Probe 1 (min)	Probe 2 (min)	Probe 1 (min)	Probe 2 (min)
1	1.520	220	120	425.8	1067.2	424.5	965.2
2	1.530	175	95	31.9	21.5	30.6	20.9
3	1.530	154	120	104.5	144.1	104.6	143.3
4	1.706	186	130	318.8	1892.2	319.1	1892.4
5	1.774	169	120	12.1	36.7	12.7	36.6
6	1.742	181	125	700,756.1	10,374,153.5	700,756.2	10,374,153.6
7	1.659	193	66	0.0	0.6	0.0	0.7
8	1.780	183	139	195,042.1	663,984.9	193,876.0	662,671.7
9	1.656	157	141	19.3	34.1	19.3	34.1
10	1.666	230	105	17,691,756.2	25,308.8	17,668,607.9	25,304.7
11	1.718	148	110	132.2	65.3	132.4	65.5
12	1.722	192	80	108.5	9417.8	107.5	9407.1
13	1.786	201	81	6.1	2.4	6.5	2.7
14	2.664	NA	98	10.8	134.8	11.2	135.1
15	2.524	149	134	7,446,173.7	475,436.4	7,380,747.9	457,077.8
16	1.878	129	97	193.8	22.2	194.1	22.9
Minimum	1.52	129	66	0	0.6	0	0.7
Maximum	2.66	230	141	>1000	>1000	>1000	>1000

[a] The average of the actual temperatures recorded by the thermocouple in the oven after it had reached cooking temperature.
[b] The elapsed time that the leg was held in the oven, as indicated by the time–temperature data gathered by the thermocouple recording oven temperature.
[c] F_p value is the severity of the heating process expressed as equivalent time in minutes at a reference temperature of 70 °C and with z values of 4.21 and 4.51 °C for type S and type C *MAP* strains, respectively.
[d] The final F_p value is the accumulated F_p value after the leg of lamb had cooled.

4. Discussion

There are no prior data on the impact of food processing methods on the inactivation of *MAP* in meat products (Eltholth et al., 2009). In this study we applied a highly accurate and reproducible method of heating a sample to study the inactivation of *MAP* in a substrate resembling the tissue fluid environment of skeletal muscle, and it is the first attempt to evaluate the efficacy of cooking in destroying *MAP* in red meat. If *MAP* is present in red meat, it is not likely to be present within myofibres, but most likely would be intracellular, possibly in monocytes or other phagocytes in blood or lymph vessels within muscle. It would be difficult to provide an *in vitro* environment which exactly represents this situation, but we attempted to represent the skeletal muscle environment, rather than use a simple laboratory buffer. Recently the physical state of muscle, that is whether it is intact or ground, was shown to be a factor in thermal resistance of *Salmonella* in beef; resistance was significantly higher in whole-muscle (Mogollon et al., 2009). It is unknown whether this would be a factor in the thermotolerance of *MAP*.

The use of the lamb skeletal muscle homogenate fluid medium as a diluent for bacterial suspensions in the most probable number counts resulted in viable counts of *MAP* that were 10 fold higher than those for suspensions of the same strains prepared in PBS (Table 3). The reasons might include declumping of *MAP* in the lamb skeletal muscle homogenate fluid, but were not investigated in this study.

There are no published data on the D_{55} value of *MAP*. Previously reported D_{60} values for *MAP* suspended in milk or lactate were 2 to 14 min (Keswani and Frank, 1998; Sung and Collins, 1998), which were of the same order of magnitude as the values obtained here. D_{65} values of 56 to 71 s were obtained in milk or lactate in a previous study (Sung and Collins, 1998); values in this study were slightly lower. D_{71} values of 13 to17 s were obtained in milk or lactate (Sung and Collins, 1998), whereas D_{70} values obtained in this study in lamb skeletal muscle homogenate fluid were lower.

Heat resistance of *M. avium* strain DSM43216 was determined in water; D_{50}, D_{55}, D_{60} and D_{70} were 17 h, 54 min, 4 min and 2.3 s, respectively (Schulze-Robbecke and Buchholtz, 1992). These values are considerably lower than those for *MAP*. In an evolutionary context *M. avium* may not require such thermal resistance as it is an environmental organism with capacity to replicate outside host animals and so regenerate segments of the population that are lost due to unfavourable conditions.

Interestingly the S strain of *MAP* appeared to be less thermotolerant than the C strain. This was apparent in the D values, which are independent of initial bacterial count. The time required for complete inactivation is less reliable; the slightly lower initial viable count for the S strain may account for differences in observed times for complete inactivation between it and the C strain.

D value is dependent on the medium and D values in milk may be higher than those in lamb skeletal muscle homogenate fluid. For

Table 6
Estimated number of log reductions[a] achieved in baking processes, calculated as Total F_p value/D_{70} value for S and C strains of *MAP* in leg-of-lamb roasts.

