1 **Whole room disinfection with hydrogen peroxide mist to control *Listeria monocytogenes* in food**

2 **industry related environments**

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4 **Trond Møretrø[1]*, Helge Fanebust[2], Annette Fagerlund[1] and Solveig Langsrud[1]**

5 [1]Nofima, Norwegian Institute of Food, Fisheries and Aquaculture Research, N-1430 Ås, Norway

6 [2] Decon-X International, Vollsveien 13C, N-1366 Lysaker, Norway

7

8 *Corresponding author

9 Address:

10 Trond Møretrø

11 Nofima, Norwegian Institute of Food, Fisheries and Aquaculture Research

12 Osloveien 1

13 N-1430 Ås,

14 Norway

15

16 Tel: +47 64970100

17 E. mail: trond.moretro@nofima.no

18

Abstract

Listeria monocytogenes surviving daily cleaning and disinfection is a challenge for many types of food industries. In this study, it was tested whether whole room disinfection (WRD) with H_2O_2 mist could kill *L. monocytogenes* under conditions relevant for the food industry. Survival of a mixture of four *L. monocytogenes* strains exposed to H_2O_2 mist was investigated in a 36 m^3 room. A commercial machine produced H_2O_2 mist by pumping a 5% H_2O_2 solution containing 0.005% silver through a nozzle, and breaking the liquid up in droplets using pressurized air.

When a suspension of bacteria in 0.9% NaCl applied on stainless steel coupons was exposed to WRD with H_2O_2 mist, a >5 log reduction (LR) of *L. monocytogenes* was observed. Similar reductions were observed in all tests with conditions between 12-20 °C, H_2O_2 concentrations of 35-80 ppm and 1-2 hour exposure. It was shown that the H_2O_2 in the mist dissolved and accumulated in the liquid on the steel, and acted against *L. monocytogenes* in the liquid phase. At high cell concentrations, the effect was reduced if cells were pregrown at highly aerated conditions. The anti-listerial effect was robust against protein and fat, but the effect was quenched by raw meat and raw salmon, probably due to high catalase activity. The effect of whole room disinfection with H_2O_2 against dried *L. monocytogenes* cells was 1-2 LR, however the effect of air-drying by itself lead to 3-4 LR. When biofilms were exposed to WRD, no surviving *L. monocytogenes* were observed on stainless steel, however for *L. monocytogenes* on a PVC conveyor belt material, there were surviving bacteria, with about 2 LR. Screening of 54 *L. monocytogenes* strains for growth susceptibility to H_2O_2 showed that their sensitivity to H_2O_2 was very similar, thus WRD with H_2O_2 are likely to be robust against strain variation in susceptibility to H_2O_2. Production of H_2O_2 mist resulted in increased room humidity, and this may limit the maximum H_2O_2 concentration achievable, especially at low temperatures. The results in this study show that whole room disinfection with H_2O_2 may have potential to control *L. monocytogenes* in the food industry, however intervention studies in the food industry are needed to verify the effect in practical use.

44

45 **Keywords**: fogging, aerosol, sanitising, hygiene, biofilm

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49 # 1. Introduction

50 *Listeria (L.) monocytogenes* is a foodborne pathogenic bacterium. The bacterium causes the disease
51 listeriosis, which has a relative low incidence, but a death rate which is among the highest of
52 foodborne infections (Swaminathan and Gerner-Smidt, 2007). In addition to the burden of the
53 disease for humans and the society, there is also considerable costs associated with *L.*
54 *monocytogenes* for the food industry, such as costs related to withdrawal of products from market,
55 and costs for control measures and analysis of *L. monocytogenes*. The majority of listeriosis cases are
56 caused by consumption of ready to eat (RTE) food like cold cuts, soft cheeses and lightly processed
57 fish products as well as fresh produce (Laksanalamai et al., 2012; Swaminathan and Gerner-Smidt,
58 2007). RTE foods are cross-contaminated with *L. monocytogenes* from the processing environment
59 during production. *L. monocytogenes* can establish itself in the processing environment. *Listeria*
60 positive environmental samples are often linked to niches that are difficult to sanitize (Møretrø and
61 Langsrud, 2004).

62 In most processing plants a manual cleaning and disinfection (C&D) process is performed daily after
63 the production process. Typically, for sanitation besides CIP systems, foaming cleaning agents and
64 disinfectants are manually applied to surfaces with rinsing steps with water in between cleaning and
65 disinfection and after the final disinfection step. In most facilities, this process lasts several hours.
66 Some areas/machines may be difficult to reach by the conventional sanitation process, and this may
67 be partly due to too little time to dismantle machines between the production shifts. In addition,
68 some type of equipment/machines may not be cleaned thoroughly as they may be sensitive to water
69 or C&D agents (Lelieveld et al., 2014). *L. monocytogenes* is frequently found in many food processing
70 plants despite the use of conventional C&D (Ferreira et al., 2014; Møretrø and Langsrud, 2004). We
71 recently reported that conventional C&D foaming agents had limited effect against *L. monocytogenes*
72 attached to conveyor belts (Fagerlund et al., 2017). An alternative to conventional manual C&D is
73 whole room disinfection (WRD) with gaseous agents (Beswick et al., 2011; Otter et al., 2013). In
74 hospitals and healthcare facilities, WRD with gaseous hydrogen peroxide (H_2O_2) has gained popularity

75 in the last decade (Doll et al., 2015; Falagas et al., 2011). Advantages with the process are that the
76 gas is distributed throughout the room, the process can be automatic, the gas does not affect
77 sensitive equipment and hydrogen peroxide is environmental friendly as it decomposes into water
78 and oxygen (Block, 2001; Linley et al., 2012; Otter et al., 2013; Unger-Bimczok et al., 2011).
79 Challenges related to the process are that an H_2O_2 gas/vapor generator is needed, that the room
80 must be sealed off and that personnel cannot enter during the disinfection process. There are in
81 principle two different technologies for H_2O_2 WRD; these are based on hydrogen peroxide vapor
82 (HPV) and aerosolized hydrogen peroxide (aHP) (Holmdahl et al., 2011). For HPV, a heat generated
83 vapor of 30-35% H_2O_2 is spread throughout the room by a high velocity air stream. With the aHP
84 technology, a solution of H_2O_2 of 5-7% is sprayed out through a nozzle that forms small droplets,
85 which evaporate and spread in the environment (Holmdahl et al., 2011; Otter et al., 2013). For some
86 aHP systems, H_2O_2 solutions with low concentrations of silver are used. Silver stabilises the H_2O_2
87 solution (Martin et al., 2015). For water disinfection, silver has also been shown to potentiate the
88 antibacterial effect of H_2O_2, but to our knowledge this is yet to been proven for WRD systems (Martin
89 et al., 2015; Pedahzur et al., 1995).

90 WRD with H_2O_2 has been extensively tested in hospitals and health care facilities. Results from *in situ*
91 use show that HPV systems have eradicated reservoirs of *Clostridium difficile*, MRSA and
92 *Acinetobacter baumannii* during outbreaks, while aHP systems resulted in reduced levels of the same
93 types of microorganisms (Falagas et al., 2011; Otter et al., 2013). But there is limited information
94 available about the effect of using H_2O_2 for WRD in the food industry. McDonnell et al. (2002) claim
95 that a HPV system was effective against *L. monocytogenes* and other bacteria relevant for food
96 processing, though this was a popularized report and few scientific details were given. However,
97 H_2O_2 vapor has been reported to effectively reduce *Listeria* spp. on vegetables (Back et al., 2014;
98 Jiang et al., 2017) and on stainless steel (Choi et al., 2012). Although the use of H_2O_2 for WRD has
99 been shown to be effective in hospitals, this cannot be directly extrapolated to the food industry, as
100 there are different environmental conditions in many food processing areas compared to

101 hospital/health care settings and different types of bacteria are relevant. Hydrogen peroxide may
102 react with organic materials, and the effect of food residues on H_2O_2 may be different than soils from
103 hospitals such as blood. Also humidity and temperature can influence the effect of H_2O_2 WRD
104 (Hultman et al., 2007; Unger-Bimczok et al., 2008) and such conditions may differ between hospitals
105 and food industries. In addition, the resistance towards H_2O_2 and other toxic reactive oxygen species
106 may vary between different bacteria. For instance, *L. monocytogenes* and many other bacteria can
107 produce the enzyme catalase which degrades H_2O_2 to O_2 and water (Azizoglu and Kathariou, 2010).
108 Thus, if H_2O_2 is to be used for WRD against *L. monocytogenes* in the food industry, information about
109 the effect against *L. monocytogenes* under food production environmental conditions are needed.

