

International Journal of Food Engineering

Volume 7, Issue 2

2011

Article 3

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Recommended Citation:

Saucier, Linda and Plamondon, Éveline (2011) "Heat Inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in Aseptically Prepared Ground Beef," *International Journal of Food Engineering*: Vol. 7 : Iss. 2, Article 3.

Available at: <http://www.bepress.com/ijfe/vol7/iss2/art3>

DOI: 10.2202/1556-3758.2062

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Heat Inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in Aseptically Prepared Ground Beef

Linda Saucier and Éveline Plamondon

Abstract

Mycobacterium avium subsp. *paratuberculosis* (Map) is the etiologic agent of Johne's disease in bovine and other ruminants. Concern for public health was raised when the organism was also suggested to be responsible for Crohn's disease in humans, although the evidence remains inconclusive. Nonetheless, limiting human exposure to Map is viewed as a proper precautionary measure. Hence, the efficacy of heat treatment to control the organism in milk has been studied but it has not been studied to the same extent in meat. In this study, aseptically prepared ground beef was obtained from beef *semimembranosus* muscle and inoculated with two strains of Map (ATCC 7080 and gN27) to determine the decimal reduction time (D-value) and temperature sensitivity (z-value) for each strain. A 25 g sample of meat was inoculated with 100 μ l of culture to a final concentration of 10^7 cfu/g. The inoculum was evenly distributed in the meat, which was spread in a thin (2 mm) layer of to maximise heat transfer. Treatments were performed at 55, 60, 65 and 70 °C for times allowing a minimum 5-log reduction. D-values decreased significantly with temperature ($P < 0.05$) ranging from 80.5 ± 6.1 minutes to 12 ± 1 seconds for both Map strains. When compared to *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 7080, D-values were significantly lower for *E. coli* ($P < 0.05$) whereas *E. faecalis* was not consistently more resistant than the two Map strains and, therefore, cannot be used as a surrogate strain for Map control with heat treatment. The z-values were not significantly different ($P > 0.05$) amongst the four strains and ranged from 5.6 ± 0.1 °C to 6.2 ± 0.3 °C. The results suggest that a low concentration of Map could be controlled with conventional cooking methods.

KEYWORDS: *Mycobacterium avium* subsp. *paratuberculosis*, D-value, z-value, meat

Author Notes: The authors thank Dr. L. Mutharia and her research team at the University of Guelph for kindly providing the strains used in this study and for cultivation guidance for Map, which is very fastidious to grow. A special thank you is also addressed to Dr. C.O. Gill and W.J. Meadus for valuable discussion on Map importance and prevalence in the food supply. This research was sponsored by a grant from *Le Fonds québécois de la recherche sur la nature et les technologies* (FQRNT) from the *Gouvernement du Québec*.

1. INTRODUCTION

Mycobacteria are small rod-shaped cells responsible for many human diseases, notably, tuberculosis and leprosy, respectively caused by *Mycobacterium tuberculosis* and *M. Leprae* (Stahl and Urbance, 1990). *M. avium* subsp. *paratuberculosis* (Map) is the causative agent of Johne's disease in ruminants, especially cattle. This chronic enteritis develops slowly and infected animals can easily go unnoticed before persistent diarrhoea and weight loss become evident, usually when the animal reaches two years of age. Thickening of the intestinal wall interferes with nutrient absorption and the negative impact of the disease on milk production is well documented (Cocito *et al.*, 1994; Collins, 1997; Manning and Collins, 2001; Collins, 2003). The gross symptoms of Crohn's disease in humans are somewhat similar to those of Johne's disease (Sartor, 2005; Chamberlin and Naser, 2006). In fact, the IS900 sequence, present in 14 to 20 copies on the Map genome (Bull *et al.*, 2000), was detected in some but not all Crohn's patient's biopsies of the intestinal mucosa (Greenstein, 2003; Autschbach *et al.*, 2005; Sechi *et al.*, 2005). Hence, although the scientific evidence remains inconclusive, limiting the exposure to Map is viewed as an appropriate precautionary measure. In terms of food safety, it is important to determine the efficacy of food processing technologies, such as heat treatment, at controlling Map in case of accidental contamination of foods.

