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1

2 **Title page**

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

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11

12 **Running title: *Humidity and L. monocytogenes***

13

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15 commercial, or not-for-profit sectors.

16

17 **Significance and Impact**

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

26

27 **Abstract**

28 *Listeria monocytogenes* is a pathogenic bacterium, with human disease and infection
29 linked to dairy products, seafood, ready-to-eat meat and raw & undercooked meats.
30 Stainless steel is the most common food preparation surface and therefore, it is
31 important to understand how food storage conditions such as surface materials,
32 temperature and relative humidity can affect survival of *L. monocytogenes*. In this study,
33 survival of *L. monocytogenes* on stainless steel was investigated at three temperatures
34 (4, 10 and 21°C), each approx. 11%, 50% and 85% humidity. Results indicate that the
35 lower the temperature, the more cells were recovered in all three humidity
36 environments, whilst medium humidity enhances survival, irrespective of temperature.
37 Lower humidity decreases recovery at all temperatures. These data support the
38 guidance noted above that humidity control is important, and that lower humidity
39 environments are less likely to support retention of viable *L. monocytogenes* on a
40 stainless steel surface.

41 **Keywords**

42 Food
43 Listeria
44 Food preparation
45 Humidity
46 Stainless steel

47 **Introduction**

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

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

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

73 Although surface characteristics such as roughness and wettability are important
74 variables when considering survival of microorganisms on steel, other environmental
75 conditions are likely to play a key role. An increase in relative humidity (RH), a measure
76 relating to amount of water vapour in the atmosphere, has been shown to prolong
77 survival of *L. monocytogenes*, as well as encourage growth when inoculated on fresh
78 produce (Likotrafiti et al. 2013), whilst a decrease in RH has demonstrated a decreased
79 survival of *L. monocytogenes* (Zoz et al. 2016). Conversely, reduction in RH has been
80 shown to enhance transfer of *L. monocytogenes* from biofilm to meat products
81 potentially due to increased capillary action within the food (Rodríguez et al. 2007).
82 Control of relative humidity in relation to control of microbial contamination in food
83 processing environments is suggested by many governments around the world (e.g. FDA
84 2009; Abu Dhabi Food Control Authority 2010; FSA 2015), and advice is available
85 (EHEDG 2006). However these documents do not recommend specific levels of RH,
86 likely due to the complex and unique nature of each food processing environment.

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

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

98 **Results and Discussion**

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

105 **Surface profiles**

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

110 **Recovery of cells from SS 304 following incubation in controlled humidity and** 111 **temperature**

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

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

118 As time and temperature increased, viability was also reduced at high humidity (figure
119 4). This decrease was statistically significant ($p < 0.05$) between 5h and 7h at 4°C and 10
120 °C ($P > 0.05$).

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

124 **Recovery of living cells from SS 316 following incubation in controlled humidity** 125 **and temperature**

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

129 **Acridine orange (AO) staining of SS 304 and SS 316 to assess retention on surface** 130 **after swabbing**

131 The average percentage coverages of cells on SS 304 and SS 316 were 74.97% and
132 65.65% respectively, when unswabbed coupons were visualised with AO. After
133 swabbing the coverage decreased significantly ($p < 0.05$). There was no significant
134 difference ($P > 0.05$) in the percentage average of cells on the surfaces, with SS 304 and
135 SS 316 presenting 2.08% and 3.59% respectively, indicating effective swabbing.

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

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

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

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

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

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

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

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

179

180 **Materials and Methods**

181 **Microorganisms**

182 *Listeria monocytogenes* Scott A, serotype 4 (kindly donated by Professor Lone Gram
183 (Danish Institute of Fisheries Research (DIFRES), Technical University of Denmark)
184 (Briers et al. 2011) was maintained on Tryptone Soya Agar (TSA) (Oxoid, Basingstoke)
185 at 5°C and inoculated into 100 ml⁻¹ Tryptone Soya Broth (TSB) (Oxoid). Cultures were
186 grown overnight (22 ± 1h) at 30°C with agitation (225 rpm). Cells were harvested by
187 centrifugation (3600 rpm, 10 min, room temperature) and washed once in 0.85% NaCl
188 (Oxoid), resuspended to optical density (540nm) of 1.0. A 1 ml⁻¹ sample from the cell
189 suspension was serially diluted, plated out onto NA and CFU counted, finding the cell
190 concentration to be 3.18 ± 0.65 x10⁹ CFU/ml⁻¹. This was used for the initial inoculum of
191 stainless steel coupons.

