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Survival of *Listeria monocytogenes* Scott A on metal surfaces: Implications for cross-contamination

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Abstract

Listeria monocytogenes is an important re-emerging pathogen which is commonly found in the environment. Many outbreaks have been associated with the contamination of food produce, often linked to cross-contamination from surfaces or equipment to prepared foodstuffs. In the present study a number of copper-base metal alloys have been used to assess the survival times of *L. monocytogenes* on different materials, in comparison with stainless steel. High concentrations (10^7) of bacteria were placed on metal coupons cut from each alloy. After defined incubation times, coupons were placed in tubes containing phosphate buffered saline and vortexed to remove the cells. Aliquots were then plated onto tryptone blood agar plates and the number of colony forming units counted. The high concentration of bacteria was used to represent a "worst-case" scenario. The results indicate that survival is greatly reduced on a copper-base alloy compared to stainless steel. Viable cells could be detected on stainless steel after 24 h incubation at room temperature. On copper, brass, aluminium bronze and silicon bronze, no viable bacteria could be detected after 60 min incubation, indicating a 5 log reduction (the detection limit of the procedure was 100 bacteria). No cells could be detected from copper nickel and copper nickel zinc alloys, after 90 min incubation. The viability stain, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), confirmed these results, with actively respiring bacteria being clearly labelled on stainless steel after 24 h. The results suggest that careful choice of surface material could reduce the potential risk of cross-contamination in industrial, commercial and domestic environments.

Keywords: *Listeria monocytogenes*; Copper alloys; Survival; Cross-contamination

1. Introduction

The Centres for Disease Control and Prevention (CDC) reported, in 1999, that listeriosis had the second highest fatality rate (20%) and the highest hospitalisation rate (90%) of all the diseases caused by foodborne pathogens which are being monitored. On average, there are approximately 2500 cases of foodborne listeriosis each year in the US, with around 500 fatalities (Mead et al., 1999). It is especially dangerous for pregnant women, the elderly and those with immunocompromised diseases such as HIV (Farber and Harwig, 1996). Beumer and Kusumaningrum (2003) report that in Europe and North America, more than half the registered food infections are contracted in homes and name *Listeria monocytogenes* as a serious risk in refrigerated produce.

L. monocytogenes is widely distributed in the environment (Weiss and Seeliger, 1975) and survives for extended periods of time in matrices such as sewage sludge spread to land (Nicholson et al., 2000; Garrec et al., 2003). Beuchat (1996) found *L. monocytogenes* to survive for 10–12 years in plant materials. Garrec et al. (2003) estimated that the spreading of treated sludge to land can add 10^6–10^8 *L. monocytogenes* per hectare per year. The pathogen has been isolated from soil (Welshimer, 1960: Van Renterghem et al., 1991; MacGowen et al., 1994), silage (Gray, 1960; Ryser et al., 1997) and surface waters (Bernagozzi et al., 1994). Some humans and animals can also be carriers (Skovgaard and Norrung, 1989; MacGowen et al., 1994).

Several studies have investigated the potential risk of contamination in food processing environments (Lawrence and

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Table 1
Alloy composition (weight %) (data supplied by the Copper Development Association Inc., US)

Alloy UNS no	Cu	Zn	Sn	Ni	Al	Mn	Fe	Cr	P	Si	Ti	Mg
Coppers												
C10200	100											
C11000	100											
C18080	99						0.1	0.5			0.1	
C19700	99						0.7		0.3			
Brasses												
C21000	95	5										
C22000	90	10										
Y90*	78	12		3		7						
Bronzes												
C51000	95		5						0.2			
C61500	90			2	8							
C63800	95				3					2		
C65500	97					1				2		
C68800	74	23			3							
Cu–Ni												
C70250	96			3						0.7		0.2
C70600	89			10			1					
C71000	79			21								
C71300	75			25								
C71500	70			30								
C72900	77		8	15								
Cu–Ni–Zn												
C73500	72	10		18								
C75200	65	17		18								
C77000	55	27		18								
Stainless steel												
S30400	0			8			74	18				

Cu=copper, Zn=zinc, Sn=tin, Ni=nickel, Al=aluminium, Mn=manganese, Fe=iron, Cr=chromium, P=phosphorus, Si=silicon, Ti=titanium, Mg=magnesium. (Wilks et al., 2005).

