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Whole room disinfection with hydrogen peroxide mist to control *Listeria monocytogenes* in food industry related environments

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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 and 20 °C, H_2O_2 concentrations of 35–80 ppm and 1–2 h 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.

1. Introduction

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

In most processing plants a manual cleaning and disinfection (C&D) process is performed daily after the production process. Typically, for sanitation besides CIP systems, foaming cleaning agents and disinfectants are manually applied to surfaces with rinsing steps with water in between cleaning and disinfection and after the final disinfection step. In most facilities, this process lasts several hours. Some areas/machines may be difficult to reach by the conventional sanitation process, and this may be partly due to too little time to dismantle machines between the production shifts. In addition, some type of equipment/machines may not be cleaned thoroughly as they may be

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sensitive to water or C&D agents (Lelieveld et al., 2014). *L. monocytogenes* is frequently found in many food processing plants despite the use of conventional C&D (Ferreira et al., 2014; Møretrø and Langsrud, 2004). We recently reported that conventional C&D foaming agents had limited effect against *L. monocytogenes* attached to conveyor belts (Fagerlund et al., 2017). An alternative to conventional manual C&D is whole room disinfection (WRD) with gaseous agents (Beswick et al., 2011; Otter et al., 2013). In hospitals and healthcare facilities, WRD with gaseous hydrogen peroxide (H_2O_2) has gained popularity in the last decade (Doll et al., 2015; Falagas et al., 2011). Advantages with the process are that the gas is distributed throughout the room, the process can be automatic, the gas does not affect sensitive equipment and hydrogen peroxide is environmental friendly as it decomposes into water and oxygen (Block, 2001; Linley et al., 2012; Otter et al., 2013; Unger-Bimczok et al., 2011). Challenges related to the process are that an H_2O_2 gas/vapor generator is needed, that the room must be sealed off and that personnel cannot enter during the disinfection process. There are in principle two different technologies for H_2O_2 WRD; these are based on hydrogen peroxide vapor (HPV) and aerosolized hydrogen peroxide (aHP) (Holmdahl et al., 2011). For HPV, a heat generated vapor of 30–35% H_2O_2 is spread throughout the room by a high velocity air stream. With the aHP technology, a solution of H_2O_2 of 5–7% is sprayed out through a nozzle that forms small droplets, which evaporate and spread in the environment (Holmdahl et al., 2011; Otter et al., 2013). For some aHP systems, H_2O_2 solutions with low concentrations of silver are used. Silver stabilises the H_2O_2 solution (Martin et al., 2015). For water disinfection, silver has also been shown to potentiate the antibacterial effect of H_2O_2, but to our knowledge this is yet to been proven for WRD systems (Martin et al., 2015; Pedahzur et al., 1995).

WRD with H_2O_2 has been extensively tested in hospitals and health care facilities. Results from *in situ* use show that HPV systems have eradicated reservoirs of *Clostridium difficile*, MRSA and *Acinetobacter baumannii* during outbreaks, while aHP systems resulted in reduced levels of the same types of microorganisms (Falagas et al., 2011; Otter et al., 2013). But there is limited information available about the effect of using H_2O_2 for WRD in the food industry. McDonnell et al. (2002) claim that a HPV system was effective against *L. monocytogenes* and other bacteria relevant for food processing, though this was a popularized report and few scientific details were given. However, H_2O_2 vapor has been reported to effectively reduce *Listeria* spp. on vegetables (Back et al., 2014; Jiang et al., 2017) and on stainless steel (Choi et al., 2012). Although the use of H_2O_2 for WRD has been shown to be effective in hospitals, this cannot be directly extrapolated to the food industry, as there are different environmental conditions in many food processing areas compared to hospital/health care settings and different types of bacteria are relevant. Hydrogen peroxide may react with organic materials, and the effect of food residues on H_2O_2 may be different than soils from hospitals such as blood. Also humidity and temperature can influence the effect of H_2O_2 WRD (Hultman et al., 2007; Unger-Bimczok et al., 2008) and such conditions may differ between hospitals and food industries. In addition, the resistance towards H_2O_2 and other toxic reactive oxygen species may vary between different bacteria. For instance, *L. monocytogenes* and many other bacteria can produce the enzyme catalase which degrades H_2O_2 to O_2 and water (Azizoglu and Kathariou, 2010). Thus, if H_2O_2 is to be used for WRD against *L. monocytogenes* in the food industry, information about the effect against *L. monocytogenes* under food production environmental conditions are needed.