Diseased animals shed the organisms in milk and in their feces (Whittington and Sergeant, 2001; Sartor, 2005). Hence, contamination of raw milk and carcass during dressing operations is bound to occur. Cross-contamination from asymptomatic animals shedding the organisms in their feces is also a major herd management challenge. The resistance of Map in the environment and to decontaminating reagents is due, at least in part, to the thick cell envelope rich in mycolic acids which constitute a hydrophobic barrier around the cell (Wick *et al.*, 2002; Barton, 2005). Consequently, Map cells in aqueous solutions have a tendency to form aggregates that probably protect the cell during heat treatment (Borrego *et al.*, 2000; Lund *et al.*, 2002). Map is more heat resistant than *Salmonella*, *Listeria*, *Coxiella*, *M. tuberculosis* and *M. bovis* (Sung and Collins, 1998; Gao *et al.*, 2002; Grant, 2005; Grant, 2006). Furthermore, the fact that Map was isolated from pasteurized milk raised the question of whether Map is capable of resisting heat treatments equivalent to commercial pasteurization (Grant *et al.*, 2002; Ellingson *et al.*, 2005). *Clostridium botulinum* is well known for the heat resistance of its bacterial spores and for its pathogenicity in causing severe food-borne illness. Consequently, it is widely recognized as the indicator organism to demonstrate contamination control in food sterilization (Ramaswamy and Marcotte, 2006). Unfortunately, an equivalent

indicator organism for cooked, but not sterilized, food has not been formally identified. *Enterococcus faecalis* is sometimes used for this purpose (Reichert *et al.*, 1979 cited by de Halleux *et al.*, 2005) but further research is needed to select a non-spore forming indicator that warrants sufficient cooking treatment, such as in meat. Whether or not some *Mycobacterium* species could serve as such an indicator remains to be demonstrated.

The lethality of a heat treatment is determined by exposing a known number of target cells to a specific temperature for various times and enumerating the survivors. Plotting the number of survivors over time at a given temperature allows the determination of the decimal reduction time (D-value), which is the time required to reduce the cell population by 90% or 1 log unit (Ramaswamy and Marcotte, 2006).

$$D = \frac{t_2 - t_1}{(\log N_0 - \log N)} \quad (1)$$

N is the microbial population at any time during the process;

N_0 is the initial population;

$t_2 - t_1$ is the heating time.

Hence, the slope (m) of a survival curve is equal to $-1/D$.

$$\log N = -1/D (t) + \log N_0 \quad (2)$$

At each temperature, the D-value for a specific organism allows the temperature sensitivity (z-value) to be calculated by plotting the \log_{10} of the D-value against the corresponding temperature. The z-value is the temperature elevation required to reduce the D-value by 90% or 1 log unit (Ramaswamy and Marcotte, 2006).

$$z = \frac{T_2 - T_1}{(\log D_{T1} - \log D_{T2})} \quad (3)$$

Where D_{T1} and D_{T2} are the D-values at temperature $T1$ and $T2$. Hence, the slope (m) is equal to $-1/z$.

$$\text{Log } D = -1/z (T) + \log D_0 \quad (4)$$

Studies on Map inactivation in meat are rare. However, Merkal *et al.* (1979) studied the control of *Mycobacterium intracellulare* complex in Wieners type sausages inoculated with infected liver and lymph node tissues carrying 10^3

to 10^4 cfu/g. The organism was not recovered (detection level of 40 cfu/g) after heat treatments that allowed the internal temperature to reach 65.5 °C or higher. Fortunately, the few studies on the incidence of Map in meat indicate that it can be isolated but only in few numbers (Javarata *et al.*, 2007; Meadus *et al.*, 2008). The aim of this study was to determine the D- and z-value of Map in aseptically prepared ground beef to determine if common cooking procedures can control Map in a meat matrix.

2. MATERIAL AND METHODS

2.1 Bacterial strains and culture conditions

M. avium subsp. *paratuberculosis* ATCC 19698 and a strain isolated from feces originating from an infected herd of cattle in Ontario, Canada (gN27) were studied and were kindly provided by Dr. Lucy Mutharia, University of Guelph, Ontario, Canada. *E. faecalis* ATCC 7090 and *Escherichia coli* ATCC 29522 were also tested for comparison – *E. faecalis* for its particular heat resistance (Incze *et al.*, 1999; Reichert *et al.*, 1979 cited by de Halleux *et al.*, 2005) and *E. coli* because it is an indicator organism commonly used to evaluate hygiene and fecal contamination in meat (Gill, 2000). Stock cultures were stored at -80 °C in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with Tyloxapol (0.05% v/v; Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) and mycobactine J (2 mg/l; The Paratuberculosis Laboratory, Allied Monitor, Fayette, MO, USA) for Map strains and in Brain Heart Infusion broth (BHI; Difco Laboratories) for *E. faecalis* and *E. coli*. Glycerol (FisherBiotech, Fairlawn, NJ, USA) was added to a final concentration of 20% as a cryoprotectant.

