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2 **Title page**

3 Effect of humidity and temperature on the survival of *Listeria monocytogenes* on
4 surfaces

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Significance and Impact

Understanding survival of potential food-borne pathogens is essential to the safe production and preparation of food. Whilst it has long been 'common knowledge' that relative humidity can affect the growth and survival of microorganisms, this study systematically describes the survival of *L. monocytogenes* on stainless steel under varying humidity and temperatures for the first time. The outcomes from this paper will allow those involved with food manufacture and preparation to make informed judgement on environmental conditions relating to humidity control, which is lacking in the food standards guidelines.

Abstract

Listeria monocytogenes is a pathogenic bacterium, with human disease and infection linked to dairy products, seafood, ready-to-eat meat and raw & undercooked meats. Stainless steel is the most common food preparation surface and therefore, it is important to understand how food storage conditions such as surface materials, temperature and relative humidity can affect survival of *L. monocytogenes*. In this study, survival of *L. monocytogenes* on stainless steel was investigated at three temperatures (4, 10 and 21°C), each approx. 11%, 50% and 85% humidity. Results indicate that the lower the temperature, the more cells were recovered in all three humidity environments, whilst medium humidity enhances survival, irrespective of temperature. Lower humidity decreases recovery at all temperatures. These data support the guidance noted above that humidity control is important, and that lower humidity environments are less likely to support retention of viable *L. monocytogenes* on a stainless steel surface.

Keywords

Food

Listeria

Food preparation

Humidity

Stainless steel

Introduction

Listeria monocytogenes is a pathogenic bacterium, with human disease and infection linked to dairy products, seafood, ready-to-eat meat and raw & undercooked meats. Listeriosis, encompassing bacterial meningitis, sepsis, endocarditis, neonatal abortion and stillbirth in humans (Schlech et al. 1983), usually presenting in those already immunosuppressed, pregnant, old or young (Scholing et al. 2007; Barocci et al. 2015). During the late 1990s there was a large outbreak of listeriosis linked primarily to consumption of pâté (McLauchlin et al. 1991). Investigations resulted in the discovery of *Listeria* in cheese and other cook-chill foods, subsequently leading to an increase in regulation surrounding chilled food storage (ACMSF 2003).

Studies on the interaction between *L. monocytogenes* and stainless steel, the most common surface used in food preparation, have found that the survival of the microorganism on the surface alters depending on contact time, temperature, nutrients, moisture and the presence of other microorganisms (Bremer et al. 2001; Poimenidou et al. 2009; Skovager et al. 2013a). Additionally, survival of *L. monocytogenes* can be decreased by introducing antimicrobial compounds such as Lauric Arginate into stainless steel *(Saini et al. 2013)*, or by coating a stainless steel surface with an antimicrobial film, for example, TiN/Ag (Skovager et al. 2013b). However, inert stainless steel is the most suitable for the food industry due to its non-toxic, easy-clean, mechanically stable and corrosion-resistant properties (EHEDG 2004). In short, if contaminated food product requires preparation prior to packaging/cooking, for

example in a food processing plant, surfaces such as stainless steel worktops or conveyor belts pose cross-contamination potential. Whilst this is not the only source of contamination, with factors such as hygiene and disinfection being important, the environmental conditions are critical to ensure there is little opportunity for growth of microorganisms on surfaces and that survival is minimal.

Although surface characteristics such as roughness and wettability are important variables when considering survival of microorganisms on steel, other environmental conditions are likely to play a key role. An increase in relative humidity (RH), a measure relating to amount of water vapour in the atmosphere, has been shown to prolong survival of *L. monocytogenes*, as well as encourage growth when inoculated on fresh produce (Likotrafiti et al. 2013), whilst a decrease in RH has demonstrated a decreased survival of *L. monocytogenes* (Zoz et al. 2016). Conversely, reduction in RH has been shown to enhance transfer of *L. monocytogenes* from biofilm to meat products potentially due to increased capillary action within the food (Rodríguez et al. 2007). Control of relative humidity in relation to control of microbial contamination in food processing environments is suggested by many governments around the world (e.g. FDA 2009; Abu Dhabi Food Control Authority 2010; FSA 2015), and advice is available (EHEDG 2006). However these documents do not recommend specific levels of RH, likely due to the complex and unique nature of each food processing environment.