110 In the present study, the effect of WRD with aerosolized H_2O_2 (aHP) was tested against *L.*
111 *monocytogenes* under food processing related conditions in a test room.

112

113 2. Materials and Methods

114

115 *2.1. Bacterial strains and cultural conditions*

116 *L. monocytogenes* was tested in WRD as a mixture of four strains. The four strains represented
117 different MLST (multilocus sequence typing) sequence types (STs): MF4536 (ST9) and MF5634
118 (ST121) from meat industry, and MF5259 (ST7) and MF3949 (ST8) from salmon industry. All strains
119 were from Møretrø et al. (2017) and had previously been found to persist in food processing plants.

120 An additional 50 *L. monocytogenes* strains were tested for catalase activity and growth sensitivity to
121 H_2O_2. This set included 22 strains from the ILSI *Listeria* strain collection (Fugett et al., 2006),
122 representing all four genetic *L. monocytogenes* lineages (I, n=8; II, n=10; III, n=2, and IV, n=2) and 28
123 strains representing a variety of strains originating from Nofima's collection of strains from
124 Norwegian food and food processing environments (lineage I, n= 2; II, n=26, mainly from Møretrø et

125 al. (2017)). All bacteria were cultivated in tryptic soy broth (TSB, Oxoid, Basingstoke, UK) and on
126 tryptic soy agar (TSA, Oxoid) at 30 °C, and overnight cultures were grown for 16-18 h in 5-ml volumes
127 in culture tubes without agitation, unless otherwise stated.

128

129 ***2.2. Whole room H_2O_2 disinfection***

130 Disinfection with H_2O_2 mist was tested out in a room at a class 3 biological hazard facility. The room
131 had a total volume of 36 m^3, with inner plastic walls and ceiling and a painted concrete floor. The
132 room contained two conveyor belt units, a stainless steel counter with sinks and some additional
133 small equipment with surfaces of stainless steel as well as a drain channel. During exposure to H_2O_2,
134 the ventilation system was blocked with an airtight shutter and the door closed and sealed with
135 adhesive tape within two minutes after starting the disinfection machine. The room could be
136 preconditioned to 12 °C or 18 °C besides ambient temperature, however the air conditioning was
137 turned off during WRD. At low temperatures, a dehumidifier (Cotech, Clas Olson, Sweden) was used
138 in the period prior to disinfection and programmed to obtain a maximum relative humidity (RH) of
139 50% at the start of disinfection. For disinfection, the room was filled with H_2O_2 mist, produced by a
140 Decon-X DX1 machine (Decon-X International, Lysaker, Norway). The machine uses a 5 % H_2O_2
141 solution containing 0.005% silver (Decon-X 520/521, Decon-X International), and sprays out small
142 droplets of H_2O_2 through a nozzle, the droplets later evaporate into H_2O_2 gas. The generator was
143 placed in a corner of the room, spraying diagonally in direction of the corner across the room. After
144 end of the disinfection cycle, the ventilation was turned on, and after 5 min the samples were
145 removed from the test room by a person wearing a protective gas mask. H_2O_2 concentration was
146 monitored by a sensor on the outside the machine and with an external H_2O_2 sensor (both sensors:
147 H2O2 CB500, Membrapor AG, Wallisellen, Switzerland) which was placed in close proximity to the
148 samples to be disinfected. Temperature and %RH were measured by sensors on the outside of the

149 machine, and also with an external logging device (Testo 175H1 temperature and humidity logger,

150 Testo Inc., Sparta, NJ, USA), which was placed together with the samples to be disinfected.

151 For the majority of the tests, the H_2O_2 mist generator was programmed to run a disinfection process

152 for a programmed time with a defined concentration of H_2O_2 in the air in the test room. A hysteresis

153 control loop was used to start and stop filling H_2O_2 into the room during the exposure phase. The

154 machine is in this mode configured with four parameters that control the disinfection process: Max

155 H_2O_2 threshold, Min H_2O_2 threshold, Max relative humidity and Process time. The machine will when

156 starting the disinfection process start to fill H_2O_2 mist into the room. When the Max H_2O_2 threshold

157 or the Max relative humidity value is reached, the machine will stop filling H_2O_2 into the room. When

158 the H_2O_2 concentration in the room falls below the Min H_2O_2 threshold value, and the humidity in the

159 room is below the Max relative humidity threshold, then the machine will again start to fill more

160 H_2O_2 mist into the room. This process continues for the programmed time duration (Process time).

161 The threshold values and process time that have been used in the present work are Max H_2O_2

162 threshold: 60-120 ppm, Min H_2O_2 threshold: 40-100 ppm, Max relative humidity: 90 %RH and Process

163 time: 53-126 min.

164

165 ***2.3. Effect of WRD with H_2O_2 against bacterial suspension on surfaces***

166 Individual overnight cultures in test tubes with 5 ml TSB, cultured at 30° C without agitation were

167 mixed in equal volumes, washed and resuspended in 0.9 % NaCl. Four drops of 10 µl of this

168 suspension (bacterial concentration 8.5-9.6 log/ml) were added a coupon of stainless steel (AISI 304,

169 2B, Norsk Stål, Nesbru, Norway) (all coupons were sterilized by autoclaving, used only once and were

170 made from new and previously unused steel plates). The coupons were treated in two different

171 ways: One set of coupons were moved to the test room within 5 min after application of the

172 bacteria, while the other set of coupons were dried for 1 hour in a safety hood, until visible dry, after

173 application of bacteria, before moving the coupons to the test room. After exposure the coupons

174 were swabbed with a sterile cotton swab (for dry coupons the swab was pre-moistened with saline),
175 and the swab was transferred to a tube with 2 ml Dey Engley Neutralizing Broth (Difco, USA). The
176 tube was vortexed and the number of surviving bacteria determined after plating to TSA (30 °C). Dry
177 and wet control coupons were placed for 2 h in a climatic cabinet (KB8400F, Termaks, Bergen,
178 Norway) at 90 %RH at the desired temperature, and otherwise treated as coupons subjected to
179 disinfection. All tests were run with 2-3 coupons as technical replicates.

180

181 ***2.4. Effect of food soils on disinfection effect***

182 To test the impact of different soiling/residues on the disinfection efficiency, the four-strain *L.*
183 *monocytogenes* mixture was made as described above and resuspended in 0.9% NaCl (control), 3%
184 Bovine serum albumin (BSA), raw or heat treated meat juice, or heat treated salmon juice. Meat juice
185 was prepared by adding 100 ml dH_2O to 100 g minced meat, followed by homogenizing in a
186 Stomacher for 1 min. The homogenate was further diluted 1:3 with dH_2O, and treated with a
187 Stomacher for two times 1 min. Heat treatment was performed at 80 °C for 30 min. Salmon juice was
188 prepared as previously described (Langsrud et al., 2015). The protein and fat content of the food
189 juices were determined by the Kjeldahl method and NMR, respectively, by a commercial analytical
190 lab. Four drops of 10 µl of the resulting suspensions were added to coupons of stainless steel (no
191 drying step) and subjected to H_2O_2 WRD (122 min process, 35-45 ppm H_2O_2, mean temperature 13
192 °C). After exposure, the number of viable *L. monocytogenes* was determined by plating to TSA as
193 described above. The experiment was performed with two coupons as technical replicates in
194 triplicate on different days.

