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ORIGINAL ARTICLE

Survival of *Salmonella* on cuts of beef carcasses subjected to dry aging

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Abstract

Aims: The aim of this study was to determine the survival of 15 different strains of *Salmonella* of selected serotypes during prolonged cold storage of beef.

Methods and Results: Fifteen strains of eight different serotypes of *Salmonella* were spiked onto fresh cuts beef portions, and the survival was followed during storage in a laboratory cooling system. Over a 14-day period, all strains were reduced significantly in numbers; however, strains of *Salmonella* Typhimurium DT104 and *Salmonella* Enteritidis PT4 and PT8 survived significantly longer than strains of the serovars Dublin, Derby, Infantis and Newport. For five selected strains, the observations were verified in a pilot plant cooling facility mimicking industrial cooling. No significant differences in reduction were found between the two cooling methods.

Conclusions: A significant reduction in *Salmonella* can be obtained by dry aging of beef during cold storage but the survival is strain dependent.

Significance and Impact of the Study: From a hygienic point of view, cold storage of unpacked beef, which is still performed in small slaughterhouses, is a good alternative to vacuum packaging.

Introduction

Salmonella enterica is an important pathogen attributable to major animal and human health consequences as well as significant economic burden on the society (Roberts *et al.* 2003). It consists of more than 2500 serotypes belonging to six subspecies; however, most serotypes associated with animal and human disease belong to *Salm. enterica* subspecies *enterica*. In Danish cattle, *Salmonella* Dublin and *Salmonella* Typhimurium are the dominating serotypes accounting for 64 and 18% of isolates from beef batches in 2009, respectively (Anonymous 2010).

Salmonella can be carried in the intestines of all production animals, and faecal contamination of meat is a major cause of human salmonellosis (Wegener *et al.* 2003; Humphrey 2004). In this respect, the slaughter process is critical as faecal contamination is virtually inevitable (Dincer and Baysal 2004). Risk assessments of different meat products have emphasized the importance of reducing the number of pathogenic bacteria on carcasses to reduce the number of food-borne infections (Cassin *et al.* 1998; Rosenquist *et al.* 2003).

In modern bulk production, maturation of vacuum-packed beef is accelerated by electro-stimulation. This is referred to as wet aging as opposed to traditional dry aging at low temperatures (Sitz *et al.* 2006). However, small slaughterhouses still use cold storage of unpacked beef for maturation. A preliminary investigation, reported as a conference report and performed with few strains and serotypes of *Salmonella*, indicated that dry aging may be a good alternative to vacuum packaging from a hygienic point of view (Aabo *et al.* 2002). Likewise, a reduction in the viable count of *Salm.* Typhimurium on pork carcasses during cold storage have been observed (Van Laack *et al.* 1993; Chang *et al.* 2003).

Because the report by Aabo *et al.* (2002) included few strains and only two serotypes of *Salmonella*, it is unknown whether this observation is of general relevance to *Salmonella* reduction on beef carcasses. Hence, the aim of this study was to investigate the survival of more *Salmonella* strains and strains of different serotypes during cold storage of beef.

Materials and methods

Bacterial strains and preparation of cultures

Table 1 lists bacterial strains. Wild-type strains of eight different serotypes were from the strain collection at the National *Salmonella* Reference Laboratory at the National Food Institute, Technical University of Denmark. The strains were mostly isolated in Denmark from cattle between the years 2000 and 2003.

Strains were grown aerobically in Lennox broth (Difco, BD A/S, Brandby, Denmark) at 37°C overnight and stored in Lennox broth containing 15% glycerol at −80°C until use. Lennox broth were inoculated with overnight cultures and grown at 37°C for 8·5 h without shaking to obtain a uniform cell density of *c.* 10^8 CFU ml^{-1}. Five millilitres was centrifugated at 4000 ***g*** for 15 min, and the pellet suspended in maximum recovery diluent (Oxoid, Greve, Denmark), adjusted to an optical density of 0·1 at 600 nm and a tenfold dilution in maximum recovery diluents was made to obtain an inoculation culture of *c.* 10^7 CFU ml^{-1}. The viable count of the inoculation culture was enumerated by making tenfold dilutions and plating on LB agar.