Gilmour, 1995; Salvat et al., 1995; Chasseignaux et al., 2002; Bornert et al., 2003; Coleman et al., 2003; Gailey et al., 2003; Gianfranceschi et al., 2003; Martinez-Gonzales et al., 2003; Wallace et al., 2003). Contamination is a serious risk in ready-to-eat foods (Salvat et al., 1995) and has led to the publication of "FSIS Risk Assessment for *Listeria* in Deli Meats" (Gallagher et al., 2003). In other studies, the risk of surface contamination has been investigated (Salvat et al., 1995; Chasseignaux et al., 2002; Gianfranceschi et al., 2003; Martinez-Gonzales et al., 2003). Contamination often occurs between "raw" and "cooked" meats and contact of cooked produce with contaminated surfaces (Lawrence and Gilmour, 1994; Salvat et al., 1995; Jay, 1996; Chasseignaux et al., 2002; Martinez-Gonzales et al., 2003). Cross-contamination during domestic washing up has also been studied (Mattick et al., 2003) and illustrates the high potential for this to occur.

A recent study by Wilks et al. (2005) found that the persistence and survival of *Escherichia coli* O157 was greatly reduced on copper alloys when compared to stainless steel. No viable bacteria could be recovered from high copper alloys after less than 90 min at room temperature. Similar effects were also observed in previous studies (Keevil et al., 1999). Other studies have found the survival of *Legionella pneumophila* to vary on different materials (Domek et al., 1984; Schoenen and Schlomer, 1989; De Veer et al., 1994; Rogers et al., 1994a,b). Copper has strong antibacterial properties but it is soft and not durable, making it unsuitable for use in food processing areas. Copper is also susceptible to acidic substances and oxidation. However more durable and resistant copper-containing alloys may offer the same antibacterial characteristics and be appropriate for use in such environments. The current study aimed to investigate the effects of different metal alloys on the survival of *L. monocytogenes* as their use could lead to important improvements in public health in food processing, domestic and healthcare environments. Bacterial survival was assessed using a combination of direct plating onto agar media and an in situ vitability staining procedure.

2. Materials and methods

2.1. Culturing

L. monocytogenes Scott A was originally supplied by the Centre for Applied Microbiology Research (CAMR, Porton Down, UK). Stocks were maintained on microbeads and stored frozen at −80 °C (Protect system, Fisher Scientific, Loughborough, UK). For each new experiment fresh cultures were grown on brain heart infusion broth (Oxoid, Basingstoke, UK) at 37 °C for 15–20 h before use (as they entered stationary phase).

2.2. Experimental method

A 20-µl aliquot of fresh culture, containing 10^7 bacteria, was placed on each coupon to be tested. The coupons were housed within a plastic container to minimise contamination from the laboratory environment and left for defined incubation times

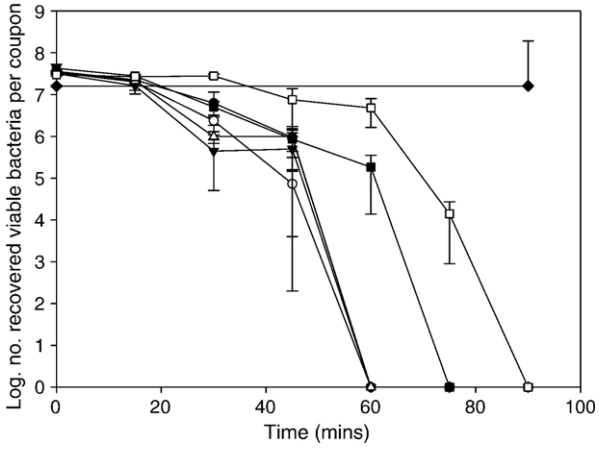

Fig. 1. Survival of *Listeria monocytogenes* on various metal alloys at room temperature. Survival has been assessed in terms of colony forming units from bacteria recovered from the metal coupons. Mean values are given as well as the standard error (shown as bars). ● Copper (UNS C10200), ▽ brass (UNS C22000), ▼ aluminium bronze (UNS C63800), ○ silicon bronze (UNS C65500), ■ copper nickel (UNS C70600), □ silver nickel (UNS C75200), ◆ stainless steel (UNS S30400).

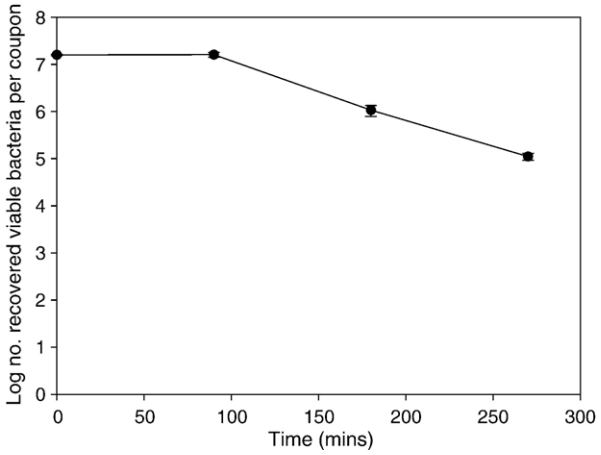

Fig. 2. Survival of *Listeria monocytogenes* on stainless steel at room temperature. Survival has been assessed in terms of colony forming units from bacteria recovered from the metal coupons. Mean values are given as well as the standard error (shown as bars).