Frozen stock cultures of *E. faecalis* ATCC 7090 and *E. coli* ATCC 29522 were sub-cultured (1% v/v) daily in BHI broth for a minimum of two and a maximum of seven consecutive days before use in experiments. Cells were incubated overnight at 37 °C. Cultures used for the heat treatments were incubated for 24 h in meat extract (2% total nitrogen (ME2); EMD Chemicals Inc., Gibbstown, NJ) or BHI broth at 37 °C. Map grows very slowly with a generation time of > 20 h (Lambrecht *et al.*, 1988; Coetsier *et al.*, 1998; Rowe and Grant, 2006) so cells were incubated for 51 days at 37 °C in Middlebrook 7H9 broth supplemented with Tyloxapol (0.05%), mycobactine J (2 mg/l), glycerol (5 g/l) and with albumin (BSA fraction V 50 g/L; EMD Chemicals Inc.), dextrose (20 g/L; Calbiochem, Darmstadt, Germany), catalase (0.04 g/L; EMD Chemicals Inc.) as supplements (ADC; 100 ml/l). Cells were centrifuged (4080 x g, 4 °C, 10 min) and washed twice with peptone water (0.1% p/v; Bacto peptone, Difco Laboratories) for *E. faecalis* and *E. coli*, and with

Middlebrook 7H9 broth supplemented with Tyloxapol but without mycobactine J for Map.

Cells were enumerated on BHI agar incubated at 37 °C for 24-48 h for *E. faecalis* and *E. coli*, and on Middlebrook 7H11 agar (Difco Laboratories) supplemented with mycobactine J (2 mg/l), glycerol (50 mg/l), ADC (100 ml/l), penicillin G (100 000 unités/l; EMD Chemicals Inc.) and chloramphenicol (50 mg/l; EMD Chemicals Inc.) for Map. Map was grown using tissue culture flasks containing a filtered septum for free flow of sterile oxygen (Falcon™ Cell Culture Flask, 25 cm², BD Biosciences, Mississauga, ON, Canada), instead of a Petri dish, to control contamination by moulds over the long incubation period (14 days and higher). The flask had a surface similar to a Petri dish and colonies were easier to count than on an agar slant in a large test tube, also commonly used for Map enumeration.

2.2 Aseptically prepared ground meat

Heat treatments were performed in aseptically prepared raw lean ground meat (Greer and Jones, 1991; Saucier and Greer, 2001). Briefly, meat was prepared from *semimembranosus* Angus AAA beef obtained from a local butcher shop. The whole piece of meat was soaked in ethanol (95%) and then set on fire. The procedure was repeated twice and a hand held welding torch was used to remove any excess ethanol. The centre core of muscle was aseptically extracted in a laminar flow hood. The initial population of microorganisms in the meat was below detection level (< 10 cfu/g). By controlling the indigenous microflora normally found on meat, cells can be enumerated using non-selective conditions, which yield better recovery of heat-injured cells.

The percentage of moisture in the meat was determined by lyophilising (model 6203-3005-OL, Virtis Co., Gardiner, NY, USA) 25 g of meat for 7 days. Fat content was measured using a Soxtec system (Soxtec system HT 1043, Tecator, Hoganas, Sweden) by procedure 991.36 of the Association of Official Analytical Chemists (AOAC, 1995); results are expressed on a wet weight basis. The total protein was determined using procedure 992.15 of the AOAC (1995) with a LECO® protein analyser (model FP-2000, Leco Corp., St-Joseph, MO, USA); results are expressed on a wet weight basis. All analyses were performed in triplicate.

2.3 Heat treatments

Heat treatments were performed as described by Sallami *et al.* (2006). Briefly, samples of aseptically prepared raw meat (25 ± 0.5 g) were aseptically weighed and placed in a 11.4 cm x 22.8 cm (18 oz.) Whirl-pak™ sampling bag (Nasco, Newmarket, ON, Canada) and inoculated to a final concentration of 10⁷ cfu/g.