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

2. Materials and methods

2.1. Bacterial strains and cultural conditions

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

An additional 50 *L. monocytogenes* strains were tested for catalase activity and growth sensitivity to H_2O_2. This set included 22 strains from the ILSI *Listeria* strain collection (Fugett et al., 2006), representing all four genetic *L. monocytogenes* lineages (I, $n = 8$; II, $n = 10$; III, $n = 2$, and IV, $n = 2$) and 28 strains representing a variety of strains originating from Nofima's collection of strains from Norwegian food and food processing environments (lineage I, $n = 2$; II, $n = 26$, mainly from Møretrø et al. (2017)). All bacteria were cultivated in tryptic soy broth (TSB, Oxoid, Basingstoke, UK) and on tryptic soy agar (TSA, Oxoid) at 30 °C, and overnight cultures were grown for 16–18 h in 5-ml volumes in culture tubes without agitation, unless otherwise stated.

2.2. Whole room H_2O_2 disinfection

Disinfection with H_2O_2 mist was tested out in a room at a class 3 biological hazard facility. The room had a total volume of 36 m^3, with inner plastic walls and ceiling and a painted concrete floor. The room contained two conveyor belt units, a stainless steel counter with sinks and some additional small equipment with surfaces of stainless steel as well as a drain channel. During exposure to H_2O_2, the ventilation system was blocked with an airtight shutter and the door closed and sealed with adhesive tape within two minutes after starting the disinfection machine. The room could be preconditioned to 12 °C or 18 °C besides ambient temperature, however the air conditioning was turned off during WRD. At low temperatures, a dehumidifier (Cotech, Clas Olson, Sweden) was used in the period prior to disinfection and programmed to obtain a maximum relative humidity (RH) of 50% at the start of disinfection. For disinfection, the room was filled with H_2O_2 mist, produced by a Decon-X DX1 machine (Decon-X International, Lysaker, Norway). The machine uses a 5% H_2O_2 solution containing 0.005% silver (Decon-X 520/521, Decon-X International), and sprays out small droplets of H_2O_2 through a nozzle, the droplets later evaporate into H_2O_2 gas. The generator was placed in a corner of the room, spraying diagonally in direction of the corner across the room. After end of the disinfection cycle, the ventilation was turned on, and after 5 min the samples were removed from the test room by a person wearing a protective gas mask. H_2O_2 concentration was monitored by a sensor on the outside the machine and with an external H_2O_2 sensor (both sensors: H2O2 CB500, Membrapor AG, Wallisellen, Switzerland) which was placed in close proximity to the samples to be disinfected. Temperature and %RH were measured by sensors on the outside of the machine, and also with an external logging device (Testo 175H1 temperature and humidity logger, Testo Inc., Sparta, NJ, USA), which was placed together with the samples to be disinfected.

For the majority of the tests, the H_2O_2 mist generator was programmed to run a disinfection process for a programmed time with a defined concentration of H_2O_2 in the air in the test room. A hysteresis control loop was used to start and stop filling H_2O_2 into the room during the exposure phase. The machine is in this mode configured with four parameters that control the disinfection process: Max H_2O_2 threshold, Min H_2O_2 threshold, Max relative humidity and Process time. The machine will when starting the disinfection process start to fill H_2O_2 mist into the room. When the Max H_2O_2 threshold or the Max relative humidity value is reached, the machine will stop filling H_2O_2 into the room. When the H_2O_2 concentration in the room falls below the Min H_2O_2 threshold value, and the humidity in the room is below the

Max relative humidity threshold, then the machine will again start to fill more H_2O_2 mist into the room. This process continues for the programmed time duration (Process time). The threshold values and process time that have been used in the present work are Max H_2O_2 threshold: 60–120 ppm, Min H_2O_2 threshold: 40–100 ppm, Max relative humidity: 90%RH and Process time: 53–126 min.