195

196 ***2.5. Disinfection of biofilms***

197 To test WRD with H_2O_2 against biofilms, *L. monocytogenes* were grown on 2 × 2 cm coupons of
198 stainless steel (AISI 304, 2B) and a PVC conveyor belt material (Forbo-Siegling Transilon; E 8/2 U0/V5
199 MT white FDA). Coupons were placed in a tilted vertical position inside a 50 ml tube. The tube with
200 the coupon was added 6 ml of the *L. monocytogenes* mix diluted in TSB (10^6 cfu ml^{-1}, final cell
201 concentration). The tubes were incubated with a slowly rocking motion (15 rpm) at 12 °C (a relevant
202 temperature for meat production (European Commission, 2004; Møretrø et. al, 2013)). After three
203 days, the medium was removed and exchanged with the same volume of new TSB, followed by
204 further incubation of the tube at 12 °C. After a total of 4 days, the coupons were washed with 10 ml
205 0.9% NaCl on each side before laying them in an empty petri dish and subjecting them to WRD with
206 H_2O_2 (process started within 5 min after washing, 122 min process, 50-60 ppm H_2O_2, mean
207 temperature 14 °C), or incubation in a humidity cabinet at 90% RH at 13.5° C for 2 h (control).
208 Coupons subjected to WRD with H_2O_2 as well as control coupons were swabbed on the side of
209 interest with cotton swabs which were transferred to glass tubes with 2 ml Dey Engley Neutralizing
210 broth and subjected to sonication for 10 min (Bransonic 3510, Bransonic Ultrasonic, The
211 Netherlands) before dilution and plating to TSA with incubation at 30 °C.

212

213 ***2.6. Measurement of H_2O_2 concentration in liquid with test strips***

214 The residual H_2O_2 concentration in liquid phase (drops of suspension or liquid on biofilm surface)
215 after WRD was measured semi-quantitatively within 5 min with Quantofix Peroxide 100/1000 strips
216 (Sigma-Aldrich) according to the manufacturer's instructions. For measuring of suspensions, the strip
217 was put in contact with the drop. For biofilm studies, strips were put in contact with wet spots, or if
218 such spots were not apparent, 10 µl 0.9% NaCl was added to the coupon, pipetted up and down a
219 couple of times and as much of the volume as possible was transferred to a H_2O_2 strip. Using the

220 strips, the concentration of the H_2O_2 solution used for WRD was determined to be 50 000 ppm (5%),

221 which is the concentration given by the manufacturer, thus confirming the test strips results.

222

223 ***2.7. Suspension test***

224 In order to verify that the liquid H_2O_2 in the drops on stainless steel had antibacterial effect, the

225 bacterial reduction in liquid H_2O_2 was tested in suspension tests. Suspension tests were performed by

226 a modified version of the Council of Europe suspension test EN1276 (Anonymous, 1987), as

227 described previously (Møretrø et al., 2003; Møretrø et al., 2009), with a 2 h exposure time. The test

228 was performed with the four strain mixture of *L. monocytogenes* with dilutions (final concentrations

229 tested 5, 2, 1, 0.5, 0.25, 0.2, 0.1 and 0.05%) of the H_2O_2 solution (Decon-X 520/521) or with pooled

230 samples of liquid retrieved from 10 µl drops of 0.9% NaCl applied on stainless steel after exposure to

231 H_2O_2 WRD.

232

233 ***2.8. Bacteriostatic growth assay***

234 Assay of the growth of single strains of *L. monocytogenes* in the presence of H_2O_2 was carried out

235 using twofold dilutions of H_2O_2 in a broth microdilution assay, performed in a Bioscreen C instrument

236 (Oy Growth Curves Ab, Ltd.). Each well was inoculated with 300 µl samples of *L. monocytogenes*

237 (overnight cultures were prepared as described in Section 2.1), diluted to approximately 10^4 cfu ml^{-1}

238 in TSB with a twofold dilution series of H_2O_2 solution (Sigma, St. Louis, USA) or Decon-X 520/521

239 (which contains 5% H_2O_2), and grown at 25°C with recording of OD_{600} every 15 minutes for 48 hours

240 with shaking before each measurement. Controls contained *L. monocytogenes* grown in TSB, and

241 blank wells contained TSB broth only. The lowest concentration of H_2O_2 able to inhibit growth of *L.*

242 *monocytogenes*, relative to controls without H_2O_2, was determined from the resulting growth curves

243 and recorded as the minimum inhibitory concentration (MIC). Duplicate wells were used for each
244 sample, and tested strains were assayed at least three times.

245 In addition to the four *L. monocytogenes* strains listed in Section 2.1 (MF4536, MF5634, MF5259 and
246 MF3949), the following 50 *L. monocytogenes* strains (phylogenetic lineage noted in parenthesis)
247 were tested in this assay: FSL J1-110, FSL J1-225, FSL R2-503, FSL J2-064, FSL N1-225, FSL J2-035, FSL
248 J1-177, FSL R2-500, MF2184, MF6554 (lineage I); EGD-e, FSL C1-056, FSL N3-031, FSL J2-063, FSL M1-
249 004, FSL C1-115, FSL J2-066, FSL J2-054, FSL J2-031, FSL J2-020, MF3638, MF3853, MF3860, MF3939, ,
250 MF3995, MF4475, MF4545, MF4554, MF4562, MF4624, MF4627, MF4712, MF4792, MF4995,
251 MF4999, MF5366, MF5369, MF5372, MF5377, MF5378, MF5630, MF6241, MF6300, MF6319,
252 MF6556 and MF6708 (lineage II), FSL J1-168 and FSL J1-031 (lineage III), and FSL J1-158 and FSL W1-
253 111 (lineage IV). Isolates with names starting with the prefix «FSL» are from the ILSI strain collection
254 (Fugett *et al*. 2006), while the strains with names starting with «MF» are obtained from Norwegian
255 food industry (Møretrø et al, 2017).

256

257 ***2.9. Catalase test***

258 Catalase activity was tested by suspending a loop from a bacterial colony in 10 µl 3% H_2O_2 (Sigma),
259 and visual observation of bubbling was used as an indicator of catalase activity (Chester, 1979).

260

261 ***2.10. Geobacillus stearothermophilus spore test***

262 Spores are often used as biological indicators to test the effect of sterilizations and disinfection. The
263 spores used are non-pathogenic and can be included for process validation in *in situ* tests where
264 pathogens cannot be used. However, it is important to verify that the pathogen of interest has
265 similar sensitivity to the bactericidal treatment as the spores used as indicators. The effect of H_2O_2
266 mist was tested against a standardized indicator of spores of *Geobacillus stearothermophilus* (Apex

267 biological indicator 4-5-6 log, Mesalabs, Bozeman, MT, USA). The indicator set consists of three steel
268 discs, with 4, 5 and 6 log of spores, respectively. After exposure to H_2O_2 the discs with spores were
269 transferred to tubes with growth media (Mesalabs) and incubated at 55 °C for 7 days. Color change
270 to yellow indicated growth of surviving spores. The viability of the spores was regularly checked by
271 incubating un-exposed disks in growth media as positive controls.

272

273 ***2.11. Calculations***

274 As a metric for the difference in viable *L. monocytogenes* on coupons before and after exposure to
275 H_2O_2, LR_{Total} was determined by subtracting the log transformed number of viable bacteria on coupon
276 after exposure from the log transformed number of bacteria applied to the coupon. As a metric for
277 the difference in viable *L. monocytogenes* before and after exposure to a control period at equal
278 conditions as cells treated with H_2O_2 (time, temperature, humidity), but without exposure to
279 disinfection, the average logarithmic reduction $LR_{Control}$, was calculated by subtracting the log
280 transformed number of viable bacteria on coupon after the control period from the log transformed
281 number of bacteria applied to the coupon. The net effect of H_2O_2 WRD exposure was then calculated
282 as: $LR_{Disinfect}=LR_{Total} - LR_{Control}$.

283

284

285 # 3. Results and Discussion

286

287 ***3.1. Whole room disinfection was effective against suspended Listeria***

288 Initial experiments using whole room disinfection (WRD) with H_2O_2 at regular room temperature
289 (18.5 °C) indicated that the methodology can kill *L. monocytogenes* suspended in thin films of water

290 on surfaces. Exposing droplets of *L. monocytogenes* to H_2O_2 mist resulted in more than 5 log
291 reduction (LR) in viable counts (counts below detection limit, <20 cfu/coupon), even at relatively
292 short exposure times (53 min) and concentrations of H_2O_2 in the range 50-80 ppm (see Table 1). The
293 LR in controls incubated in a humidity cabinet at 90% RH was <1 log, thus the reduction observed
294 after WRD was mainly a result of H_2O_2 exposure ($LR_{Total} \approx LR_{Disinfect}$).