The meat was hand massaged to evenly distribute the cells in the meat matrix. The meat was then flattened into a 2 mm thick layer for maximum heat transfer. Inoculated bags were heated in a high precision (± 0.001 °C) circulating programmable water bath (Cole-Palmer Polystat Heated Circulating Bath, Cole-Parmer Canada Inc., Anjou, QC, Canada) at 50 to 70 ± 0.1 °C for varying times, but long enough to obtain a minimum 5-log reduction in cell number. The temperature of the meat was followed using a data logger equipped with a type T thermocouple (Food tracker MultiPaq21, Datapaq Inc., Wilmington, MA, USA) and data were analysed using Datapaq Insight software (Datapaq Inc.). After treatment, samples were cooled to 37 °C in an iced water bath (10 s) and immediately transferred to a Stomacher bag to avoid cold shock. Meat samples were immediately homogenised in 225 ml of 0.1% (p/v) peptone water for 2 min (Seward Ltd, West Sussex, UK) for *E. faecalis* and *E. coli* cell enumeration and with Middlebrook 7H9 broth supplemented with Tyloxapol but without mycobactine J for Map. Meat samples were inoculated and treated one at a time. All experiments at each time-temperature couple were repeated three times.

2.4 Statistical Analysis

D- and z-values were compared by analysis of variance (ANOVA) using the General Linear Model (GLM) procedures of SAS (version V6.10; SAS Institute, 2002) at $\alpha = 5\%$. The Kruskal-Wallis test was used to account for the heterogeneity of the variance between data at different temperatures (discrimination by ranks). Whenever factor effects were detected, a multiple comparisons technique was used to locate differences among factors levels. A Bonferroni correction was used to control the risk of declaring false differences at $\alpha = 5\%$. Linear regressions on survival curves were calculated using EXCEL software (Microsoft Office for MacIntosh, 2004) and were all ≥ 0.85 .

3. RESULTS AND DISCUSSION

3.1 Meat proximate composition

It took four *semimembranosus* whole muscle (40 Kg in total) to produce enough aseptically prepared ground meat to carry out experiments in triplicate at each time-temperature couple, for each microorganism studied. The average composition of the meat was $72.7 \pm 2.2\%$ humidity, $7.5 \pm 1.4\%$ fat and $23.9 \pm 1.5\%$ protein.

3.2 Efficacy of heat transfer

Only treatments providing a 5-log reduction, or more, in viable cells were used to determine the D- and z-values. Treatment time started only when the target temperature was reached, which took 52 to 106 s. Times ranging from 6.2 h, 1.6 h, 5.9 min and 0.9 min were necessary to obtain a 5-log reduction at 55, 60, 65 and 70 °C, respectively for Map ATCC 19698 and gN27 (Table 1). More severe treatment provided greater than 5-log-reductions. Heat treatment at 70 °C was too severe for *E. coli* since a 5-log reduction was obtained in less than 15 s. Furthermore, at 50 °C, heat treatments were not effective for Map and *E. faecalis*. For example, it took *E. faecalis* 48 h at 50 °C to reach a 5-log cell-count reduction (data not shown) which is not a practical cooking time for industrial processing. These experimental parameters are important to avoid survival curves presenting a shoulder and tailing (Murphy *et al.*, 2000; Juneja and Sofos, 2002). It is important that the temperature rises quickly to avoid cell adaptation and induction of bacterial stress response to the heat treatment (Yura *et al.*, 2000; Seyer *et al.*, 2002).

3.3 D-values

For *E. coli*, the maximum D-value was observed in cells prepared in BHI broth and tested in meat treated at 50 °C; the D-value reached 4563 ± 160 s. The minimal D-value was observed at 65 °C for cells prepared in ME2, with a value of 15.2 ± 0.4 s. For *E. faecalis*, the maximal and minimal D-values were 3519 ± 263 s at 55 °C and 9 ± 2 s at 70 °C both in ME2/meat. For the two Map strains, the maximal D-value was 4828 ± 368 s at 55 °C (Map gN27) and the minimal value was 12 ± 1 s (Map ATCC 19698) at 70 °C (Table 2). No interaction between microorganisms, media or temperature was observed, except at 65 °C where an interaction was detected between the liquid medium used to grow the cells prior to heat treatment and the microorganism tested, according to the non-parametric analysis of variance ($P = 0.003$). The maximum D-value was obtained in BHI/meat for *E. coli* whereas it was minimal for *E. faecalis* in the same medium. Bonneferroni comparisons indicated that the liquid medium used to prepare the inoculum was the only parameter significantly influencing ($P < 0.05$) the D-value of *E. coli* compared to *E. faecalis* at 65 °C (Figure 1). The growth conditions prior to heat treatment are known to be important since they influence the physiological state of the organism (Naim *et al.*, 2003). But in our experiments, the medium used to prepare the cells for the heat treatments had a limited effect, even though the pH of BHI (7.7) is quite different from ME2 (6.4). Acid adaptation is known to increase thermal resistance (Sharma *et al.*, 2005).