Beef portions and inoculation procedure

Cuts of flank from warm carcasses of slaughtered cattle were placed in plastic bags and transported to the laboratory in insulated containers within 2 h of slaughter. In the laboratory, portions of 10 × 10 cm were prepared and placed on wet filter paper in separate plastic trays. The sample surface, the upper side of the beef portion, i.e. 10 × 10 cm, constituted mainly of muscle tissue; however, some adipose and connective tissues were present. One hundred microlitres of the inoculation culture (10^7 CFU ml^{-1}) was spread over the sample surface with a sterile spreader giving an initial concentration of *Salmonella* of *c.* 10^4 CFU cm^{-2} of the sample surface.

Table 1 *Salmonella* serovars and strains used in the study

Serotype	Phage type	Reference ID*/Lab ID†	Source‡	Year
Salmonella Bovismorbificans		74-14162/MS20858	Faeces, calf	2002
Salmonella Derby		73-12078/MS20852	Afterbirth, calf foster	2001
Salmonella Dublin		72-13581-1/MS14334	Organs, calf	2000
Salm. Dublin		73-23093-1/MS17264	Minced beef	2001
Salm. Dublin		73-22691-1/MS17265	Faeces, cattle	2001
Salm. Dublin		73-23125-1/MS17266	Surface swabs, cattle	2001
Salmonella Enteritidis	PT4	73-12491/MS20847	Faeces, cow	2001
Salm. Enteritidis	PT8	74-10194/MS20848	Faeces, calf	2002
Salmonella Idikan		75-21816/MS20856	Isolate, cattle	2003
Salmonella Infantis		75-10748/MS20855	Intestinal, calf	2003
Salmonella Newport		74-20059/MS20857	Faeces, cattle	2002
Salmonella Typhimurium	DT12	75-13825/MS20842	Faeces, cow	2003
Salm. Typhimurium	DT104	72-22419-1/MS14329	Faeces,	2000
Salm. Typhimurium	DT104	72-13024/MS14332	Faeces, cow	2000
Salm. Typhimurium	DT104	-/MS15295	Spanish pork	2000
Salm. Typhimurium	DT104	-/MS15294	Pork tenderloin	2000
Salm. Typhimurium	DT104	-/MS15700	German tenderloin	2000
Salm. Typhimurium	DT120	75-14665/MS20843	Lungs, calf	2003
Salm. Typhimurium	DT193	74-12849/MS20844	Faeces, cow	2002

All *Salm.* Typhimurium DT104 strains were multidrug resistant, except from strain MS15294, which was fully sensitive to the test panel of the National Reference Laboratory for *Salmonella*.

*Reference ID from the strain collection at the National Reference Laboratory for *Salmonella* and zoonotic *Escherichia coli*, National Food Institute, Technical University of Denmark. Dr Dorthe Lau Baggesen provided the strains.

†Laboratory ID from the strain collection at Department of Microbiology and Risk Assessment, National Food Institute, Technical University of Denmark.

‡The strains were of Danish origin unless otherwise stated.

Storage conditions

The survival of *Salmonella* during cooling was investigated over a period of 14 days in two different cooling systems: (i) a conventional, upright refrigerator (Gram K625 NMRH, Vojens, Denmark) was used as a laboratory model, and (ii) a refrigerated container of *c.* 16 m^3 served as a slaughterhouse pilot plant cooler. In the latter, cooling was performed with and without blast with an air velocity of $0{\cdot}6 \pm 0{\cdot}1$ ms^{-1} and 0 ms^{-1}, respectively. The blast was produced in an air tunnel and was restricted to the periods with compressor activity. Under all cooling conditions, the temperature and humidity were monitored with loggers (Ebro EBI-2TH-611, Ingolstadt, Germany). In the container experiments, air velocity was monitored using a vane anemometer with data logger (Testo 400; Testo, Buhl & Bønsøe A/S, Smørūm, Denmark). In the conventional refrigerator, the temperature was 1 ± 2°C with the relative humidity varying between 70 and 100% whereas in the refrigerated container, the temperature was $3{\cdot}5 \pm 1{\cdot}5$°C and the relative humidity varied between 75 and 100%. To mimic water transport to the surface from deeper muscle layers and to avoid extensive drying of the meat samples, the filter paper, on which the meat was placed, was kept wet during the experimental period by the addition of 10 ml of sterile milliQ water every other day. The position of the inoculated beef cuts in the cooler was randomized.