The ability for *L. monocytogenes* not only to survive but also to grow across a relatively wide temperature range, often described in the literature as between 2°C to 45°C, means that refrigerated food is not necessarily protected from microbial colonisation by *L. monocytogenes* (Gandhi and Chikindas 2007). Given the variety of surface materials, temperatures and RH combinations possible in the manufacture, transport and consumption of food, it is important therefore to understand the effect of temperature and RH on the survival of *L. monocytogenes* on surfaces. This study will investigate the

survival of *L. monocytogenes* on stainless steel in three different humidity-controlled environments, selected as examples of the possible range of humidity in a food processing location (although not all are likely to be encountered - approx. 11%, 50% and 85%), at three different temperatures.

Results and Discussion

The aim of this study was to investigate the survival of *L. monocytogenes* on stainless steel over time with respect to temperature and humidity. The experiment used *L. monocytogenes* in its planktonic state as inoculum. Biofilm is unlikely to form in this environment because good hygiene practice should remove the possibility of *L. monocytogenes* building a biofilm on a food preparation surface. The focus was survival since growth was unlikely.

Surface profiles

The average Ra value for SS 304 was 42.65nm whilst the average Ra value for SS 316 was 41.12nm. There was no significant difference ($P>0.05$) in the Ra values between the two surface types, but surfaces were visually different, with SS 304 appearing smoother with fewer defects compared to SS 316 (figure 1).

Recovery of cells from SS 304 following incubation in controlled humidity and temperature

The viability of cells recovered from the sample in low humidity decreased as time and temperature increased (figure 2). After one hour, no cells were recovered from any surface.

At medium humidity (figure 3), as temperature increased, viability decreased, although this is less obvious than at low humidity. At 4°C there was no decrease in survival, indeed the opposite was observed, with the number of cells recovered increasing.

As time and temperature increased, viability was also reduced at high humidity (figure 4). This decrease was statistically significant ($p<0.05$) between 5h and 7h at 4°C and 10 °C ($P>0.05$).

Overall, it appears a medium level of humidity is optimum for survival of *L. monocytogenes* on SS 304, with the change of humidity being most important in supporting survival irrespective of temperature.

Recovery of living cells from SS 316 following incubation in controlled humidity and temperature

No cells were recovered at low humidity/21°C on SS 316 after incubation (figure 5). Cells recovered after incubation at high humidity/4°C (figure 5) reduced following a similar trend to that observed on SS 304.

Acridine orange (AO) staining of SS 304 and SS 316 to assess retention on surface after swabbing

The average percentage coverages of cells on SS 304 and SS 316 were 74.97% and 65.65% respectively, when unswabbed coupons were visualised with AO. After swabbing the coverage decreased significantly ($p<0.05$). There was no significant difference ($P>0.05$) in the percentage average of cells on the surfaces, with SS 304 and SS 316 presenting 2.08% and 3.59% respectively, indicating effective swabbing.

During the study it was observed that samples incubated at medium or high humidity became wet, despite being dried before incubation, likely due to the water vapour in the environment. It has been shown previously that the presence of moisture on a surface can loosen cells from a surface and increase the number of cells recovered by swabbing (Verran et al. 2010), which is a possible explanation for the varied counts recovered. It is also possible that as the inoculum is rehydrated, any cell division initiated might continue, increasing the number of recovered cells.

A critique of this methodology is the equal drying time and conditions each sample received prior to incubation in different temperatures and humidity. Whilst it was important in this study to control the drying conditions to be able to draw comparisons, the authors acknowledge that within a real life scenario it is possible that contamination will 'dry' dependant on the ambient humidity it is stored in, which is likely to vary the survival time of the microorganism.

Findings show that the lower the temperature, the more cells are recovered from steel when incubated in any of the three humidity environments. Not many cells are retained on the surface, so essentially viability is indicated by recovery. Interestingly, studies on survival of *L. monocytogenes* on biotic surfaces, for example Likotrafiti et al. (2013), have shown that a reduced temperature decreases the number of recovered cells when in low humidity environments.

Results relating to SS 316 show no significant difference between survival in relation to temperature and humidity, with very few cells remaining on the surface after swabbing. These data indicate that the application of a finish to steel (for example, bright annealed) did not affect ease of cleanliness.