295 Food processing facilities are often kept at 12-14 °C to limit bacterial growth, and it is well known
296 that the bactericidal efficacy of chemical disinfectants decreases with lower temperature
297 (Kostenbauder, 1991). Nevertheless, H_2O_2 fogging seemed to have high bactericidal activity, even at
298 lower temperatures. As for the experiments at 18.5°C, also more than 5 LR of *L. monocytogenes* on
299 stainless steel was observed for WRD with a mean temperature of 13.5°C against cells in suspension
300 on stainless steel (Table 1). In one of the experiments at 13.5 °C, a suspension of *L. monocytogenes*
301 on coupons of a polyurethane coated conveyor belt material was exposed to WRD, and >5 LR was
302 also observed in this test. To our knowledge there are no earlier reports on the effect of H_2O_2 WRD at
303 temperatures below 20 °C. Ochiai et al. (2017) reported that *L. monocytogenes* were more resistant
304 to liquid H_2O_2 when grown at 20 °C compared to 30 °C so as the precultivation in the present study
305 was at 30 °C, we cannot rule out that the effect would be lower if the cells had been pregrown at
306 lower temperature.

307

308 ***3.2. H_2O_2 works through accumulation in liquid phase during WRD***

309 Several studies have demonstrated that drying after cleaning will have an additional inactivation
310 effect on microbes, and it is recommended to keep processing facilities as dry as possible (Tompkin,
311 2002; Tompkin et al., 1999; US Food and Drug Administration 2017). From a microbiological point of
312 view, one could expect that drying followed by disinfection would lead to an additive inactivation
313 effect and even a synergistic effect due to stressed cells (Koutsoumanis et al., 2003; Lehrke et al.,
314 2011). We were therefore surprised to find that WRD with H_2O_2 performed on *L. monocytogenes*

315 dried on surfaces resulted in significantly lower killing effect than when the cells were present in

316 suspensions (Table 1).

317 These results led us to hypothesize that gaseous H_2O_2 may dissolve in the suspension with the

318 bacteria and be active against the bacteria as liquid H_2O_2. It is known from literature that gaseous

319 and liquid H_2O_2 may act through different mechanisms (Finnegan et al., 2010), but there is a

320 disagreement in the literature whether a dry or a humid disinfection process is the most effective

321 (Hultman et al., 2007; Linley et al., 2012; Unger-Bimczok et al., 2008). To test if H_2O_2 accumulated in

322 the liquid phase during the WRD exposure, droplets of 0.9 % NaCl (10 µl each, with and without

323 bacteria) were applied on stainless steel, and exposed to WRD. The H_2O_2 concentration in the

324 droplets immediately after the WRD process was around 10 000 ppm H_2O_2. To confirm that H_2O_2

325 accumulating in the drops during WRD had an antibacterial effect, droplets (without bacteria)

326 exposed to WRD were pooled and bactericidal activity tested against *L. monocytogenes* in a

327 suspension test. More than 5 LR of *L. monocytogenes* was obtained after 2 h exposure and similar

328 reduction was found for diluted H_2O_2 solution used for WRD at H_2O_2 concentrations of ≥0.2 %.

329 Together, these results support the hypothesis that H_2O_2 dissolves in the liquid during WRD and acts

330 against *L. monocytogenes* in the liquid phase.

331

332 ***3.3. No difference in sensitivity towards H_2O_2 for the tested L. monocytogenes strains***

333 The minimum inhibitory concentration (MIC) was determined both for the pure H_2O_2 solution from

334 Sigma and the H_2O_2 solution containing silver used for WRD, and found to be 125 ppm for both

335 solutions for the four *L. monocytogenes* strains used in the four-strain cocktail in WRD experiments

336 as well as the other 50 strains tested (representative growth curves are shown in Supplementary

337 Figure S1). Furthermore, all 54 *L. monocytogenes* strains were confirmed to be catalase-positive.

338 These results indicate that the results obtained in WRD with H_2O_2 for the four-strain mixture is

339 relevant also for other *L. monocytogenes* strains, and that the WRD disinfection with H_2O_2 is robust
340 against strain variations.
341

342 ***3.4. Peroxide disinfection was robust against organic materials***

343 Since H_2O_2 is a highly reactive compound, we expected that the presence of organic materials would
344 significantly reduce its bactericidal activity (Russell, 1992), but this did not seem to be the case. Even
345 when suspended in 3% BSA, which is a concentration ten times higher than what is used to simulate
346 heavily soiled areas in standard disinfection tests (Anonymous, 1987, 2001), more than 5 LR was
347 obtained for *L. monocytogenes* after WRD. Since the soil in food processing environments is rather
348 complex, we challenged the disinfection system even further, exposing *L. monocytogenes* to WRD
349 suspended in salmon juice (1.2% protein, 0.22% fat, autoclaved) and meat juice (0.8% protein, 0.11%
350 fat, heat treated 80 °C, 30 min). Even in these complex soils, full reduction of *L. monocytogenes* (>5
351 LR) was obtained. Thus, the disinfection process was robust against proteins and fats in soil relevant
352 for production of cooked ready-to-eat salmon and meat products. Finally, we exposed *L.*
353 *monocytogenes* suspended in raw meat juice or raw salmon juice to WRD, and the bactericidal effect
354 was significantly reduced as only 0.9 LR and 0.7 LR was obtained, for raw meat juice and raw salmon
355 juice, respectively. The H_2O_2 concentration was measured in the drops on the steel coupons after
356 WRD. The H_2O_2 concentration in drops with 0.9% NaCl, BSA, autoclaved salmon juice and heat
357 treated meat juice was >1000 ppm, while the H_2O_2 concentration in raw meat juice was as low as 5
358 ppm, and in raw salmon juice about 200 ppm. The neutralizing effect of raw meat and salmon was
359 likely due to factors that was inactivated by heat. Raw meat is reported to have catalase activity
360 (Bekhit et al., 2013), and the salmon and meat juices (without bacteria added) were tested for
361 catalase activity. When 10 µl of raw meat or salmon juice were added to 10 µl 3% H_2O_2, bubbling was
362 observed, indicating catalase activity. No bubbling was observed when the same test was performed
363 with heat treated meat juice nor with salmon juice. Thus catalase activity of the raw meat and

364 salmon juices may have resulted in degradation of H_2O_2 and in the decreased disinfection effect by

365 H_2O_2 in presence of raw meat and salmon juice.

366

367 ***3.5. In high numbers, aerobically grown bacteria may protect themselves***

368 When *L. monocytogenes* in suspension on stainless steel was exposed to WRD with H_2O_2, as

369 described above, the disinfection was effective (>5 LR, number of viable cells below detection limit)

370 even at as high cell numbers as 8 log cfu per coupon. In these tests, *L. monocytogenes* was cultivated

371 in test tubes without shaking before application to the coupons. To test whether the cultivation

372 conditions could influence the sensitivity of *L. monocytogenes* to WRD with H_2O_2, *L. monocytogenes*

373 was cultivated overnight in baffled Erlenmeyer flasks with shaking at 150 rpm, which are conditions

374 which result in higher oxygen concentration in the culture medium. The cell counts in the flasks

375 varied between the experiments. When the tested cell counts were high (8.1-8.2 log per coupon,

376 three experiments) there were no reduction (< 0.3 LR) of *L. monocytogenes* exposed to WRD as

377 suspension at stainless steel. However in experiments with lower numbers of cells (6.6-7.2 log cfu per

378 coupon, three experiments), > 5 LR was observed. In addition, a further control experiment with

379 culturing in test tube with agitation (150 rpm) (8.1 log cfu applied per coupon) resulted in only 1 LR

380 after WRD. We measured the residual H_2O_2 concentration in the suspensions at stainless steel after

381 WRD, and the concentration was <50 ppm in suspensions made from cultures grown with agitation

382 and >700 ppm for suspensions made from cultures from test tubes without agitation. Thus, *L.*

383 *monocytogenes* cultivated under aerobic conditions seemed to degrade H_2O_2 at high cell

384 concentrations. *L. monocytogenes* is a catalase-positive bacterium and it may be speculated that

385 increased expression of the catalase gene (*kat*) under aerobic conditions may explain the lower

386 bactericidal effect and residual H_2O_2 concentrations in the suspensions after disinfection. This is

387 supported by earlier studies demonstrating that the expression of *kat* in *L. monocytogenes* is higher

388 during aerobic than anaerobic conditions (Muller-Herbst et al., 2014), and that cells grown under

389 aerobic conditions are considerably more resistant towards H_2O_2 than cells grown during low levels

390 of oxygen (Boura et al., 2016).