In the laboratory experiments, the investigated beef samples originated from two different slaughterhouses (I and II). They were analysed in two different experimental rounds with two replicates (two beef portions) per time point. In the pilot plant cooler experiments, the beef samples originated from slaughterhouse II and the survival of selected *Salmonella* strains were investigated in one round with three replicates (three beef cuts) per time point.

Bacterial counts

At each time point, i.e. 1 h, at day 6 and day 14, the entire meat sample was transferred to a stomacher bag and stomached for 2 min in 100 ml of buffered peptone water (BPW; Oxoid) using a Stomacher 400 Laboratory Blender (Seward Medical, London, UK). For direct counts, tenfold dilutions in BPW were prepared and 100 μl was plated on Xylose Lysine Decarboxylase Agar (XLD; Oxoid) and Drigalski Agar (Bromthymol blue lactose agar, SSI, Copenhagen, Denmark). Agar plates were incubated at 37°C for 18–24 h. Direct counts were verified by checking selected suspect colonies for swarming on Modified Semi-solid Rappaport Vassiliadis medium with 20·0 mg l^{-1} novobiocin after incubation for 18–24 h at 42°C. In total, ten colonies were verified by sero- and phagetyping. Randomly selected unspiked meat samples served as controls for naturally occurring *Salmonella* contamination and were always shown to be negative.

Survival curves and statistics

Survival curves were fitted by regression analyses of $\log_{10}$ transformed CFU cm^{-2} with or without random effects using PROC MIXED within the SAS® statistical software 9.1 (SAS Institute, Inc., Cary, NC, USA). Data below the detection limit were set to one-half of the detection limit (one data point). Otherwise, missing data were replaced by estimates (six data points). The estimates were constructed from the regression line based on the remaining points. The regression line was determined from more than 30 pair-wise observations accounting for errors on both axes.

The regression lines were examined for goodness of fit by plotting the residuals *vs* the predicted values as well as a QQ-plot and Kolmogorow–Smirnov test for normality of the residuals. Duncan's multiple-range test was implemented to investigate possible clusters of the reduction coefficients (slopes) between the 15 strains. The reduction coefficients were calculated from the significant estimates of *Time* plus interactions with *Time*. The standard deviation of the reduction coefficient (SD_{reduce}) was calculated from the variance of the *Time* estimate, of the interactions with *Time* and from their covariance. To estimate the variance of *D*-values (−1/slope), a first-order Taylor approximation was used. A significance level of $P \leq 0{\cdot}05$ was used for the Duncan grouping.

Two statistical analyses were applied to investigate (i) whether survival varied between *Salmonella* strains and (ii) whether significantly different levels of reductions were observed between the two different cooling systems. In both instances, the change in concentration of the bacteria over time was examined. In the analysis of survival between strains, the factors analysed were *Time*, *Strain* and *Slaughterhouse* (I and II), plus their interactions (two- and three-way interactions). The factor *Slaughterhouse* was considered confounded with a day-block effect, because the meat from the two slaughterhouses was analysed on two different days, e.g. two biological replicates. In the second analysis, the factors were *Cooling* (refrigerator, container with air velocity and container without air velocity), *time* and *strain*, plus their interactions. *P*-values were considered significant when they were below 0·05.