(time zero equals the moment when bacteria were placed on the coupon). After the required incubation time at room temperature (20±1 °C), the coupons were transferred into sterile 50-ml centrifuge tubes containing 10-ml of autoclaved phosphate buffered saline (PBS, pH 7.3) (Oxoid, Basingstoke, UK) and 2-mm diameter glass beads (sterilised by autoclaving) (VWR, Poole, UK). These were then mixed thoroughly using a vortex mixer for 1 min. Serial dilutions were made in PBS.

A 50-µl aliquot of each dilution was pipetted onto a tryptone blood agar plate (Oxoid, Basingstoke, UK) which had been prewarmed to 37 °C. This was then spread over the surface of the plate using a sterile, disposable, plastic L-shaped spreader. Agar plates were incubated, lid down, at 37 °C, under microaerophilic conditions (Campygen sachet, Oxoid, Basingstoke, UK) for 18–24 h before colonies were counted. Microaerophilic conditions were used for the recovery of possibly injured cells. All experiments were replicated at least three times. This procedure gave a detection limit of 100 bacteria by counting colony forming units. Additional experiments were carried out at the time points where no viable bacteria could be detected. These involved filtering the total 10-ml volume of PBS onto a 0.2-µm pore diameter membrane, which was then transferred onto a tryptone blood agar plate and incubated as previously described.

2.3. Copper alloys and stainless steel

Alloys of various copper content and other metals, as well as stainless steel, were provided by the Copper Development Association, New York (for composition, see Table 1). A total of 25 metal alloys were tested which fall into seven groups; coppers, brasses, aluminium bronze alloys, silicon bronze alloys, copper nickel alloys, nickel silvers and stainless steel. The data from a representative alloy from each group will be presented here.

2.4. Epifluorescence microscopy techniques

Bacteria were also observed directly on the various coupons using the bacterial vitability stain, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Polysciences Inc., Warrington, US). The methods used followed the procedure provided by the company. CTC is a monotetrazolium redox dye which, when biologically reduced produces a fluorescent insoluble formazan. This means that actively respiring bacterial cells fluoresce bright red after the application of this dye (Rodriguez et al., 1992). *L. monocytogenes* contaminated coupons were placed in sterile Petri dishes and 1-ml CTC (final concentration 4.0 mM) added. The Petri dishes were incubated, in the dark, at 37 °C for 2 h. Following incubation, excess stain was removed and the coupon gently flooded with filter-sterilised deionised water. The coupons were examined under episcopic differential interference contrast (EDIC) and epifluorescence illumination on a Nikon Eclipse ME600 microscope (Best Scientific, Swindon, UK), using long working distance objectives (Keevil, 2003). EDIC illumination allows the imaging of opaque samples and so in this case was used to examine the coupons without the removal of bacterial cells.

3. Results

It was found that *L. monocytogenes* survived for different periods of time on different metal alloys. The detection method for this direct plating procedure was 100 bacteria representing a maximum 5 log reduction in cell number from the initial 10^7 inoculum. The efficiency of cell removal (by vortex mixing with glass beads) was assessed by comparing direct plate counts with microscopic counts at time zero. It was found that bacteria could be removed efficiently using this procedure (results not shown).

The alloy UNS C102000 is composed of 100% copper (Table 1) and no viable bacteria (assessed as colony forming units, cfu) could be detected after 60 min incubation at room temperature (representing a 5 log reduction) (Fig. 1). The same pattern was observed for alloys UNS C22000, UNS C63800 and UNS C65500 (Fig. 1). UNS C22000 is a brass alloy containing 90% copper (Table 1). Alloy, UNS C63800 is an aluminium bronze alloy and contains 95% copper (Table 1). The remaining member of this group, UNS C65500, is a silicon bronze with a composition of 96% copper (Table 1).