However, the data suggest that "medium" humidity enhances survival, irrespective of temperature, presumably because of a decrease in stress to cells. Lower humidity decreases recovery at all temperatures, whilst high humidity decreases recovery at high temperatures, presumably due to an increase in stress.

It is likely *in situ* that humidity will be controlled within the food industry environments, however, as discussed in the introduction, humidity control is not dictated by legislation, and is therefore likely to be variable across the sector. Low and high humid environments can be uncomfortable and potentially dangerous to human health (Davis et al. 2016), and therefore a humidity closer to 50% is more likely. However, in a food processing environment, personnel are not the focus: the results of this study suggest

this is the least favourable option for reducing viable *L. monocytogenes* on stainless steel.

It is likely that environments may where food is prepared and/or stored with no humidity control. Whilst no specific guidance could be found for humidity control in such circumstances, it is recognised as one measure for the control of bacterial contamination. Our data support the guidance referenced earlier that humidity control is important, and that lower humidity environments are less likely to support retention and survival of viable *L. monocytogenes* on a stainless steel surface. It is likely that storage will always be at a low temperature, so humidity control is critical if the low temperature itself increases survival.

Materials and Methods

Microorganisms

Listeria monocytogenes Scott A, serotype 4 (kindly donated by Professor Lone Gram (Danish Institute of Fisheries Research (DIFRES), Technical University of Denmark) (Briers et al. 2011) was maintained on Tryptone Soya Agar (TSA) (Oxoid, Basingstoke) at 5°C and inoculated into 100 ml^{-1} Tryptone Soya Broth (TSB) (Oxoid). Cultures were grown overnight (22 ± 1h) at 30°C with agitation (225 rpm). Cells were harvested by centrifugation (3600 rpm, 10 min, room temperature) and washed once in 0.85% NaCl (Oxoid), resuspended to optical density (540nm) of 1.0. A 1 ml^{-1} sample from the cell suspension was serially diluted, plated out onto NA and CFU counted, finding the cell concentration to be 3.18 ± 0.65 $x10^9$ CFU/ml^{-1}. This was used for the initial inoculum of stainless steel coupons.

Preparation of stainless steel

Bright annealed 304 stainless steel (SS 304) and 2B 316 stainless steel (SS 316) (Outokumpu, Sheffield, UK) were cut into coupons (2cm x 2cm x 1mm) using a guillotine. The steel coupons were soaked in 96% ethanol overnight to remove/inactivate microorganisms and remove grease from the surface (BSSA n.d.), after which they were rinsed with distilled water and air dried for one hour in a class two cabinet (BH-EN 2003, Faster, Cornaredo).

White light profilometry

A MicroXAM (phase shift) surface mapping microscope (ADE; Omniscan, Wrexham) with an analogue to digital (AD) phase shift controller (Omniscan) was coupled with an image analysis system (Mapview AE 2.17; Omniscan) to visualise the surface and provide Ra values.

Humidity control

Humidity was controlled using saturated salt solutions contained within a desiccator chamber (250mm diameter, Fischer Scientific, Loughborough UK). Salts used were; lithium chloride (Fischer Scientific) to achieve a low humidity approximately 11%RH, magnesium chloride (Fischer Scientific) to achieve a medium humidity approximately 50%RH and potassium sulphate (Fischer Scientific) to achieve a high humidity approximately 85%RH (Rockland 1960). Water was added to the salts until a slushy mixture filled the bottom of the chamber. The saturated salt solution was left in the chamber for 24 hours prior to the start of the experiment to allow the desired RH to be attained. Relative humidity and temperature were monitored with a mobile USB data logger (RHT10, Extech Instruments, Boston, USA).

The effect of humidity and temperature on the survival of *Listeria monocytogenes* on stainless steel

Stainless steel coupons were inoculated with $10\mu l^{-1}$ of standardised *Listeria monocytogenes* Scott A planktonic cell suspension, and spread across the surface using a

sterile pipette tip. Coupons were left to dry for 30 minutes in a class two cabinet at room temperature prior to being placed in the desiccator containing the appropriate saturated salt solution on a platform approximately 4cm above the salt solution. The desiccator was then placed inside an incubator at the appropriate temperature. At each sample time, each coupon was swabbed with a moist swab which was placed in $10ml^{-1}$ of 0.85% saline and diluted to 10^{-8}. Dilutions were plated out onto TSA, incubated for 24h at 30°C and colonies counted.