Results

In our study, prolonged dry aging in a conventional refrigerator facility caused a statistically significant ($P < 0{\cdot}001$) reduction in all 15 *Salmonella* strains analysed (Table 2) over the 14-day study period. Cuts of beef were

Table 2 Reduction coefficients and *D*-values for survival of fifteen *Salmonella* strains on beef cuts during 14-day storage in the conventional refrigerator (Exp. 1)

Strain	Strain ID	Reduction coefficient* ($\log_{10}$/day)	*D*-value$_{1 \pm 2°C}$† (days)	Duncan grouping‡			
Salmonella Typhimurium DT120	MS20843	−0·216 ± 0·016	4·63 ± 0·33	A			
Salmonella Newport	MS20857	−0·215 ± 0·017	4·64 ± 0·37	A	B		
Salmonella Dublin	MS17266	−0·204 ± 0·017	4·89 ± 0·41	A	B		
Salmonella Infantis	MS20855	−0·204 ± 0·017	4·91 ± 0·41	A	B		
Salm. Typhimurium DT12	MS20842	−0·201 ± 0·017	4·96 ± 0·42	A	B		
Salm. Typhimurium DT193	MS20844	−0·191 ± 0·017	5·22 ± 0·46	A	B		
Salm. Dublin	MS17264	−0·185 ± 0·017	5·40 ± 0·50	A	B		
Salmonella Derby	MS20852	−0·184 ± 0·017	5·43 ± 0·50	A	B		
Salm. Dublin	MS17265	−0·176 ± 0·017	5·68 ± 0·55	A	B	C	
Salm. Dublin	MS14334	−0·168 ± 0·014	5·96 ± 0·50	A	B	C	D
Salmonella Bovismorbificans	MS20858	−0·162 ± 0·017	6·19 ± 0·66	A	B	C	D
Salmonella Idikan	MS20856	−0·161 ± 0·016	6·22 ± 0·60	A	B	C	D
Salmonella Enteritidis PT8	MS20848	−0·158 ± 0·017	6·34 ± 0·68		B	C	D
Salm. Typhimurium DT104	MS14329	−0·126 ± 0·017	7·94 ± 1·07			C	D
Salm. Enteritidis PT4	MS20847	−0·113 ± 0·017	8·88 ± 1·34				D

*The reduction coefficients were calculated from the significant estimates of *Time* plus *Strain*Time*. Reduction coefficient ± SD_{Reduce}.
†The $D_{1 \pm 2°C}$-values were calculated from (−1/reduction coefficients), and the variation was estimated by a first-order Taylor approximation. $D_{1 \pm 2°C} \pm SD_{D\text{-value}}$.
‡Duncan's multiple-range tests were performed on reduction coefficients (slopes). Means with same letters are not significantly different at a 0·05 level.

obtained from two different slaughter-housed and spiked and analysed in different rounds of analysis (two biological replicates). Results from these two rounds did not differ significantly ($P > 0·05$), indicating a good reproducibility of our results.

The reduction coefficients varied from $-0·216$ $\log_{10}$ day^{-1} (*Salm.* Typhimurium DT120) to $-0·113$ $\log_{10}$ day^{-1} (*Salmonella* Enteritidis PT4). This corresponds to a range in *D*-values (time to obtain one $\log_{10}$ reduction) at 1 ± 2°C between 4·6 and 8·9 days (Table 2). Duncan's multiple-range test revealed that the strains grouped into four (Duncan groups A, B, C and D) and that serotype was not the decisive factor for survival capability meaning that intraserotype variation was bigger than interserotype variation. Three strains of three serotypes (Dublin, Bovismorbificans and Idikan) were included in all four Duncan groups, indicating the survival of these strains were not significantly different from any of the other strains. The Duncan group D comprised of the multidrug-resistant (MDR) *Salm.* Typhimurium DT104 and *Salm.* Enteritidis PT4 and PT8. These strains had significantly lower reduction coefficients and higher *D*-values compared to the strains in the A and B group. Group C comprised of one strain of *Salm.* Dublin and one strain of *Salm.* Enteritidis and the three strains that grouped with all four Duncan groups. Groups A and B consisted of strains of serotypes *Salmonella* Derby, *Salm.* Dublin, *Salmonella* Infantis and *Salmonella* Newport. These strains showed the lowest survival ability.