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

Individual overnight cultures in test tubes with 5 mL TSB, cultured at 30 °C without agitation were mixed in equal volumes, washed and resuspended in 0.9% NaCl. Four drops of 10 µL of this suspension (bacterial concentration 8.5–9.6 log/mL) were added a coupon of stainless steel (AISI 304, 2B, Norsk Stål, Nesbru, Norway) (all coupons were sterilized by autoclaving, used only once and were made from new and previously unused steel plates). The coupons were treated in two different ways: One set of coupons were moved to the test room within 5 min after application of the bacteria, while the other set of coupons were dried for 1 h in a safety hood, until visible dry, after application of bacteria, before moving the coupons to the test room. After exposure the coupons were swabbed with a sterile cotton swab (for dry coupons the swab was pre-moistened with saline), and the swab was transferred to a tube with 2 mL Dey Engley Neutralizing Broth (Difco, USA). The tube was vortexed and the number of surviving bacteria determined after plating to TSA (30 °C). Dry and wet control coupons were placed for 2 h in a climatic cabinet (KB8400F, Termaks, Bergen, Norway) at 90%RH at the desired temperature, and otherwise treated as coupons subjected to disinfection. All tests were run with 2–3 coupons as technical replicates.

2.4. Effect of food soils on disinfection effect

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

2.5. Disinfection of biofilms

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

2.6. Measurement of H_2O_2 concentration in liquid with test strips

The residual H_2O_2 concentration in liquid phase (drops of suspension or liquid on biofilm surface) after WRD was measured semi-quantitatively within 5 min with Quantofix Peroxide 100/1000 strips (Sigma-Aldrich) according to the manufacturer's instructions. For measuring of suspensions, the strip was put in contact with the drop. For biofilm studies, strips were put in contact with wet spots, or if such spots were not apparent, 10 µL 0.9% NaCl was added to the coupon, pipetted up and down a couple of times and as much of the volume as possible was transferred to a H_2O_2 strip. Using the strips, the concentration of the H_2O_2 solution used for WRD was determined to be 50,000 ppm (5%), which is the concentration given by the manufacturer, thus confirming the test strips results.

2.7. Suspension test

In order to verify that the liquid H_2O_2 in the drops on stainless steel had antibacterial effect, the bacterial reduction in liquid H_2O_2 was tested in suspension tests. Suspension tests were performed by a modified version of the Council of Europe suspension test EN1276 (Anonymous, 1987), as described previously (Møretrø et al., 2003; Møretrø et al., 2009), with a 2 h exposure time. The test was performed with the four strain mixture of *L. monocytogenes* with dilutions (final concentrations 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 samples of liquid retrieved from 10 µL drops of 0.9% NaCl applied on stainless steel after exposure to H_2O_2 WRD.

2.8. Bacteriostatic growth assay

Assay of the growth of single strains of *L. monocytogenes* in the presence of H_2O_2 was carried out using twofold dilutions of H_2O_2 in a broth microdilution assay, performed in a Bioscreen C instrument (Oy Growth Curves Ab, Ltd.). Each well was inoculated with 300 µL samples of *L. monocytogenes* (overnight cultures were prepared as described in Section 2.1), diluted to approximately 10^4 cfu mL^{-1} in TSB with a twofold dilution series of H_2O_2 solution (Sigma, St. Louis, USA) or Decon-X 520/521 (which contains 5% H_2O_2), and grown at 25 °C with recording of OD_{600} every 15 min for 48 h with shaking before each measurement. Controls contained L. *monocytogenes* grown in TSB, and blank wells contained TSB broth only. The lowest concentration of H_2O_2 able to inhibit growth of *L. monocytogenes*, relative to controls without H_2O_2, was determined from the resulting growth curves and recorded as the minimum inhibitory concentration (MIC). Duplicate wells were used for each sample, and tested strains were assayed at least three times.

In addition to the four *L. monocytogenes* strains listed in Section 2.1 (MF4536, MF5634, MF5259 and MF3949), the following 50 *L. monocytogenes* strains (phylogenetic lineage noted in parenthesis) were tested in this assay: FSL J1-110, FSL J1-225, FSL R2-503, FSL J2-064, FSL N1-225, FSL J2-035, FSL J1-177, FSL R2-500, MF2184, MF6554 (lineage I); EGD-e, FSL C1-056, FSL N3-031, FSL J2-063, FSL M1-004, FSL C1-115, FSL J2-066, FSL J2-054, FSL J2-031, FSL J2-020, MF3638, MF3853, MF3860, MF3939, MF3995, MF4475, MF4545, MF4554, MF4562, MF4624, MF4627, MF4712, MF4792, MF4995, MF4999, MF5366, MF5369, MF5372, MF5377, MF5378, MF5630, MF6241,

MF6300, MF6319, MF6556 and MF6708 (lineage II), FSL J1-168 and FSL J1-031 (lineage III), and FSL J1-158 and FSL W1-111 (lineage IV). Isolates with names starting with the prefix ≪FSL≫ are from the ILSI strain collection (Fugett et al., 2006), while the strains with names starting with ≪MF≫ are obtained from Norwegian food industry (Møretrø et al., 2017).