Variables investigated were low, medium and high humidity, each at 4°C, 10°C and 21°C on SS 304. Low humidity and 21°C and high humidity and 4°C were investigated on SS 316. All temperatures were maintained to within 1°C, except at sampling time when temperature could vary ±3°C. Sampling was carried out at 0h, 1h, 5h, 7h and 24h hours. Three replicates of each surface were tested at each time point. Experiments were repeated once.

Bacterial staining to assess swabbing effectiveness adapted from Airey and Verran (2007).

Cells retained on sample coupons, pre and post swabbing, were stained with acridine orange (Sigma, Dorset) (0.03% in 2% glacial acetic acid) (VWR, Lutterworth), and the surfaces were rinsed and dried before examination with epifluorescence microscopy (x100) (Nikon Eclipse E600; Nikon UK Ltd, Surry). Ten random fields of each replicate surface were examined. The percentage of an area of each microscopic field covered by cells was calculated by using cell F software (Olympus Soft Imaging Solutions). The experiment was repeated once.

Data analysis

Data were analysed in SPSS® 21 for Windows (IBM, USA) and Excel® 2013 (Microsoft, USA). Statistically significant differences were tested for using a one-way ANOVA. Data

are presented as percentage changes compared to the CFU ml^{-1} recovered from steel sample before incubation. Initial recovered CFU ml^{-1} can be found in the caption for the corresponding figure.

Conflict of Interest

No conflict of interest declared

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252 References
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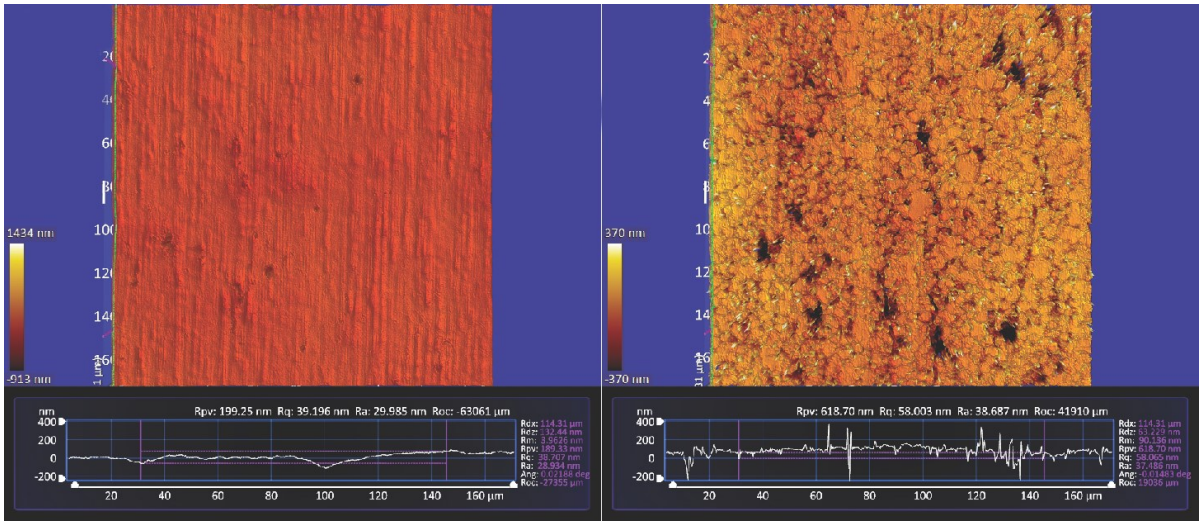


Figure 1 –Example WLP images of SS 304 (left) and SS 316 (right) taken at x50 magnification.