192 **Preparation of stainless steel**

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

199 **White light profilometry**

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

203 **Humidity control**

204 Humidity was controlled using saturated salt solutions contained within a desiccator
205 chamber (250mm diameter, Fischer Scientific, Loughborough UK). Salts used were;
206 lithium chloride (Fischer Scientific) to achieve a low humidity approximately 11%RH,
207 magnesium chloride (Fischer Scientific) to achieve a medium humidity approximately
208 50%RH and potassium sulphate (Fischer Scientific) to achieve a high humidity
209 approximately 85%RH (Rockland 1960). Water was added to the salts until a slushy
210 mixture filled the bottom of the chamber. The saturated salt solution was left in the
211 chamber for 24 hours prior to the start of the experiment to allow the desired RH to be
212 attained. Relative humidity and temperature were monitored with a mobile USB data
213 logger (RHT10, Extech Instruments, Boston, USA).

214 **The effect of humidity and temperature on the survival of *Listeria monocytogenes*** 215 **on stainless steel**

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

218 sterile pipette tip. Coupons were left to dry for 30 minutes in a class two cabinet at room
219 temperature prior to being placed in the desiccator containing the appropriate
220 saturated salt solution on a platform approximately 4cm above the salt solution. The
221 desiccator was then placed inside an incubator at the appropriate temperature. At each
222 sample time, each coupon was swabbed with a moist swab which was placed in 10ml⁻¹
223 of 0.85% saline and diluted to 10⁻⁸. Dilutions were plated out onto TSA, incubated for
224 24h at 30°C and colonies counted.

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

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

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

240 **Data analysis**

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

243 are presented as percentage changes compared to the CFU ml⁻¹ recovered from steel
244 sample before incubation. Initial recovered CFU ml⁻¹ can be found in the caption for the
245 corresponding figure.