Saucier and Plamondon: Heat Inactivation of Map in Ground Beef

Table 1. Minimal time required to obtain a 5-log reduction of viable cells at each temperature (°C).

Microorganisms	50°C		55°C		60°C		65°C		70°C	
	Time (h)	Log cfu/g	Time (h)	Log cfu/g	Time (min)	Log cfu/g	Time (min)	Log cfu/g	Time (min)	Log cfu/g
Map ATCC 19698	NE ^a	-	5.0 h	5.0	66.0 min	4.8	3.9 min	4.9	0.9 min	4.8
gN27	NE	-	6.2 h	5.0	96.0 min	5.0	5.9 min	4.8	1.0 min	4.8
<i>E. coli</i> ATCC 25922 (BHI/Meat)	6.6 h	5.4	0.9 h	4.8	4.0 min	4.8	1.4 min	4.9	TS	TS
<i>E. coli</i> ATCC 25922 (ME2/Meat)	NE	-	1.0 h	5.2	4.9 min	5.0	1.4 min	5.6	TS	TS
<i>E. faecalis</i> ATCC 7090 (BHI/Meat)	NE	-	4.6 h	5.5	60.0 min	5.3	9.1 min	5.2	1.0 min	5.2
<i>E. faecalis</i> ATCC 7090 (ME2/Meat)	NE	-	4.5 h	5.0	55.9 min	5.1	10.1 min	5.3	0.6 min	5.0

^a NE = Not effective, TS = Too severe.

Table 2. Thermal resistance of target microorganisms in terms of D- and z-values evaluated in aseptically prepared ground beef at different temperatures (°C).

Strains	Medium ^a	D-value (s)					z-value (°C)
		50°C	55°C	60°C	65°C	70°C	
Map^c ATCC 19698	7H9/Meat	NE	4255 ± 665	905 ± 88	58 ± 11	12 ± 1	5.7 ± 0.1
Map gN27	7H9/Meat	NE	4828 ± 368	1290 ± 104	76 ± 2	13.1 ± 0.3	5.6 ± 0.1
<i>E. coli</i> ATCC 25922	BHI/Meat	4563 ± 160 ^b	730 ± 42	62 ± 12	19 ± 3	TS	6.2 ± 0.1
	ME2/Meat	NE	761 ± 55	61 ± 1	15.2 ± 0.4	TS	5.9 ± 0.1
<i>E. faecalis</i> ATCC 7080	BHI/Meat	NE	3452 ± 405	802 ± 115	116 ± 11	11.7 ± 0.6	6.2 ± 0.3
	ME2/Meat	NE	3519 ± 263	725 ± 68	127 ± 13	9 ± 2	5.9 ± 0.2

^a Growth medium/medium used for heat treatment.

^b Average ± standard deviation; NE = Not effective, TS = Too severe.

^c Map is a fastidious slow grower that requires a particular medium to grow; it cannot grow in BHI or ME2.