2.9. Catalase test

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

2.10. Geobacillus stearothermophilus spore test

Spores are often used as biological indicators to test the effect of sterilizations and disinfection. The spores used are non-pathogenic and can be included for process validation in *in situ* tests where pathogens cannot be used. However, it is important to verify that the pathogen of interest has similar sensitivity to the bactericidal treatment as the spores used as indicators. The effect of H_2O_2 mist was tested against a standardized indicator of spores of *Geobacillus stearothermophilus* (Apex biological indicator 4-5-6 log, Mesalabs, Bozeman, MT, USA). The indicator set consists of three steel discs, with 4, 5 and 6 log of spores, respectively. After exposure to H_2O_2 the discs with spores were transferred to tubes with growth media (Mesalabs) and incubated at 55 °C for 7 days. Color change to yellow indicated growth of surviving spores. The viability of the spores was regularly checked by incubating unexposed disks in growth media as positive controls.

2.11. Calculations

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

3. Results and discussion

3.1. Whole room disinfection was effective against suspended Listeria

Initial experiments using whole room disinfection (WRD) with H_2O_2 at regular room temperature (18.5 °C) indicated that the methodology can kill *L. monocytogenes* suspended in thin films of water on surfaces. Exposing droplets of *L. monocytogenes* to H_2O_2 mist resulted in > 5 log reduction (LR) in viable counts (counts below detection limit, < 20 cfu/coupon), even at relatively short exposure times (53 min) and concentrations of H_2O_2 in the range 40–80 ppm (see Table 1). The LR in controls incubated in a humidity cabinet at 90% RH was < 1 log, thus the reduction observed after WRD was mainly a result of H_2O_2 exposure ($LR_{Total} \approx LR_{Disinfect}$).

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

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	18.5 ± 0.2	> 5[e,f]	3.7	1.3	2.4
60–80	53		> 5	3.5	0.9	2.6
60–80	83		> 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	13.5 ± 0.5	> 5	> 5	0.3	> 5
35–42	122		> 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.

3.2. H_2O_2 works through accumulation in liquid phase during WRD

Several studies have demonstrated that drying after cleaning will have an additional inactivation effect on microbes, and it is recommended to keep processing facilities as dry as possible (Tompkin, 2002; Tompkin et al., 1999; US Food and Drug Administration, 2017). From a microbiological point of view, one could expect that drying followed by disinfection would lead to an additive inactivation effect and even a synergistic effect due to stressed cells (Koutsoumanis et al., 2003; Lehrke et al., 2011). We were therefore surprised to find that WRD with H_2O_2 performed on *L. monocytogenes* dried on surfaces resulted in significantly lower killing effect than when the cells were present in suspensions (Table 1).

These results led us to hypothesize that gaseous H_2O_2 may dissolve in the suspension with the bacteria and be active against the bacteria as liquid H_2O_2. It is known from literature that gaseous and liquid H_2O_2 may act through different mechanisms (Finnegan et al., 2010), but there is a disagreement in the literature whether a dry or a humid disinfection process is the most effective (Hultman et al., 2007; Linley et al., 2012; Unger-Bimczok et al., 2008). To test if H_2O_2 accumulated in the liquid phase during the WRD exposure, droplets of 0.9% NaCl (10 µL each, with and without bacteria) were applied on stainless steel, and exposed to WRD. The H_2O_2 concentration in the droplets immediately after the WRD process was around 10,000 ppm H_2O_2. To confirm that H_2O_2 accumulating in the drops during WRD had an antibacterial effect, droplets (without bacteria) exposed to WRD were pooled and bactericidal activity tested against *L. monocytogenes* in a

suspension test. > 5 LR of *L. monocytogenes* was obtained after 2 h exposure and similar reduction was found for diluted H_2O_2 solution used for WRD at H_2O_2 concentrations of ≥0.2%. Together, these results support the hypothesis that H_2O_2 dissolves in the liquid during WRD and acts against *L. monocytogenes* in the liquid phase.