246

247 **Conflict of Interest**

248 No conflict of interest declared

249

250

251

252 References

253

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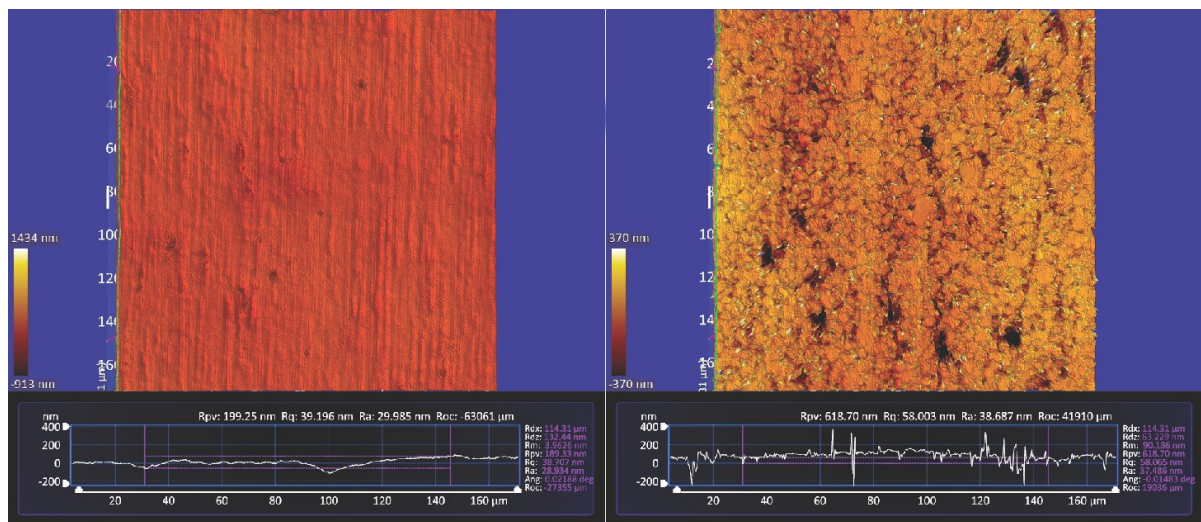
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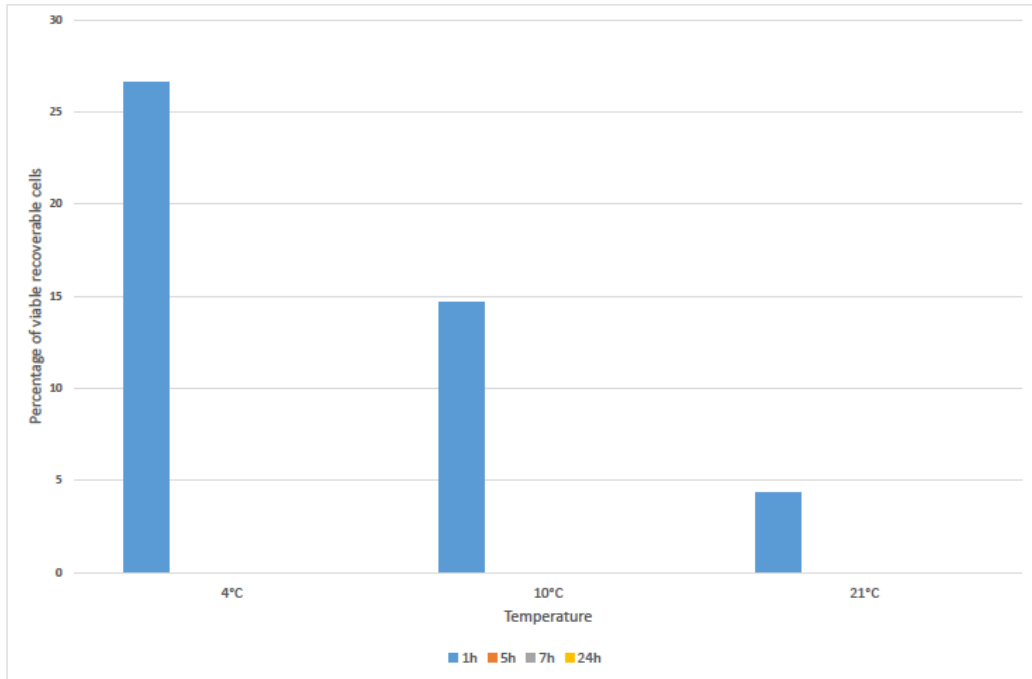


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 321

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

324

325



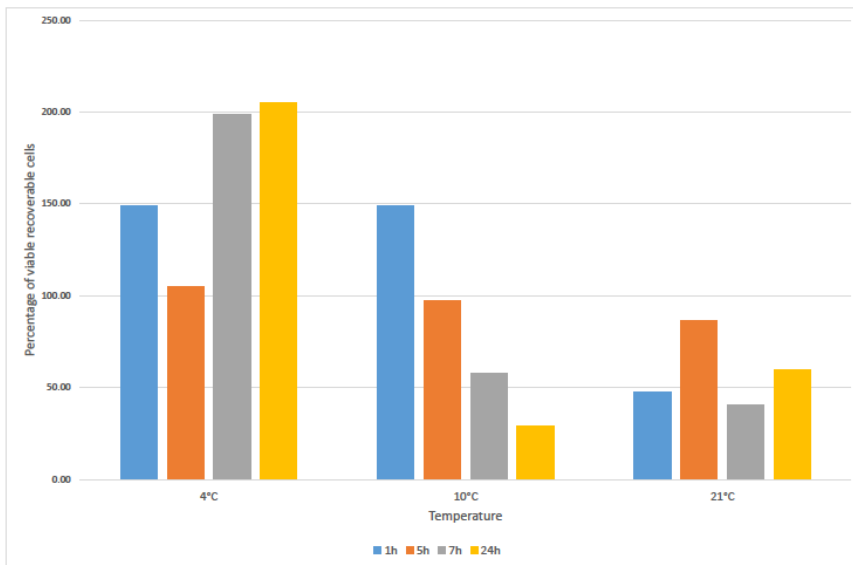
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328 Figure 2 – Percentage of viable cells recovered from SS 304 over 24 hours in a low
 329 humidity environment (approximately 11%RH) at three different temperatures (4°C,
 330 10°C and 21°C). Percentages are based on the number of recovered cells before applying
 331 treatment: 4°C = 2.4×10^4 cfu/ml, 10°C = 3.37×10^4 cfu/ml, 21°C = 7.27×10^4 cfu/ml. n=30
 332 for each time point.