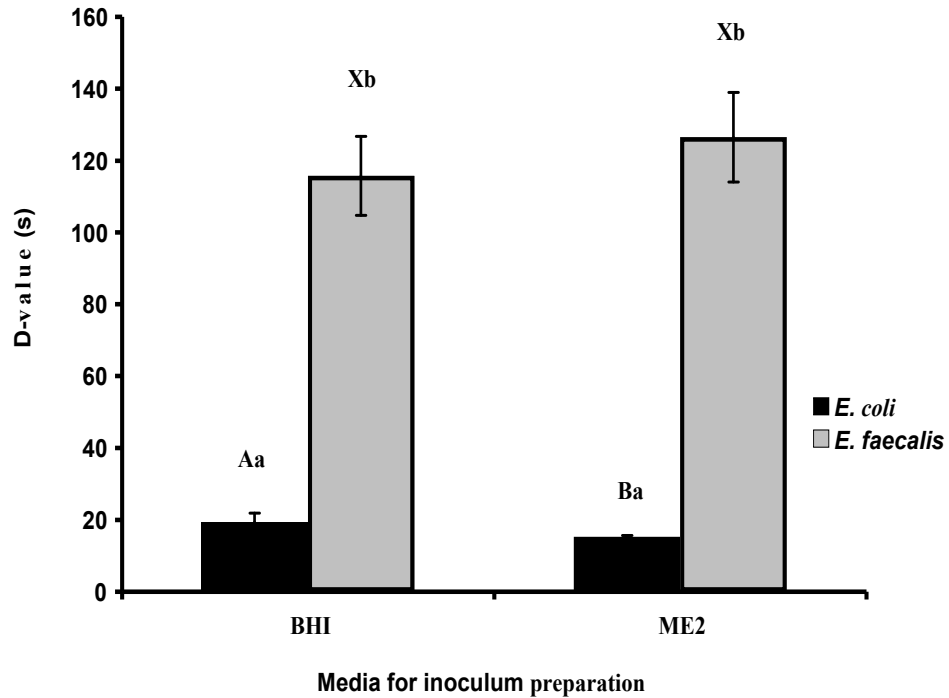


Figure 1. Interaction between the medium in which the inoculum was prepared before heat treatment and the microorganism tested for the D-value evaluated in aseptically prepared ground beef at 65 °C ($P = 0.003$). For a specific medium, results with a similar lowercase letter are not significantly different ($P > 0.05$). Capital letters are used for a specific strain, i.e., AB for *E. coli* and X for *E. faecalis*.

Table 3. Effect of temperature on the D-value obtained for each stain studied in aseptically prepared ground beef.

Strains	Temperature (°C)								
	50 vs 55	50 vs 60	50 vs 65	55 vs 60	55 vs 65	55 vs 70	60 vs 65	60 vs 70	65 vs 70
MAP ATCC 19698	NE	NE	NE	** (*)	**	**	NS (**)	NS (**)	NS (**)
gN27	NE	NE	NE	**	**	**	* (**)	* (**)	NS (**)
<i>E. coli</i> (BHI/Meat)	** ^a	**	**	**	**	TS ^a	NS (*) ^{ab}	TS	TS
<i>E. coli</i> (ME2/Meat)	NE	NE	NE	**	**	TS	NS (**)	TS	TS
<i>E. faecalis</i> (BHI/Meat)	NE	NE	NE	**	**	**	* (**)	* (**)	NS (**)
<i>E. faecalis</i> (ME2/Meat)	NE	NE	NE	**	**	**	*	* (**)	NS (**)

^a * (P < 0.05); ** (P < 0.01); NS: Not significant; NE: Not Effective; TS: Too Severe.

^b The symbols in parenthesis represent the probability (P) of the non-parametric analysis of variance (discrimination by ranks). If only one symbol is presented, the results of the parametric and the non-parametric analysis of variance were similar.

3.4 Temperature effect

Results from the parametric analysis of variance were validated by a non-parametric analysis (discrimination by ranks) to account for extreme or aberrant values or for the lack of homogeneity of the variance. As the heating temperature increased, the standard deviation associated with each D-value decreased, justifying the use of a non-parametric analysis of variance (Tables 2 and 3). For each microorganism, all D-values were significantly different from each other (Bonferroni test) according to the non-parametric analysis of variance when the treatment temperature increased ($P < 0.05$). As the temperature increased, the D-value decreased significantly for each microorganism studied (Table 3). Doyle and Schoeni (1984), Ahmed *et al.* (1995) and Line *et al.* (1991) evaluated the D-value of enterohemorrhagic *E. coli* in ground beef and they observed a reduction in D-value with temperature increase. The D-values we obtained for *E. coli* were different but the organism we used was not a pathogenic strain. The calculated D-values obtained were lower in the case of Doyle and Schoeni (1984), higher compared to Ahmed *et al.* (1995) and higher at 51.7 and 57.2 °C but lower at 62.7 °C compared to the results obtained by Line *et al.* (1991). Differences in meat composition, strains and devices used for the heat treatment (laboratory test tube vs. Whirl-pak™ sampling bag) are likely to account for such differences. Chung *et al.* (2007) demonstrated that heat transfer was quicker in capillary tubes (3 mm) than in laboratory test tubes (13 mm), generating smaller D-values since the high temperature would affect cells in the center of the sample more rapidly. Furthermore, Juneja *et al.* (1997) used a method similar to ours, with a smaller Whirl-Pak™ (18 oz.; 11.4 X 22.8 cm) and only 3 g of ground meat containing 10% fat. *E. coli* O157:H7 cells were also prepared in BHI broth. The slightly higher fat content and the difference in *E. coli* strains would likely explain the higher values they obtained at 55, 60 and 65 °C. Fat is known to protect the cell by reducing the water activity and thermal conductivity of the matrix (Ahmed *et al.*, 1995; Doyle and Schoeni; 1984). Furthermore, constituents of the food matrix can absorb part of the heat which reduces heat transfer to the cells (Gordon and Ahmad, 1991; Marcotte *et al.*, 2008). Hence, when assessing the D- and z-value for industrial process validation, it is imperative to use a food matrix as similar as possible to the food product in the validation assay.