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

The minimum inhibitory concentration (MIC) was determined both for the pure H_2O_2 solution from Sigma and the H_2O_2 solution containing silver used for WRD, and found to be 125 ppm for both solutions for the four *L. monocytogenes* strains used in the four-strain cocktail in WRD experiments as well as the other 50 strains tested (representative growth curves are shown in Supplementary Fig. S1). Furthermore, all 54 *L. monocytogenes* strains were confirmed to be catalase-positive. These results indicate that the results obtained in WRD with H_2O_2 for the four-strain mixture is relevant also for other *L. monocytogenes* strains, and that the WRD disinfection with H_2O_2 is robust against strain variations.

3.4. Peroxide disinfection was robust against organic materials

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

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

When *L. monocytogenes* in suspension on stainless steel was exposed to WRD with H_2O_2, as described above, the disinfection was effective (> 5 LR, number of viable cells below detection limit) even at as high cell numbers as 8 log cfu per coupon. In these tests, *L. monocytogenes* was cultivated in test tubes without shaking before application to the coupons. To test whether the cultivation conditions could influence the sensitivity of *L. monocytogenes* to WRD with H_2O_2, *L. monocytogenes* was cultivated overnight in baffled Erlenmeyer flasks with shaking at 150 rpm, which are conditions which result in higher oxygen concentration in the culture medium. The cell counts in the flasks varied between the experiments. When the tested cell counts were high (8.1–8.2 log per coupon, three experiments) there were no reduction (< 0.3 LR) of *L. monocytogenes* exposed to WRD as suspension at stainless steel. However in experiments with lower numbers of cells (6.6–7.2 log cfu per coupon, three experiments), > 5 LR was observed. In addition, a further control experiment with culturing in test tube with agitation (150 rpm) (8.1 log cfu applied per coupon) resulted in only 1 LR after WRD. We measured the residual H_2O_2 concentration in the suspensions at stainless steel after WRD, and the concentration was < 50 ppm in suspensions made from cultures grown with agitation and > 700 ppm for suspensions made from cultures from test tubes without agitation. Thus, *L. monocytogenes* cultivated under aerobic conditions seemed to degrade H_2O_2 at high cell concentrations. *L. monocytogenes* is a catalase-positive bacterium and it may be speculated that increased expression of the catalase gene (*kat*) under aerobic conditions may explain the lower bactericidal effect and residual H_2O_2 concentrations in the suspensions after disinfection. This is supported by earlier studies demonstrating that the expression of *kat* in *L. monocytogenes* is higher during aerobic than anaerobic conditions (Muller-Herbst et al., 2014), and that cells grown under aerobic conditions are considerably more resistant towards H_2O_2 than cells grown during low levels of oxygen (Boura et al., 2016).

3.6. WRD showed bactericidal effect on biofilms

Exposure of *L. monocytogenes* biofilms on stainless steel to hydrogen peroxide in the present study resulted in a reduction of bacterial numbers larger than the detection limit of the method. The initial cell numbers of untreated control varied from 2.8 to 5 log cfu per coupon between the three replicates and the respective LRs were > 1.6, > 2.5 and > 3.7. *L. monocytogenes* grown as biofilms have been reported to show reduced susceptibility to hydrogen peroxide (Robbins et al., 2005; Yun et al., 2012; Zameer and Gopal, 2010) compared to their planktonic counterparts. On the other hand, hydrogen peroxide attacks biofilm structures and can reduce the presence of biofilms through detachment combined with a killing effect at higher concentrations (Christensen et al., 1990; Rushdy and Othman, 2011). The experiments were not designed to determine whether *L. monocytogenes* in biofilms were more sensitive than suspended bacteria. However, the results indicated higher reduction of biofilm bacteria than what was found for bacteria dried on steel. The H_2O_2 concentration of the biofilms after WRD exposure was > 700 ppm. The biofilms were humid when exposed to WRD, and the detection of residual H_2O_2 in the biofilms indicated that the action of H_2O_2 against *L. monocytogenes* biofilms was through H_2O_2 dissolved in the liquid surrounding the biofilms, similar to that seen for suspensions of *L. monocytogenes*, as described above.