391

392 ***3.6. WRD showed bactericidal effect on biofilms***

393 Exposure of *L. monocytogenes* biofilms on stainless steel to hydrogen peroxide in the present study

394 resulted in a reduction of bacterial numbers larger than the detection limit of the method. The initial

395 cell numbers of untreated control varied from 2.8 to 5 log cfu per coupon between the three

396 replicates and the respective LRs were >1.6, >2.5 and >3.7. *L. monocytogenes* grown as biofilms have

397 been reported to show reduced susceptibility to hydrogen peroxide (Robbins et al., 2005; Yun et al.,

398 2012; Zameer and Gopal, 2010) compared to their planktonic counterparts. On the other hand,

399 hydrogen peroxide attacks biofilm structures and can reduce the presence of biofilms through

400 detachment combined with a killing effect at higher concentrations (Christensen et al., 1990; Rushdy

401 and Othman, 2011). The experiments were not designed to determine whether *L. monocytogenes* in

402 biofilms were more sensitive than suspended bacteria. However, the results indicated higher

403 reduction of biofilm bacteria than what was found for bacteria dried on steel. The H_2O_2

404 concentration of the biofilms after WRD exposure was >700 ppm. The biofilms were humid when

405 exposed to WRD, and the detection of residual H_2O_2 in the biofilms indicated that the action of H_2O_2

406 against *L. monocytogenes* biofilms was through H_2O_2 dissolved in the liquid surrounding the biofilms,

407 similar to that seen for suspensions of *L. monocytogenes,* as described above.

408 WRD with H_2O_2 seemed to be at least as effective in reducing biofilms alone as exposure to regular

409 cleaning agents followed by disinfection with commercial quaternary ammonium compound or

410 peracetic acid based disinfectants. When WRD with H_2O_2 was tested against *L. monocytogenes*

411 biofilms grown on PVC conveyor belt material, the logarithmic reduction was on average 2.4 (log cfu

412 per coupon was 4.4 for the control) for the smooth front side of the conveyor belt, and 2.6 (log cfu

413 per coupon was 6.3 for the control) on the backside of the conveyor belt coupon. In a previous study

414 with coupons from the same type of conveyor belt, cleaning and foaming disinfection with
415 quaternary ammonium compounds or peracetic acid had limited effect (< 1 LR) against a biofilm on
416 the backside of the conveyor of a mixture of *L. monocytogenes*. In that study, also the thickest
417 biofilm was found on the backside of the belt, and *L. monocytogenes* were observed to be located in
418 between the threads of the woven belt (Fagerlund et al., 2017).

419 Future studies should be considered with testing of H_2O_2 WRD against mixed species biofilms with *L.*
420 *monocytogenes* and bacteria dominating in the food industry like *Pseudomonas*, *Acinetobacter* etc.
421 (Fagerlund et al., 2017; Møretrø and Langsrud, 2017).

422

423 ***3.7. H_2O_2 was effective also against Geobacillus spores***

424 A commercial spore test designed to verify the effect of H_2O_2 WRD, was included in some of the
425 experiments. The spore test with *Geobacillus stearothermophilus* was placed next to the samples
426 with *L. monocytogenes*. In all the experiments performed, at least 5 LR of spores were observed. The
427 log reductions obtained were similar to the test performed with *L. monocytogenes* suspension
428 applied as wet drops on stainless steel, thus the spore-test may be a suitable indicator to evaluate
429 the disinfection process in the industry. The spore test may also be used to investigate the
430 distribution of the H_2O_2 under WRD in industry, e.g. diffusion of gas inside equipment, and can thus
431 be used to design a disinfection process (time, concentration) that can be effective against *L.*
432 *monocytogenes* in specific niches in the food industry.

433

434 ***3.8. Technical issues and process optimization for H_2O_2 WRD***

435 The H_2O_2 mist generator tested in the present study has previously been used in health care and
436 hospital settings and was in the current project optimized for use at conditions relevant for the food
437 industry. The machine was initially run for three disinfection cycles, with a total run time of 3.5 h,

438 which was the setup for the machine that was commonly used in health care and hospital settings at
439 the time we started this project. In this case the amount of H_2O_2 introduced by the machine to the
440 test room was calculated by the machine based on inputs of the volume of the room and the desired
441 H_2O_2 room concentration. When these settings were employed in our test room at ambient
442 temperature (~20°C), the resulting H_2O_2 concentration during the process was in the range 40-140
443 ppm. These experimental conditions resulted in 100% relative humidity (%RH) and visible fogging
444 inside the room early in the experiment, followed by a decrease in the H_2O_2 concentration in the
445 room in the last phase of the experiment. The humidity was higher during exposure in the test room
446 than in experiments previously performed in health care settings. The reason was most likely that the
447 test room did not contain textiles, paper, wood etc. that may absorb H_2O_2 and humidity. As H_2O_2
448 might cause corrosion problems at 100% RH, and since it may be difficult to obtain a high enough
449 H_2O_2 concentration in the room at such humidity, the setup of the machine was changed from the
450 three cycles to a single exposure phase where a hysteresis control loop was used to start and stop
451 filling of H_2O_2 into the room during the exposure phase. Another reason for changing the process was
452 to reduce the process time.

453 In the new setup, the machine was programmed to produce H_2O_2 only when the H_2O_2 concentration
454 in the room was measured to be within the range 40-80 ppm. For tests with start temperature of 12
455 °C, an accumulation of H_2O_2 levels to >60 ppm, led to a humidity in the test room of 100 %RH. Based
456 on this, the machine was reprogrammed again to cease H_2O_2 production at RH > 90%. Under further
457 tests with start temperature at 12 °C, this programming of the machine led to a H_2O_2 concentration
458 during exposure of 35-50 ppm (example of process parameters shown in Figure 1). The revised setup,
459 using both relative humidity and H_2O_2 concentration as thresholds, was robust against changes in
460 room temperature and humidity. As high humidity in the environment can limit the maximum H_2O_2
461 concentration obtained, WRD may be considered performed in potentially humid rooms in dryer
462 periods, e.g. at the end of the weekend. Performing the disinfection in periods with lower humidity
463 will also led to lower consumption of the disinfectant.

464 The silver in the H_2O_2 solution seemed not to have a major antilisterial effect. The H_2O_2 solution used
465 contains 0.005% silver. Silver is known to be antibacterial towards *L. monocytogenes* at
466 concentrations as low as 0.002% (Belluco et al., 2016). In water disinfection tests, silver has been
467 shown to potentiate the effect of liquid H_2O_2 against *Escherichia coli* and *Pseudomonas aeruginosa*
468 (Martin et al., 2015; Pedahzur et al., 1995). In the present study, however, we observed no difference
469 in the MIC for H_2O_2 acting on *L. monocytogenes* when H_2O_2 assayed alone, compared to in the H_2O_2
470 solution containing silver. Furthermore, the antibacterial effect in the WRD experiments concur with
471 the residual H_2O_2 concentration in bacterial suspensions. Therefore, H_2O_2 is most probably the
472 dominant active compound in the WRD tests performed in the present study.

473

474 ***3.9. Potential of WRD with hydrogen peroxide to combat Listeria in the food industry***

475 *L. monocytogenes* is primarily a challenge for food producers that make ready-to-eat food that will
476 be consumed without prior heat treatment at the consumer stage. Particularly, cooked food that is
477 stored refrigerated for a long time in modified atmosphere such as deli meats/fish and soft cheeses,
478 are often involved in food borne outbreaks. The main contamination source for such foods is the
479 food production line after heat treatment and before packaging, where the pathogen can establish
480 both on the equipment and the environment. *L. monocytogenes* is typically associated with and
481 isolated from humid niches that are difficult to reach by ordinary manual C&D processes and it has
482 been suggested that high survival can partly be explained by formation of resistant biofilms (Møretrø
483 and Langsrud, 2004). To be superior to present manual disinfection processes, WRD should eliminate
484 both *L. monocytogenes* present in small puddles and smaller droplets of rinsing water left on surfaces
485 of equipment, floor and walls after cleaning, as well as those remaining and growing in humid and
486 dirty niches that are difficult to reach. The technology should also be effective at low temperatures
487 and against a wide variety of *Listeria* strains. The results from the present study suggest that WRD
488 with hydrogen peroxide meet several of these criteria. The process appeared relatively robust to

489 changes in temperature and could reduce bacteria within a timeframe that is consistent with daily
490 disinfection processes. The experiments indicated that H_2O_2 WRD potentially target *L.*
491 *monocytogenes* in humid niches by dissolving in the liquid phase. We also found that the method was
492 robust against the presence of relevant organic material at the concentrations and exposure times
493 tested. The strain variation with regard to sensitivity to hydrogen peroxide was low, indicating that
494 the results obtained most likely would be similar using other strains.