Five strains were selected for testing the effect of cooling on survival in a pilot plant cooler. *Salmonella* Enteritidis PT8 and *Salm.* Typhimurium DT104 represented strains with a high *D*-value, whereas strains of *Salm.* Dublin, *Salm.* Newport and *Salm.* Infantis were selected because of their low *D*-values. Again, a significant reduction over time was observed ($P < 0·0001$) with a significant difference between strains ($P < 0·001$) (Table 3). No significant difference in bacterial reduction between the conventional refrigerator and the pilot plant cooling system was observed ($P > 0·05$). The Duncan's multiple-range test clustered the strains by their survival ability in two groups. Reduction coefficients of *Salm.* Dublin and *Salm.* Newport (group A) were statistically different from that of *Salm.* Enteritidis PT8 and *Salm.* Typhimurium DT104 (group B). The strain of *Salm.* Infantis clustered with both groups (Table 3). *Salmonella* Typhimurium DT104 appeared to be a good survivor on beef cuts in the pilot plant, as we had also shown earlier in the laboratory model. To rule out that this was a unique observation with an unusual strain, we tested four additional *Salm.* Typhimurium DT104 strains. They had $D_{3·5 \pm 1·5°C}$-values between 6·72 and 11·01 days, and hence, these too showed a high survival capability (data not shown).

Discussion

Our study confirms preliminary observations reported by Aabo *et al.* (2002) that dry aging at low temperature

Table 3 Reduction coefficients and *D*-values for survival of five selected *Salmonella* strains on beef cuts during 14-day storage in the combined model of conventional refrigerator and pilot plant cooling system. Pooled data from Exp. 1 (slaughterhouse II) and Exp. 2

Strain	Strain ID	Reduction coefficient* (log_{10}/day)	*D*-value† (days)	Duncan grouping‡	
Salmonella Dublin	MS14334	−0·190 ± 0·0117	5·25 ± 0·323	A	
Salmonella Newport	MS20857	−0·179 ± 0·0122	5·59 ± 0·381	A	
Salmonella Infantis	MS20855	−0·163 ± 0·0122	6·15 ± 0·463	A	B
Salmonella Typhimurium DT104	MS14329	−0·143 ± 0·0122	6·99 ± 0·597	A	B
Salmonella Enteritidis PT8	MS20848	−0·124 ± 0·0122	8·07 ± 0·796		B

*The reduction coefficients were calculated from the significant estimates of *Time* plus *Strain*Time*. Reduction coefficient ± SD_{Reduce}.
‡The *D*-value of the combined model was calculated from (−1/reduction coefficients), and the variation was estimated by a first-order Taylor approximation. *D*-value ± $SD_{D\text{-value}}$.
†Duncan's multiple-range test was performed on reduction coefficients (slopes). Means with same letters are not significantly different.

causes a significant reduction in *Salmonella* on beef carcasses. Further, we showed that this is general phenomena for strains of different serotypes. Our observations are consistent with Cutter and Rivera-Betancourt (2000) also showing reductions during dry aging, whereas Luiten *et al.* (1982) and Skandamis *et al.* (2002) did not obtain reductions by storage at 5 and 10°C. Scott and Vickery (1939) suggested that desiccation is the major factor responsible for bacterial reduction during cold storage of meat. Thus, the discrepancy between these survival studies may be related to different levels of desiccation rather than to the differences in cooling temperature.