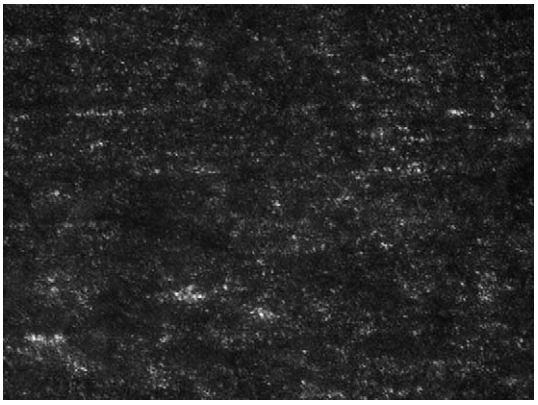

Fig. 3. Micrograph showing copper (UNS C10200) covered with a mat of bacterial cells, the coupon had been left for 90 min at room temperature. Episcopic differential interference contrast (EDIC) image showing a dense mat of bacteria on the surface of the copper coupon. Mag. ×500.

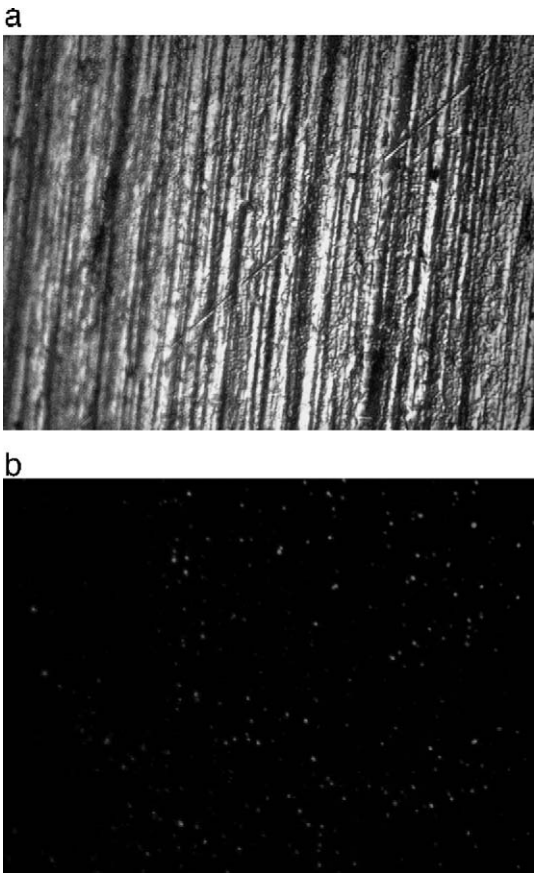

Fig. 4. a and b. Micrographs showing stainless steel (UNS S30400) covered with *L. monocytogenes* after 24 h incubation at room temperature. a. EDIC image showing a dense mat of bacteria on the surface of the stainless steel coupon. b. Epifluorecsence image showing bacteria labelled with the viability stain, CTC. Mag. ×500.

The copper nickel alloy, UNS C70600, exhibited a 2 log decrease in viable bacterial numbers after 60 min incubation, with no further viable bacteria being detected after 75 min (Fig. 1). This alloy contains 89% copper. The remaining copper alloy, UNS C75200 which is a nickel silver alloy, contains only 65% copper. After 60 min incubation on this alloy, there had only been a half log reduction in bacterial numbers, followed by a 3 log decrease after 75 min incubation. No further viable bacteria could be detected after 90 min incubation at room temperature (Fig. 1). Both these two groups of alloys showed an initial slight reduction in cell numbers followed by a decrease to no detection of viable bacteria. This could result from the drying of the sample (at room temperature the aliquot of bacterial culture had usually dried within 90 min) or may represent a lag time before the antibacterial action of the copper components can have an effect. In all cases a high inoculation level was used to represent a "worst-case" scenario, the lag time maybe increased due to the number of bacteria present, with immediate effect on the cells in direct contact with the metal followed by diffusion through the remaining sample.

For all alloys assessed, the filtration method described in the Materials and Methods section was used to check the time point where no viable cells could be detected. In all cases, this was the same as when the direct plating method was used.

In contrast, stainless steel (UNS C30400) showed no significant reduction in bacterial numbers over the 90 min incubation time. Extended incubation time experiments showed a less than 2 log decrease after 270 min exposure at room temperature (Fig. 2).

These results are supported by the microscopic observations made using the viability stain, CTC. When spiked coupons of the alloy UNS C10200, which had been left at room temperature for 90 min, were examined using the EDIC microscope, it was possible to see the thick mat of bacteria indicating that the cells had not been removed during the incubation and washing procedure (Fig. 3). However, when the same coupons were examined using the epifluorescence optics, no labelling was observed, indicating that none of these bacterial cells were actively respiring (results not shown). Stainless steel coupons gave different results after being left at room temperature for the same time period (90 min). Fig. 4a and b show the EDIC and epifluorescence images from a stainless steel coupon which had been left at room temperature for 24 h. It is clear that many of the bacteria were actively respiring causing reduction of the CTC and fluorescence of the cells and hence, had remained viable.