Leg of lamb sample	No. log reductions for S strain		No. log reductions for C strain	
	Probe 1	Probe 2	Probe 1	Probe 2
1	17,032.0	42,688.0	14,150.0	32,173.3
2	1276.0	859.1	1020.0	696.3
3	4180.0	5765.0	3486.7	4775.4
4	12,752.0	75,688.6	10,636.7	63,080.6
5	484.0	1467.8	423.3	1220.6
6	28,030,244.0	414,966,138.7	23,358,540.1	345,805,118.6
7	0.0	24.0	0.0	23.3
8	7,801,685.2	26,559,394.9	6,462,533.4	22,089,055.1
9	771.7	1362.9	643.3	1136.7
10	707,670,248.0	1,012,352.4	588,953,596.3	843,488.9
11	5287.1	2610.5	4413.4	2183.8
12	4339.6	376,710.2	3584.0	313,570.5
13	243.2	96.3	217.6	90.3
14	432.7	5392.1	372.3	4504.0
15	297,846,949.6	19,017,456.8	246,024,930.0	15,235,927.6
16	7751.8	887.2	6470.4	762.0
Minimum	0	24	0	23
Maximum	>1000	>1000	>1000	>1000
No samples with >12 log reduction	15	16	15	16

[a] Log reductions of *MAP* calculated using experimentally determined (see Section 3.3, Table 4) D_{70} values of 1.5 s and 1.8 s for the S and C strains, respectively.

example D_{71} in milk/lactate was 13 to 17 s (Sung and Collins, 1998) compared to D_{70} 1.5 to 1.8 s in lamb skeletal muscle homogenate fluid. However, these comparisons may not be valid due to differences in methodology. The PCR thermocycler and thin- walled plastic tubes that were used in the present study allowed for very accurate temperature control and very rapid heat exchange compared to the methods used in earlier studies. Furthermore, estimates of *D* value based on bacterial counts using the BACTEC culture method are not influenced by clumping of *MAP* (Sung and Collins, 1998).

z-value for *MAP* has not commonly been reported. However, data from several studies using milk as the substrate were combined in a meta-analysis to provide an estimate of 7.43 °C (Stabel et al., 2001). In a separate study in milk it was 8.6 °C (Pearce et al., 2001). Values obtained in this study for lamb homogenate fluid were lower: 4.21 °C for S strain *MAP* and 4.51 °C for C strain.

The F_p method is commonly used by the food processing industry when establishing process schedules for pasteurised and sterilised packaged foods and relies upon calculation of the cumulative lethal effect of all time–temperature combinations at the points of temperature measurement in the product. F_p values provided a direct and quantifiable measure of the severity of the heat treatments that were used to cook the leg-of-lamb roasts in a recent trial (unpublished). Using F_p, the severity of thermal processes can be compared with that achieved by the USDA Food Safety Inspection Service guideline of 71.1 °C at which temperature sufficient lethality of target pathogens is considered to be achieved instantaneously. An extension of the F_p method is to calculate the number of decimal reductions the process would deliver with respect to destruction of specified target microorganisms, in this case strains S and C of *MAP*. The wide range in the final F_p values among the leg-of-lamb samples in this study can be attributed to a combination of the following factors: average oven temperatures ranging from 129 to 230 °C, cooking times ranging from 66 to 134 min and the logarithmic rate of change in accumulation of F_p value across a temperature range of *z* °C. For instance, whereas the lowest of the maximum temperatures was 59.6 °C (Probe 1 with sample 7) the highest of the maximum core temperatures was 98.3 °C (Probe 1 with sample 10). At these two extremes the lethal rates of destruction (relative to that at 70 °C) range from 0.003 to 5,273,394 and from 0.005 to 1,883,409 for strains S and C, respectively. As a corollary of the wide range in F_p values delivered by the cooking processes, there was a correspondingly wide range in the number of log reductions achieved for both strains of *MAP*. Notwithstanding that extrapolation over such broad temperature ranges (or multiples of *z* values) is not the usual practice when comparing maximum lethal rates of destruction for "like" samples, the results nevertheless demonstrate the impact that widely different core temperatures can have on final F_p values and the number of log reductions.

The severity of the heat process applied to 15 of 16 leg-of-lamb roasts in domestic cooking would have been more than sufficient to have eliminated all *MAP* regardless of strain. However, the least severe of all processes (Probe 1 with sample 7) for which the maximum core temperature was 59.6 °C, failed to bring about a single log reduction in the population of the S strain *MAP*. To place these results in a broader context, the data showed that all treatments other than that for Probe 1 with sample 7 exceeded the most stringent demands of Good Manufacturing Practice. Of those processes that can be considered adequate, the least process (Probe 2 with sample 7), which delivered 24.0 and 23.3 log reductions for strain S and C *MAP*, respectively, lies between 3.4 and 3.6 times that required by USDA with respect to elimination of *Salmonella* in ready-to-eat beef products. Furthermore, it delivers more than twice the decimal reductions required for elimination of mesophilic *Clostridium botulinum* in commercial heat processed shelf-stable low-acid canned foods.

Assuming that the lamb homogenate fluid used as a substrate in these experiments is a valid representation of the substrate *in vivo*, routine cooking of meat will destroy *MAP* organisms if they are present in moderate to high levels of contamination. The data determined using baking as a model cooking method are generally applicable as core temperatures during baking are lower than surface temperatures during baking, grilling or frying. In practical terms, high levels of decimal reduction of pathogens including *MAP* are readily achieved in domestic and commercial cooking without modifying the commonly used techniques. However, accurate advisory information to avoid undercooking of red meat may need to be made more available to consumers. As indicated Safe cooking practices may be commonplace but it is difficult to find recommendations from an authoritative source for use by domestic consumers (unpublished).

Although the results of this study should provide consumers with a high degree of confidence that there is very low risk of viable *MAP* being consumed with cooked red meat, it is important that other aspects of food hygiene are also addressed to avoid contamination, particularly not bringing uncooked meat, or utensils or surfaces used to prepare uncooked meat, into contact with cooked meat. These recommendations are of course generic and apply to a wide range of potential foodborne pathogens. It is important to note that *MAP* is a slow growing microorganism with a generation time of about 2 days. Furthermore it multiplies only within host macrophages. Therefore it is most unlikely to be able to replicate if it were present as a contaminant of meat, unlike many other foodborne bacterial pathogens.

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