3.5 Strain effects

The D-values were compared two by two (Bonferroni test) between each strain at each treatment temperature (Table 4). Treatments at 50 °C were not effective for *E. faecalis* and Map, and too severe at 70 °C for *E. coli* and therefore could not be compared (Table 2). *E. coli* was the least resistant of the organisms tested; D-values were the lowest when compared to *E. faecalis* and both Map strains

($P < 0.05$; Table 4). At 70 °C, when strains were compared two by two (Table 4), no significant differences ($P > 0.05$) were observed, indicating that the temperature was high enough to overcome any intrinsic resistance possessed by the organisms that may have provided an advantage for one strain or another. Similarly, the two Map strains were not significantly different at 55 °C, indicating that the temperature was not high enough to discriminate between any intrinsic resistances the strains may have had, compared to one another. But at 60 and 65 °C, the D-values for the environmental strain (gN27) were higher ($P < 0.05$ for the parametric analysis of variance) than the D-values for the laboratory strains (ATCC 19698), suggesting that the former was more heat resistant. When the D-values for *E. faecalis* ATCC 7080 were compared to Map ATCC 19896, they were significantly different only at 65 °C ($P < 0.05$; Table 4) and at that temperature *E. faecalis* was more resistant. When the D-values for *E. faecalis* ATCC 7080 were compared to the other Map strain, gN27, they were significantly lower at 55 and 60 °C but higher at 65 °C suggesting that the components and attributes that help the organism to overcome the lethal effect of heat treatment behave differently according to the temperature and the strain they are associated with. For *E. faecalis* ATCC 7080 to be considered a suitable surrogate organism to study the efficacy of heat treatment, including control of the fastidious Map strains, it should have exhibited a constantly higher D-value up to a temperature high enough to control them all in a relatively short time, here 70 °C. Hence, *E. faecalis* ATCC 7080 is not a suitable candidate for that purpose although it is recognized to be one of the most heat resistant non-spore formers (Sanz Perez *et al.*, 1982; Magnus *et al.*, 1988; Franz *et al.*, 1999). Houben (2003) studied the heat resistance of vancomycin resistant *E. faecalis* in ground pork mixed with NaCl and NaNO₂ using cells cultivated in a mix of tryptone dextrose yeast extract, meat extract and peptonised milk. Heat treatments were performed in Whirlpak™ sampling bags. At 60 °C, these authors obtained D-values ranging from 594 to 846 s, compared to 725 ± 68 and 802 ± 115 s obtained in ME2/Meat and BHI/Meat, respectively, in our experiments (Table 2).

Table 4. Strain effect on the D-values at each treatment temperature^a.

Temperature (°C)	Stains tested					
	<i>E. coli</i> vs <i>E. faecalis</i>	<i>E. coli</i> vs Map ATCC 19698	<i>E. coli</i> vs Map gN27	<i>E. faecalis</i> vs Map ATCC 19698	<i>E. faecalis</i> vs Map gN27	Map ATCC 19698 vs Map gN27
50	NE	NE	NE	NE ^{ab}	NE	NE
55	*(NS)	*(NS)	*(NS)	NS	0.04 (NS)	NS
60	*(NS)	*	*	NS	*	*(NS)
65	*	*	*	*	*	*(NS)
70	TS	TS	TS	NS	NS	NS

^a Results in ME2/Meat for *E. coli* and *E. faecalis* were used for comparison with the two Map strains.

^b * (P < 0.05); ** (P < 0.01); NS: Not significant; NE: Not Effective; TS: Too Severe.

^c The symbols in parenthesis represent the probability (P) of the non-parametric analysis of variance (discrimination by ranks). If only one symbol is presented, the results of the parametric and the non-parametric analysis of variance were similar.

In general, Gram positive bacteria, like *E. faecalis*, are more resistant to heat treatment than Gram negative bacteria such as *E. coli* (Yousef and Juneja, 2003). The cell wall of Gram positive bacteria contains a large quantity of teichoic acid. This compound is absent in Gram negative organisms. It is a polymer of glycerol or ribitol linked by a phosphate group and glycerol is known for its cryoprotectant properties (Prescott *et al.*, 1995). Furthermore, *E. faecalis* produces a viscous gel-like substance similar to polysaccharide found in biofilms. This substance allows the cells to agglomerate together providing protection against antimicrobial systems, including heat treatment (Donelli *et al.*, 2004).