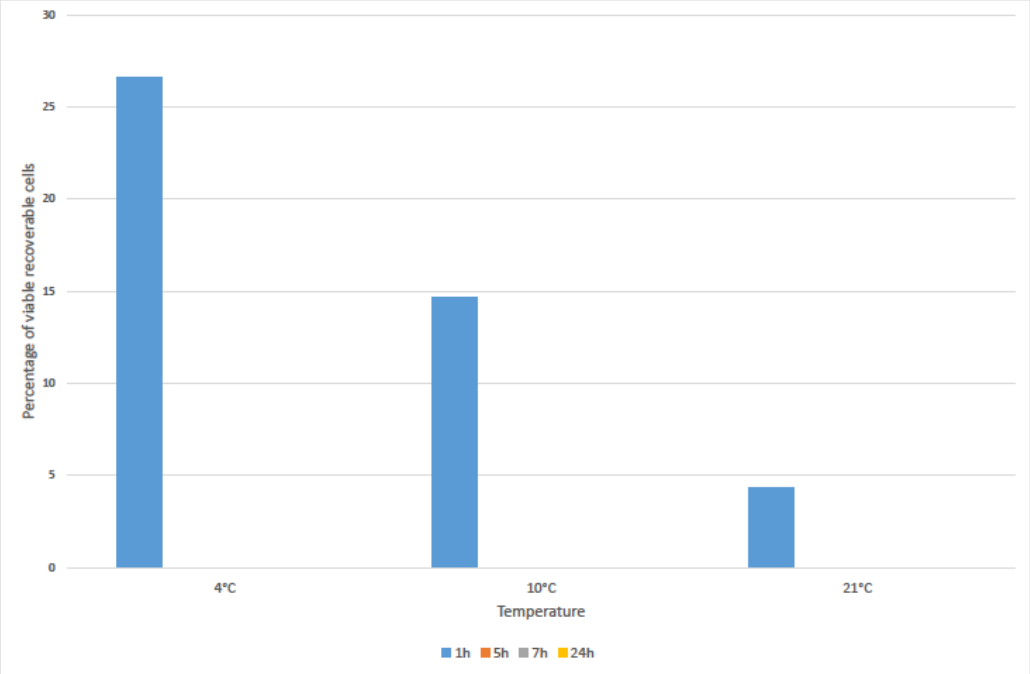
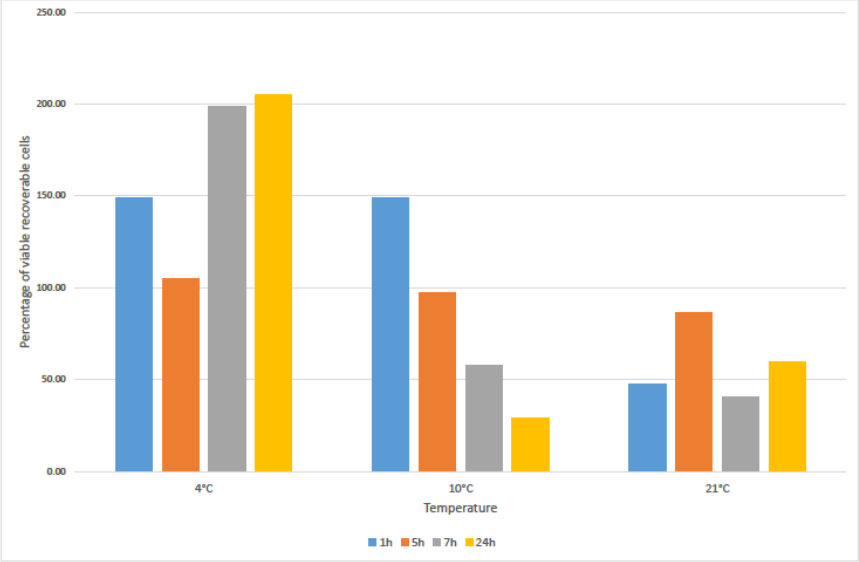


Figure 2 – Percentage of viable cells recovered from SS 304 over 24 hours in a low humidity environment (approximately 11%RH) at three different temperatures (4°C, 10°C and 21°C). Percentages are based on the number of recovered cells before applying treatment: 4°C = 2.4x104 cfu/ml, 10°C = 3.37x104 cfu/ml, 21°C = 7.27x104 cfu/ml. n=30 for each time point.



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337 Figure 3 - Percentage of viable cells recovered from SS 304 over 24 hours in a medium
338 humidity environment (approximately 52%RH) at three different temperatures (4°C,
339 10°C and 21°C). Percentages are based on the number of recovered cells before applying
340 treatment: 4°C = 7.93x103 cfu/ml, 10°C = 2.01x104 cfu/ml, 21°C = 3.96x104 cfu/ml.
341 n=30 for each time point.