333

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335



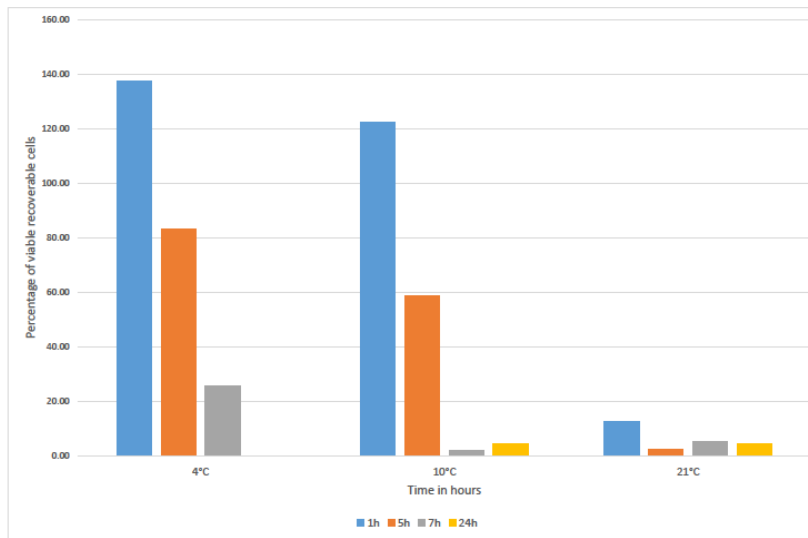
336

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 = 7.93×10^3 cfu/ml, 10°C = 2.01×10^4 cfu/ml, 21°C = 3.96×10^4 cfu/ml.
 341 n=30 for each time point.

342

343

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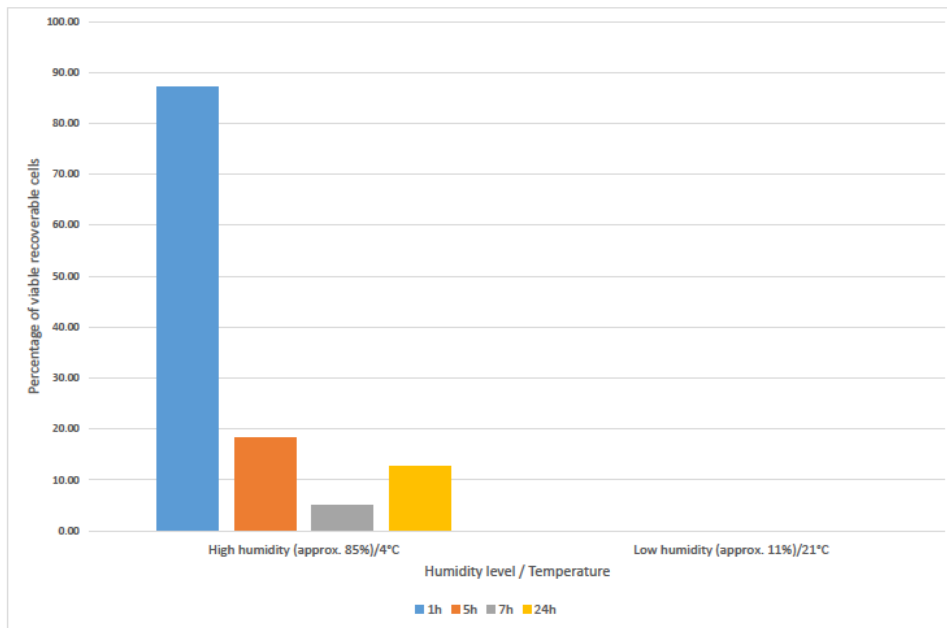


345

346 Figure 4 - Percentage of viable cells recovered from SS 304 over 24 hours in a high
347 humidity environment (approximately 86%RH) at three different temperatures (4°C,
348 10°C and 21°C). Percentages are based on the number of recovered cells before applying
349 treatment: 4°C = 2.93×10^5 cfu/ml, 10°C = 9.09×10^5 cfu/ml, 21°C = 7.89×10^4 cfu/ml.
350 n=30 for each time point.

351

352



353

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 = 1.83×10^4 cfu/ml, low humidity/21°C = 6×10^4 cfu/ml.
 358 n=30 for each time point.

359