495 The investigation also revealed some limitations and challenges with WRD that must be overcome to
496 obtain effective disinfection. The hydrogen peroxide could be neutralized by active enzymes from
497 raw materials (e.g. raw meat or raw fish) or bacteria if present in high numbers. Since *L.*
498 *monocytogenes* is primarily a problem post heat treatment, most soiling will have low enzymatic
499 activity. It remains to be clarified if bacteria in the production environment are in such numbers and
500 in a state where they produce catalase in amounts that will neutralize hydrogen peroxide. It has been
501 reported that biofilms can adapt to hydrogen peroxide in laboratory conditions (Yun et al., 2012), but
502 to which degree this mechanism has significance in practical settings is not clear. Ideally, the
503 disinfection process should work also in dry conditions. Under dry conditions *L. monocytogenes* died
504 off due to air drying. Previously we found better survival of *L. monocytogenes* dried in BHI than when
505 the cells were dried in 0.9% NaCl in the present study, and it is known that presence of organic
506 material may increase desiccation tolerance (Møretrø et al., 2013). Thus it is not clear whether the
507 limited effect of H_2O_2 WRD against dry cells will be of importance in practical situations, however *L.*
508 *monocytogenes* are not commonly isolated from dry niches in the food industry. Finally, the results in
509 the present study were obtained in a small test room, and there may be a challenge for the H_2O_2
510 gas/mist to reach all niches in a complex and larger production environment, and concentration and
511 exposure times have to be optimized by practical testing in the industry.

512

513 ## 5. Conclusions

514 This study showed that a WRD system with H_2O_2 was effective against *L. monocytogenes* in
515 suspension on open surfaces at conditions relevant for food production. WRD systems with H_2O_2 may
516 be a tool to control *L. monocytogenes* in the food industry, however testing in the food industry is
517 necessary to verify the effect under practical conditions.

518

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524

525 ## Declaration of Interest

526 Helge Fanebust is employed by Decon-X International. The authors declare no other conflict of
527 interest regarding publication of this paper.

528

529

530 ## Figure Legend

531 **Figure 1**. Process parameters for WRD experiment with generator programmed to cease H_2O_2
532 production when relative humidity %RH >90%. Mean temperature 13.5 °C. One of the H_2O_2 sensors
533 and the %RH sensor were placed directly on the outside of the mist generator, while the other H_2O_2

534 sensor and the temperature sensor were placed approximately 2 m away from the generator, 80 cm
535 above floor.

536

537 Supplementary Figure legend

538 **Supplementary Figure S1:** Growth curves from bacteriostatic growth assays performed in a
539 Bioscreen C instrument, used to determine the minimum inhibitory concentration (MIC) towards
540 H_2O_2. Three replicate experiments are shown for each of the four *L. monocytogenes* strains MF3949,
541 MF5259, MF5634, and MF4536. Results are representative also for a panel of 50 additional *L.*
542 *monocytogenes* strains as detailed in *Materials and Methods*. Samples were grown in 300µl volumes
543 of TSB growth medium containing Decon-X 520/521 at the H_2O_2 concentrations detailed in the
544 legend shown in the upper left panel (0, 63, 125, and 250 µg/ml H_2O_2). All strains show inhibition of
545 growth through an increased length of the lag phase at 125µg/ml H_2O_2.

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549 ## References

550 Anonymous, 1987. Test methods for the antimicrobial activity of disinfectants in food hygiene.
551 Council of Europe, Strasbourg.
552 Anonymous, 2001. NS-EN 13697:2001: Quantitative non-porous surface test for the evaluation of
553 bactericidal and/or fungicidal activity of chemical disinfectants used in food, industrial, domestic and
554 institutional areas. Test method and requirements without mechanical action. European Committee
555 for standardization.
556 Azizoglu, R.O., Kathariou, S., 2010. Temperature-dependent requirement for catalase in aerobic
557 growth of *Listeria monocytogenes* F2365. Applied and Environmental Microbiology 76, 6998-7003.
558 Back, K.H., Ha, J.W., Kang, D.H., 2014. Effect of hydrogen peroxide vapor treatment for inactivating
559 *Salmonella* Typhimurium, *Escherichia coli* O157:H7 and *Listeria monocytogenes* on organic fresh
560 lettuce. Food Control 44, 78-85.
561 Bekhit, A.E.A., Hopkins, D.L., Fahri, F.T., Ponnampalam, E.N., 2013. Oxidative processes in muscle
562 systems and fresh meat: Sources, markers, and remedies. Compr. Rev. Food Sci. Food Saf. 12, 565-
563 597.
564 Beswick, A.J., Farrant, J., Makison, C., Gawn, G., Frost, G., Crook, B., Pride, J., 2011. Comparison of
565 multiple systems for laboratory whole room fumigation. Appl. Biosaf. 16, 139-157.
566 Block, S.S., 2001. Peroxygen compounds, in: Block, S.S. (Ed.), Disinfection, sterilization and
567 preservation, 5 ed. Lippincott Williams & Wilkins, Philadelphia, pp. 185-204.
568 Boura, M., Keating, C., Royet, K., Paudyal, R., O'Donoghue, B., O'Byrne, C.P., Karatzas, K.A.G., 2016.
569 Loss of SigB in *Listeria monocytogenes* strains EGD-e and 10403S confers hyperresistance to
570 hydrogen peroxide in stationary phase under aerobic conditions. Appl. Environ. Microbiol. 82, 4584-
571 4591.
572

573 Chester, B., 1979. Semiquantitative catalase test as an aid in identification of
574 oxidative and nonsaccharolytic gram-negative bacteria. J. Clin. Microbiol. 10, 525-528.
575 Choi, N.Y., Baek, S.Y., Yoon, J.H., Choi, M.R., Kang, D.H., Lee, S.Y., 2012. Efficacy of aerosolized
576 hydrogen peroxide-based sanitizer on the reduction of pathogenic bacteria on a stainless steel
577 surface. Food Control 27, 57-63.
578 Christensen, B., Trønnes, H.N., Vollan, K., Smidsrød, O., Bakke, R., 1990. Biofilm removal by low
579 concentrations of hydrogen peroxide. Biofouling 2, 165-175.
580 Doll, M., Morgan, D.J., Anderson, D., Bearman, G., 2015. Touchless technologies for decontamination
581 in the hospital: A review of hydrogen peroxide and uv devices. Curr. Infect. Dis. Rep. 17.
582 European Commission, 2004. Commission Regulation of the European Parliament and of the Council
583 of 29 April 2004 laying down specific hygiene rules for food of animal origin, 853/2004/EC. Official
584 Journal, L 139, 30.4.2004.
585 Fagerlund, A., Møretrø, T., Heir, E., Briandet, R., Langsrud, S., 2017. Cleaning and disinfection of
586 biofilms composed of *Listeria monocytogenes* and background microbiota from meat processing
587 surfaces. Appl. Environ. Microbiol. 83, e01046 17.
588 Falagas, M.E., Thomaidis, P.C., Kotsantis, I.K., Sgouros, K., Samonis, G., Karageorgopoulos, D.E., 2011.
589 Airborne hydrogen peroxide for disinfection of the hospital environment and infection control: a
590 systematic review. J. Hosp. Infect. 78, 171-177.
591 Ferreira, V., Wiedmann, M., Teixeira, P., Stasiewicz, M.J., 2014. *Listeria monocytogenes* persistence in
592 food-associated environments: Epidemiology, strain characteristics, and implications for public
593 health. J. Food Protect. 77, 150-170.
594 Finnegan, M., Linley, E., Denyer, S.P., McDonnell, G., Simons, C., Maillard, J.Y., 2010. Mode of action
595 of hydrogen peroxide and other oxidizing agents: differences between liquid and gas forms. J.
596 Antimicrob. Chemother. 65, 2108-2115.