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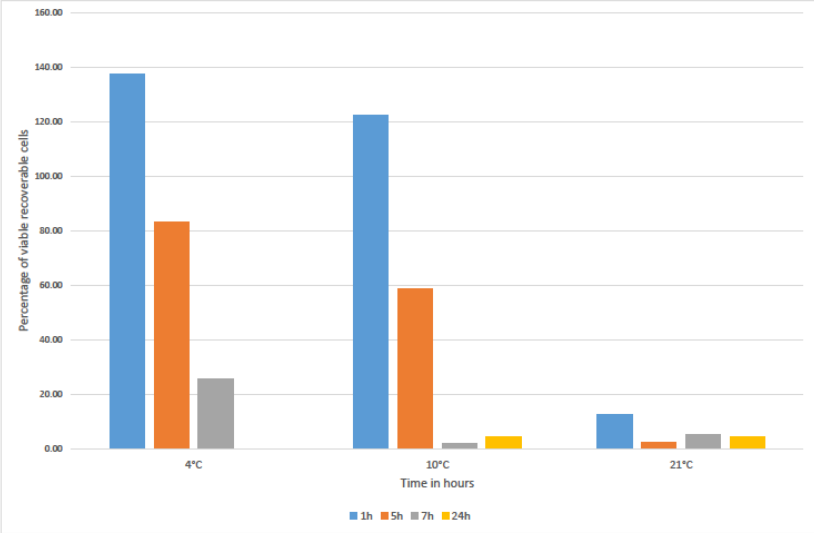
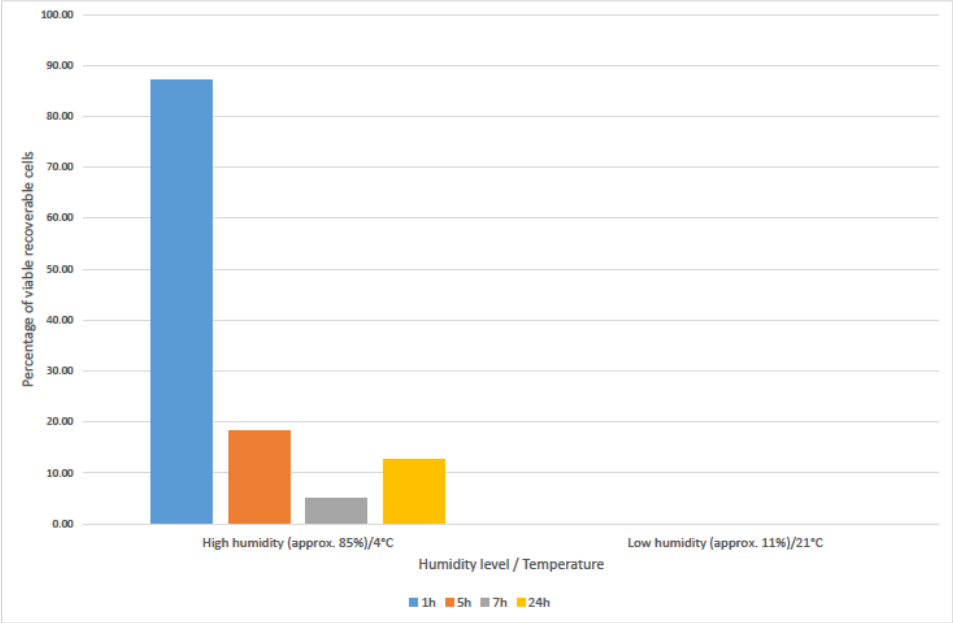


Figure 4 - Percentage of viable cells recovered from SS 304 over 24 hours in a high humidity environment (approximately 86%RH) at three different temperatures (4°C, 10°C and 21°C). Percentages are based on the number of recovered cells before applying treatment: 4°C = 2.93x105 cfu/ml, 10°C = 9.09x105 cfu/ml, 21°C = 7.89x104 cfu/ml. n=30 for each time point.



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354 Figure 5 – Percentage of viable cells recovered from SS 316 over 24 hours in either a
355 high humidity and low temperature environment or a low humidity high temperature
356 environment. Percentages are based on the number of recovered cells before applying
357 treatment: high humidity/4°C = 1.83x104 cfu/ml, low humidity/21°C = 6x104 cfu/ml.
358 n=30 for each time point.

359