Our data show that strains of *Salmonella* differed significantly in survival on beef cuts, both in the laboratory and in the pilot plant cooling system. *Salmonella* Dublin and *Salm.* Typhimurium are the two most important serotypes in relation to beef production in Denmark (Anonymous 2010). Of these, the strains of *Salm.* Dublin showed the lowest survival ability and the strains mostly clustered in the AB group. The low survival of these strains on meat products might cause contamination levels to be reduced to a level below the infective doses. Hence, this observation may help explain why *Salm.* Dublin is so rarely isolated from humans, despite being the most commonly isolated serotype from beef (Anonymous 2010). Strains of *Salm.* Typhimurium showed large diversity and spread out to all four Duncan groups. However, MDR *Salm.* Typhimurium DT104 showed a significantly better survival capability than strains of other definitive types of this serotype and grouped together with strains of *Salm.* Enteritidis PT4 and PT8 (Table 2). One could speculate that success of MDR *Salm.* Typhimurium DT104 as well as *Salm.* Enteritidis PT4 and PT8, which are the types of *Salmonella* associated with large pandemics (Buchrieser *et al.* 1997; Cloeckaert and Schwarz 2001), is attributable to the better survival in the food chain. *Salmonella* Enteritidis strains of the same phage type show relatively low genetic variation, but high phenotypic variation. Previous observations have indicated that the successful clones have particular traits that allow them to survive in the food chain compared to other clones, for example, a high ability to form biofilm and to form capsule (Morales *et al.* 2005; Pan *et al.* 2009). We did not include representatives of less successful clones of *Salm.* Enteritidis, but we did demonstrate a good survival capability for the two successful clones investigated. *Salmonella* Typhimurium DT104 is a diverse type; however, the MDR strains seem to belong to one clone (Lan *et al.* 2009). Efforts to understand the success of this clone has focussed on virulence factors (Allen *et al.* 2001; Fratamico 2003) and stress adaptation (Fratamico 2003). None of these factors distinguished MDR *Salm.* Typhimurium DT104 from non-DT104 strains. Based on our results, it may be that a better survival through the slaughter process may play a role in selection of this clone.

The relative difference in survival ability we observed between *Salm.* Dublin and *Salm.* Typhimurium is consistent with previous reports. Kerr and Sheridan (2002) found a change in *Salmonella* isolation pattern on carcasses from prior chilling to after chilling. *Salmonella* Typhimurium increased from 78% of the positive samples to 91% when comparing samples taken before and after chilling, while *Salm.* Dublin was reduced 18–2% in the same samples.

Five strains were selected for testing in a pilot plant cooling system to investigate whether the laboratory experiments reflected effects that could be seen under more realistic conditions. The bacteria *D*-values did not differ between the conventional refrigerator and the pilot plant cooling system. Moreover, the statistical analysis clustered strains by their survival ability in two groups showing the same relative survival capability as seen in the laboratory experiments. This strongly indicates that

the more comprehensive testing we performed in the model system (standard refrigerator) was of relevance to the real situation.

The lack of difference in survival between the conventional kitchen refrigerator and the pilot plant cooler was somewhat surprising as mean temperatures were lower in the conventional kitchen refrigerator (1 ± 2°C) compared to 3·5 ± 1·5°C in the pilot plant cooler. Contrary to our results, Chang *et al.* (2003) found a difference in reduction in *Salm.* Typhimurium ATCC 14028 on pork bellies when including a rapid chilling to −20°C for 3 h followed by 21 h at 4°C compared to 24 h at 4°C. They concluded that the rapid chilling to −20°C causes a potential cold shock, which is lethal for the cell. Their finding could, however, also be influenced by a potential increased desiccation during blast chilling.

In conclusion, our results show that significant reduction in concentrations of *Salmonella* can be obtained passively by dry aging of meat. Dry aging is thus an alternative intervention method to chemical and physical decontamination and may be the most cost effective method for small slaughterhouses. However, it is important to realize that other pathogens may not be inactivated to the same extent as *Salmonella* by dry aging. For example, it has been shown that *Campylobacter* ssp. and *Listeria monocytogenes* survive with higher *D*-values than shown by us for *Salmonella* (Van Laack *et al.* 1993; Chang *et al.* 2003).

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