597 Fugett, E., Fortes, E., Nnoka, C., Wiedmann, M., 2006. International life sciences institute North
598 America *Listeria monocytogenes* strain collection: Development of standard *Listeria monocytogenes*
599 strain sets for research and validation studies. J.Food Protect. 69, 2929-2938.
600 Holmdahl, T., Lanbeck, P., Wullt, M., Walder, M.H., 2011. A head-to-head comparison of hydrogen
601 peroxide vapor and aerosol room decontamination systems. Infect. Control Hosp. Epidemiol. 32, 831-
602 836.
603 Hultman, C., Hill, A., McDonnell, G., 2007. The physical chemistry of decontamination with gaseous
604 hydrogen peroxide. Pharm. Eng. 27, 22-32.
605 Jiang, Y.B., Sokorai, K., Pyrgiotakis, G., Demokritou, P., Li, X.H., Mukhopadhyay, S., Jin, T., Fan, X.T.,
606 2017. Cold plasma-activated hydrogen peroxide aerosol inactivates *Escherichia coli* O157:H7,
607 *Salmonella* Typhimurium, and *Listeria innocua* and maintains quality of grape tomato, spinach and
608 cantaloupe. Int. J. Food Microbiol. 249, 53-60.
609 Kostenbauder, H.B., 1991. Physical factors influencing the activity of antimicrobial agents, in: Block,
610 S.S. (Ed.), Disinfection, sterilization and preservation, 4th ed. Lea & Febiger, Philadelphia, pp. 59-71.
611 Koutsoumanis, K.P., Kendall, P.A., Sofos, J.N., 2003. Effect of food processing-related stresses on acid
612 tolerance of *Listeria monocytogenes*. Appl. Environ. Microbiol. 69, 7514-7516.
613 Laksanalamai, P., Joseph, L.A., Silk, B.J., Burall, L.S., Tarr, C.L., Gerner-Smidt, P., Datta, A.R., 2012.
614 Genomic characterization of *Listeria monocytogenes* strains involved in a multistate listeriosis
615 outbreak associated with cantaloupe in US. Plos One 7.
616 Langsrud, S., Moen, B., Møretrø, T., Løype, M., Heir, E., 2015. Microbial dynamics in mixed culture
617 biofilms of bacteria surviving sanitation of conveyor belts in salmon-processing plants. J. Appl.
618 Microbiol. 120, 366-378.
619 Lehrke, G., Hernaez, L., Mugliaroli, S.L., von Staszewski, M., Jagus, R.J., 2011. Sensitization of *Listeria*
620 *innocua* to inorganic and organic acids by natural antimicrobials. Lwt-Food Sci. Technol. 44, 984-991.

621 Linley, E., Denyer, S.P., McDonnell, G., Simons, C., Maillard, J.Y., 2012. Use of hydrogen peroxide as a
622 biocide: new consideration of its mechanisms of biocidal action. J. Antimicrob. Chemother. 67, 1589-
623 1596.
624 Lelieveld, H.L.M., Holah, J., Napper, D., 2014. Hygiene in Food Processing. Principles and Practice. 2nd
625 ed. Woodward Publishing Ltd., Cambridge, UK.
626 Martin, N.L., Bass, P., Liss, S.N., 2015. Antibacterial properties and mechanism of activity of a novel
627 silver-stabilized hydrogen peroxide. Plos One 10.
628 McDonnell, G., Grignol, G., Antloga, K., 2002. Vapor phase hydrogen peroxide decontamination of
629 food contact surfaces. Dairy Food Eviron. Sanit.. 22, 868-873.
630 Muller-Herbst, S., Wustner, S., Muhlig, A., Eder, D., Fuchs, T.M., Held, C., Ehrenreich, A., Scherer, S.,
631 2014. Identification of genes essential for anaerobic growth of *Listeria monocytogenes*. Microbiol.-
632 SGM. 160, 752-765.
633 Møretrø, T., Langsrud, S., 2004. *Listeria monocytogenes*: Biofilm formation and persistence in food
634 processing environments. Biofilms 1, 107-121.
635 Møretrø, T., Langsrud, S., 2017. Residential bacteria on surfaces in the food industry and their
636 implications for food safety and quality. Compr. Rev. Food Sci. Food Saf. 16, 1022-1041.
637 Møretrø, T., Langsrud, S., Heir, E., 2013. Bacteria on meat abattoir meat production process surfaces
638 after sanitation: Characterisation of survival properties of *Listeria monocytogenes* and the
639 commensal bacterial flora. Adv. Microbiol. 3, 255-264.
640 Møretrø, T., Midtgaard, E., Nesse, L., Langsrud, S., 2003. Susceptibility of *Salmonella* isolated from
641 fish feed factories to disinfectants and air-drying at surfaces. Vet. Microbiol. 94, 207-217.
642 Møretrø, T., Schirmer, B.C.T., Heir, E., Fagerlund, A., Hjemli, P., Langsrud, S., 2017. Tolerance to
643 quaternary ammonium compound disinfectants may enhance growth of *Listeria monocytogenes* in
644 the food industry. Int. J. Food Microbiol. 241, 215-224.

645 Møretrø, T., Vestby, L.K., Nesse, L.L., Storheim, S.E., Kotlarz, K., Langsrud, S., 2009. Evaluation of
646 efficacy of disinfectants against *Salmonella* from the feed industry. J. Appl. Microbiol. 106, 1005-
647 1012.
648 Ochiai, Y., Yamada, F., Yoshikawa, Y., Mochizuki, M., Takano, T., Hondo, R., Ueda, F., 2017. Sequential
649 transition of the injury phenotype, temperature-dependent survival and transcriptional response in
650 *Listeria monocytogenes* following lethal H_2O_2 exposure. Int. J. Food Microbiol. 259, 52-58.
651 Otter, J.A., Yezli, S., Perl, T.M., Barbut, F., French, G.L., 2013. The role of 'no-touch' automated room
652 disinfection systems in infection prevention and control. J. Hosp. Infect. 83, 1-13.
653 Pedahzur, R., Lev, O., Fattal, B., Shuval, H.I., 1995. The interaction of silver ions and hydrogen-
654 peroxide in the inactivation of *Escherichia coli* - a preliminary evaluation of a new long-acting residual
655 drinking-water disinfectant. Water Sci. Technol. 31, 123-129.
656 Robbins, J.B., Fisher, C.W., Moltz, A.G., Martin, S.E., 2005. Elimination of *Listeria monocytogenes*
657 biofilms by ozone, chlorine, and hydrogen peroxide. J. Food Protect. 68, 494-498.
658 Rushdy, A.A., Othman, A.S., 2011. Bactericidal efficacy of some commercial disinfectants on biofilm
659 on stainless steel surfaces of food equipment. Ann. Microbiol. 61, 545-552.
660 Russell, A.D., 1992. Factors influencing the efficacy of antimicrobial agents, in: Russell, A.D., Hugo,
661 W.B., Ayliffe, G.A.J. (Eds.), Principles and practice of disinfection, preservation and strerilization.
662 Blackwell scientific publications, Oxford, pp. 89-113.
663 Swaminathan, B., Gerner-Smidt, P., 2007. The epidemiology of human listeriosis. Microbes Infect. 9,
664 1236-1243.
665 Tompkin, R.B., 2002. Control of *Listeria monocytogenes* in the food-processing environment. J. Food
666 Protect. 65, 709-725.
667 Tompkin, R.B., Scott, V.N., Bernard, D.T., Sveum, W.H., Gombas, K.S., 1999. Guidelines to prevent
668 post-processing contamination from *Listeria monocytogenes*. Dairy Food Environ. San. 19, 551-562.