4. Discussion

The current study has examined the antibacterial properties of a range of metal alloys against the emerging pathogen, *L. monocytogenes*. A total of 25 alloys has been tested which can be separated into seven groups; coppers, brasses, aluminium bronze alloys, silicon bronze alloys, copper nickel alloys, nickel silvers and stainless steel. All experiments were carried out at room temperature (20±1 °C). The results from a representative alloy from each group have been presented here. The data obtained clearly shows that the survival and persistence of *L. monocytogenes* does differ for different surface materials. In general, the results with this Gram positive pathogen follow the same pattern as a previous study using the same metal alloys but examining survival of the gram negative *E. coli* O157 (Wilks et al., 2005).

Stainless steel is widely used as a surface material in a number of environments where there is a risk of bacterial cross-contamination occurring. This ranges from food handling industries, abattoirs and domestic kitchens to hospital environments and to potable water supply systems. Stainless steel is often the preferred material because of its durability, ease of cleaning, resistance to chemical degradation, and corrosion resistance. However, previous studies have shown that stainless steel may not be the most suitable metal with regard to its poor antibacterial properties (Kuhn, 1983; Robine et al., 2002; Wilks et al., 2005). Wilks et al. (2005) found *E. coli* O157 to survive for more than 28 days at refrigeration and room temperatures on stainless steel. In the same study, the authors found that alloys containing copper did have significant antibacterial characteristics. In the current study, *L. moncytogenes* was found to be viable after 24 h incubation on stainless steel under normal atmospheric conditions. Studies have examined the adherence of *L. monocytogenes* and other foodborne pathogens to stainless steel (Hood and Zottola, 1997), indicating that the attachment efficiency could be related to the media in which the bacteria are growing. There is also evidence that the

surface roughness and grade of stainless steel can have an impact on surface contamination (Boulange-Petermann, 1996; Hilbert et al., 2003; Meyer, 2003). A number of studies comment on the fact that developed biofilms are more difficult to remove using standard disinfectants and detergents (Sharma and Anand, 2002; Jessen and Lammert, 2003; Kusumaningrum et al., 2003; Meyer, 2003). It is therefore essential to minimise the risk of biofilm formation by using good sanitation procedures. However, the ability to select suitable materials which have inherent antibacterial properties could be a useful additional control, especially when standard hygiene procedures fail.

The current study has shown how different metal alloys can exhibit different antibacterial characteristics and this could be exploited in the future manufacture and design of equipment used in high risk settings. Unalloyed copper is thought to be soft and not as durable as many of the other alloys. They also have a poor tarnish and corrosion resistance. However, copper alloys containing nickel, tin and/or other alloying elements overcome some of these physical limitations, but their potential antibacterial characteristics are only now being investigated (Wilks et al., 2005).

The antibacterial characteristics of copper itself have been studied (Nies, 1999) and cause damage to the cells by the production of hydroperoxide radicals (Rodriguez-Montelongo et al., 1993) and disruption of the cell membrane (Suwalsky et al., 1998). Gordon et al. (1994) found the effects of copper at micromolar concentrations, on the growth of various bacteria, to range from undetectable to complete inhibition. Likewise experiments examining the effects of copper toxicity on the sulphate-reducing bacterium, *Desulfovibrio desulfuricans*, found reductions in total cell protein, lengthening of lag times, lower specific growth rates and even complete inhibition of growth (Sani et al., 2001). The current study and previous work (Wilks et al., 2005) have shown how various copper-containing alloys can reduce bacterial survival significantly. These studies have found alloys containing at least 65% copper have greater antibacterial properties. Alloys such as the copper nickels and copper nickel zinc alloys, commonly called nickel silvers exhibit this increased antibacterial property, but also have the added advantage of greater anticorrosion and anti-tarnishing characteristics and so would be suitable for use in industrial, commercial and domestic environments.

It is clear that whilst controls which have been employed in recent years have reduced the number of *L. monocytogenes* infections in Europe and North America (McLauchlin, 1996; De Valk et al., 2000), this pathogen continues to pose a serious risk to public health. The risk of cross-contamination in food production remains of great concern and although recent controls have been implemented, any measures which could reduce the risk should be examined. This study does suggest that by changing the material used for surfaces and equipment, it is possible to reduce the risk of contamination which can occur when hygiene procedures are not employed correctly. The results are similar to those from the previous study examining the survival of *E. coli* O157 (Wilks et al., 2005) and work is ongoing to investigate the effects on other pathogens causing serious public health concerns.

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