3.6 z-values

The z-values for the four strains studied ranged from 5.6 ± 0.1 to 6.2 ± 0.3 °C (Table 2) and were not significantly different from one another (P > 0.05), indicating that they exhibited similar temperature sensitivity. Hence, increasing the temperature will have a similar effect on the absolute variation of the decimal

reduction time (\log_{10} D-value) in aseptically prepared ground meat. In other words, our experimental conditions required a temperature increase of 5.5 to 6.5 °C to reduce the D-value by one log unit. The z-value is a thermoresistance characteristic of each microorganism. It is less variable than the D-value and usually reaches between 4 and 7 °C for vegetative cells and 10 °C for bacterial spores (Mafart, 1991; Brennan, 2006). For enterohemorrhagic *E. coli*, Ahmed *et al.* (1995) obtained a z-value of 4.78 °C in ground meat with 7% fat and Line *et al.* (1991) obtained 7.3 °C in ground meat with 2% fat, with both using laboratory test tubes for the experiment, whereas Juneja *et al.* (1997) obtained a z-value of 6.0 °C using a Whirl-Pak™ sampling bag. In pork mixed with salt and nitrite, Houben (2003) obtained a z-value of 10 °C for *E. faecalis*, which is higher than our results (Table 2).

3.7 Cooking as a means to control Map

The thermal resistance of Map has been studied to some extent in milk but not in meat. A D-value of 21.8 s was obtained in milk at 65 °C (Sung and Collins, 1998), compared to 58 ± 11 °C and 76 ± 2 °C for Map ATCC 19698 and gN27, respectively (Table 2), in meat, indicating that meat is a more protective matrix against heat treatment than milk. The z-value was higher in milk (7.11 °C) compared to our results in meat (Table 2). At a concentration of 10^5 cfu/ml, pasteurisation at 72 °C for 15 s was not sufficient to control Map since cells could still be detected at 4 to 16 cfu/ml after treatment (Grant *et al.*, 1999). But Rademaker *et al.* (2007) obtained a D-value of 1.2 s at 72 °C by inoculating milk with the feces of an animal known to have paratuberculosis. This provided a log reduction of 12.5D in 15 s. Nevertheless, a reduction of 4 to 5 logs should be sufficient to control Map in milk (Lund *et al.*, 2002; McDonald *et al.*, 2005). In a Wieners type emulsion, Merkat *et al.* (1979) observed that a cooking time of 2 min at 71.1 °C was required to obtain a 12D reduction in *M. avium*, a slow growing *Mycobacterium*. Our results with Map indicate that 2.4 to 2.6 min would be required at 70 °C and 1.1 to 1.6 min at 71.1 °C to obtain a 12D reduction in ground beef. In another experiment with *M. bovis*, again in a Wieners type emulsion (10% fat), D-values of 3480 and 240 s were obtained at 55 and 60 °C, respectively, compared to 4255 ± 665 s and 950 ± 88 s for Map ATCC 19698, and 4828 ± 368 and 1290 ± 104 for Map gN27 in our study (Table 2; Merkat *et al.*, 1980). Here again, the stains used and the composition of the meat matrix probably account for the variation observed between studies.

Cooking at 71 °C for 10 s has been established as sufficient to control *E. coli* O157:H7 in beef patties and has become a standard practice in the industry (United States Department of Agriculture; USDA, 2004). According to our results, this cooking parameter allowed a 1.2 to 1.8D reduction in viable cells, which might not seem like much. But Jaravata *et al.* (2007) could not detect any

Map, at a detection level of 10^1 ufc/g, in any of the 200 ground beef samples tested. So the incidence in ground beef is considered low. Carcass pasteurisation has also been introduced to control *E. coli* O157:H7 in beef (Gill, 2000). Using steam at 90 °C for 10 s, the surface of the carcass can reach 73.9 °C according to Minihan *et al.* (2003). At such temperature, the calculated D-value for both Map strains tested would be between 1.8 and 2.6 s. So in few seconds, several log reductions in viable cells can be obtained. Again, Meadus *et al.* (2008) indicated that Map can be detected from a significant number of beef carcasses on the dressing line but in low numbers, which become diluted even further during grinding. As a result, because Map is present in relatively low numbers on beef carcasses, the usual heat treatments used in commercial applications seem adequate to control Map, provided that the efficacy of heat treatments is monitored adequately through a HACCP (Hazard Analysis and Critical Control Points) or quality control program at the processing level. On farms, a surveillance program to monitor the control of Map and proper herd management are mandatory to ensure that healthy animals will be slaughtered and that Map counts on meat remain low.

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