669 Unger-Bimczok, B., Kosian, T., Kottke, V., Hertel, C., Rauschnabel, J., 2011. Hydrogen peroxide vapor
670 penetration into small cavities during low-temperature decontamination cycles. J. Pharm. Innov. 6,
671 32-46.
672 Unger-Bimczok, B., Kottke, V., Hertel, C., Rauschnabel, J., 2008. The influence of humidity, hydrogen
673 peroxide concentration, and condensation on the inactivation of *Geobacillus stearothermophilus*
674 spores with hydrogen peroxide vapor. J Pharm. Innov. 3, 123-133.
675 US Food and Drug Administration, 2017. Control of *Listeria monocytogenes* in Ready-To-Eat Foods:
676 Guidance for Industry Draft Guidance.
677 Yun, H.S., Kim, Y., Oh, S., Jeon, W.M., Frank, J.F., Kim, S.H., 2012. Susceptibility of *Listeria*
678 *monocytogenes* biofilms and planktonic cultures to hydrogen peroxide in food processing
679 environments. Biosci. Biotechnol. Biochem. 76, 2008-2013.
680 Zameer, F., Gopal, S., 2010. Impact of hydrogen peroxide on growth and survival of *Listeria*
681 *monocytogenes* biofilms. J. Chem. 7, 1008-1012.

Table 1. Effect of H_2O_2 whole room disinfection against *Listeria monocytogenes* suspension applied on stainless steel

			Inactivation: Listeria in drop	Inactivaton: Listeria dried on surface		
H_2O_2- (ppm)[a]	Time (min)	Temperature (°C)[b]	LR_{Total}[c,d]	LR_{Total}[c]	$LR_{Disinfect}$[c]	$LR_{Control}$[c]
60-80	53		> 5[e,f]	3.7	1.3	2.4
60-80	53		> 5	3.5	0.9	2.6
60-80	83	18.5 ± 0.2	> 5	3.8	1.5	2.3
80-90	83		> 5	4.3	1.3	3.0
40-90	123		> 5	4.0	0.8	3.2
60-90	123	16.4	> 5			
35-42	126		> 5	> 5	0.3	> 5
35-42	122	13.5 ±0.5	> 5			
50-55	123		> 5			

[a]Level during exposure, after the first 5-10 min filling phase

[b]Mean temperature

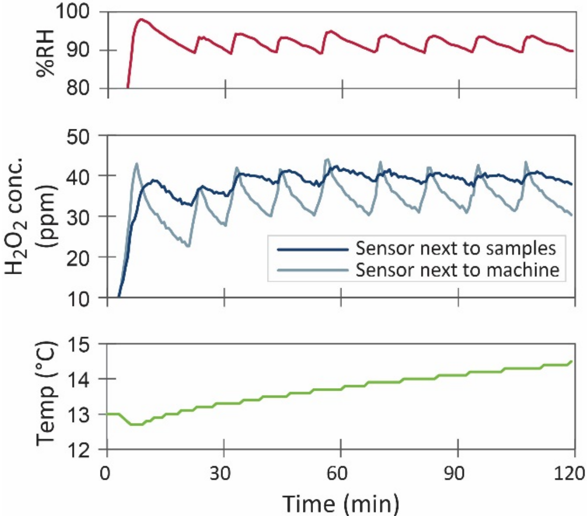
[c]LR_{Total} log reduction compared to number of applied cells; $LR_{Control}$: log reduction in control compared to number of applied cells. $LR_{Disinfect} = LR_{Total} - LR_{Control}$

[d]$LR_{Total} \approx LR_{Disinfect}$ for experiments with drops. $LR_{Control}$ was not included in all experiments with drops as the reduction in the control was insignificant

[e]Log reductions (LR) for mixture of four *L. monocytogenes* strains cultured in test tubes without shaking. Applied cells per coupon was within the range 7.1-8.2 log for the different experiments

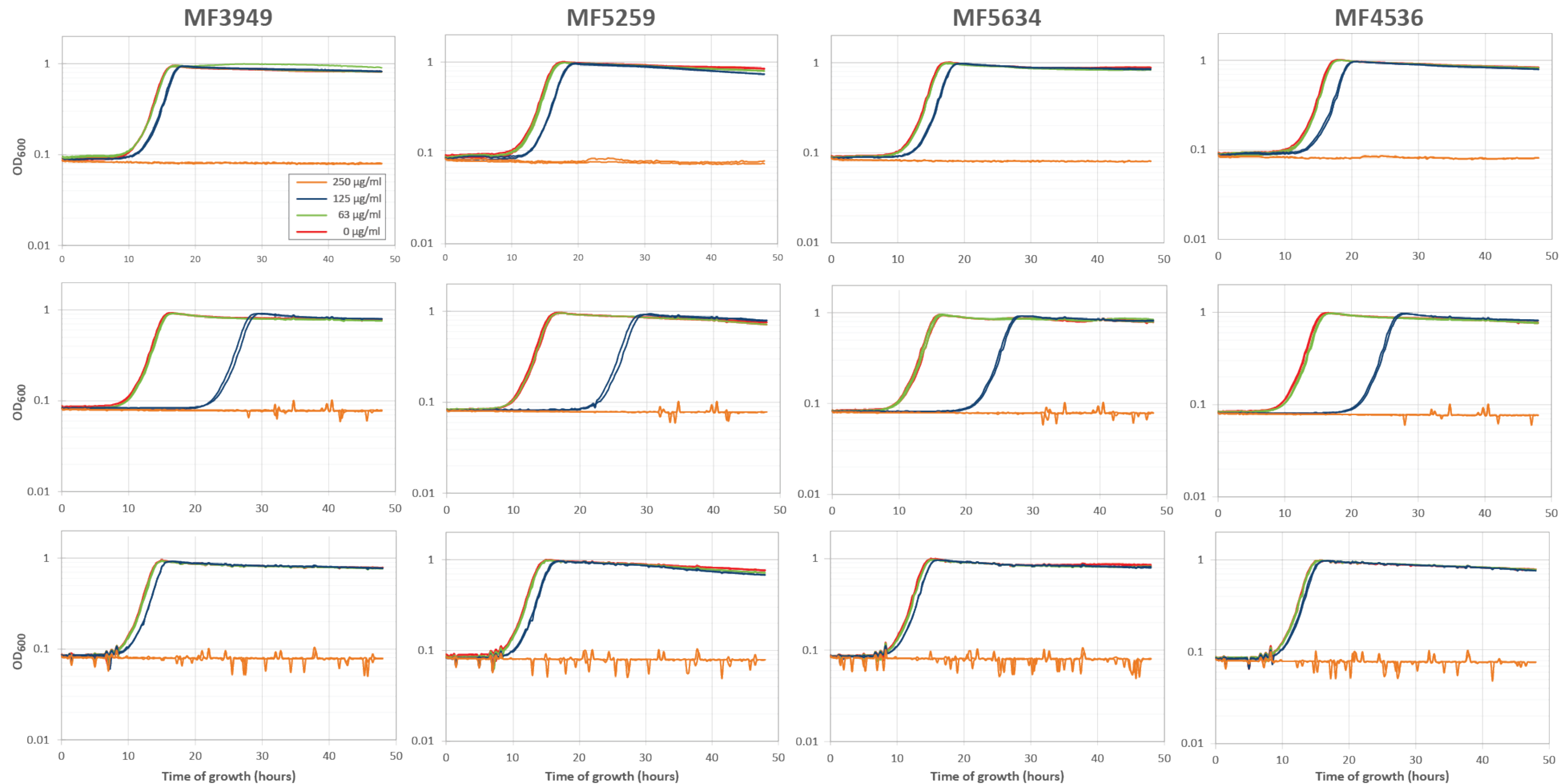
[f]“>” indicates that the number of bacteria viable bacteria was below detection limit, <20 cfu/coupon

Fig 1.



Supplementary data

Møretrø T, Fanebust H, Fagerlund A, and Langsrud S (2018). Whole room disinfection with hydrogen peroxide mist to control *Listeria monocytogenes* in food industry related environments. *Int J Food Microbiol.*



Supplementary Figure S1: Growth curves from bacteriostatic growth assays performed in a Bioscreen C instrument, used to determine the minimum inhibitory concentration (MIC) towards H_2O_2. Three replicate experiments are shown for each of the four *L. monocytogenes* strains MF3949, MF5259, MF5634, and MF4536. Results are representative also for a panel of 50 additional *L. monocytogenes* strains as detailed in *Materials and Methods*. Samples were grown in 300µl volumes of TSB growth medium containing Decon-X 520/521 at the H_2O_2 concentrations detailed in the legend shown in the upper left panel (0, 63, 125, and 250 µg/ml H_2O_2). All strains show inhibition of growth through an increased length of the lag phase at 125µg/ml H_2O_2.