WRD with H_2O_2 seemed to be at least as effective in reducing biofilms alone as exposure to regular cleaning agents followed by disinfection with commercial quaternary ammonium compound or peracetic acid based disinfectants. When WRD with H_2O_2 was tested against *L. monocytogenes* biofilms grown on PVC conveyor belt material, the logarithmic reduction was on average 2.4 (log cfu per coupon was 4.4 for the control) for the smooth front side of the conveyor belt, and 2.6 (log cfu per coupon was 6.3 for the control) on the backside of the conveyor belt coupon. In a previous study with coupons from the same type of conveyor belt, cleaning and foaming disinfection with quaternary ammonium compounds or peracetic acid had limited effect (< 1 LR) against a biofilm on the backside of the conveyor of a mixture of *L. monocytogenes*. In that study, also the thickest biofilm was found on the backside of the belt, and *L. monocytogenes* were observed to be located in between the threads of the woven belt (Fagerlund et al., 2017).

Future studies should be considered with testing of H_2O_2 WRD against mixed species biofilms with *L. monocytogenes* and bacteria dominating in the food industry like *Pseudomonas*, *Acinetobacter* etc.

(Fagerlund et al., 2017; Møretrø and Langsrud, 2017).

3.7. H_2O_2 was effective also against Geobacillus spores

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

3.8. Technical issues and process optimization for H_2O_2 WRD

The H_2O_2 mist generator tested in the present study has previously been used in health care and hospital settings and was in the current project optimized for use at conditions relevant for the food industry. The machine was initially run for three disinfection cycles, with a total run time of 3.5 h, which was the setup for the machine that was commonly used in health care and hospital settings at the time we started this project. In this case the amount of H_2O_2 introduced by the machine to the test room was calculated by the machine based on inputs of the volume of the room and the desired H_2O_2 room concentration. When these settings were employed in our test room at ambient temperature (~20 °C), the resulting H_2O_2 concentration during the process was in the range 40–140 ppm. These experimental conditions resulted in 100% relative humidity (%RH) and visible fogging inside the room early in the experiment, followed by a decrease in the H_2O_2 concentration in the room in the last phase of the experiment. The humidity was higher during exposure in the test room than in experiments previously performed in health care settings. The reason was most likely that the test room did not contain textiles, paper, wood etc. that may absorb H_2O_2 and humidity. As H_2O_2 might cause corrosion problems at 100% RH, and since it may be difficult to obtain a high enough H_2O_2 concentration in the room at such humidity, the setup of the machine was changed from the three cycles to a single exposure phase where a hysteresis control loop was used to start and stop filling of H_2O_2 into the room during the exposure phase. Another reason for changing the process was to reduce the process time.

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

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

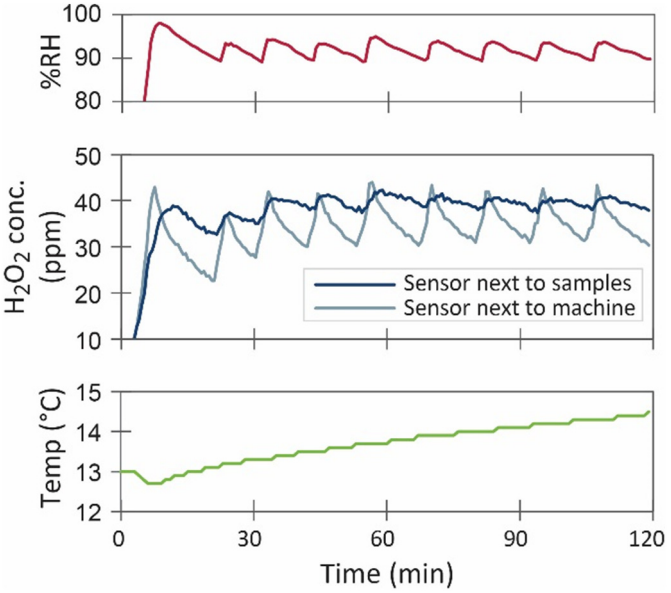

Fig. 1. Process parameters for WRD experiment with generator programmed to cease H_2O_2 production when relative humidity %RH > 90%. Mean temperature 13.5 °C. One of the H_2O_2 sensors and the %RH sensor were placed directly on the outside of the mist generator, while the other H_2O_2 sensor and the temperature sensor were placed approximately 2 m away from the generator, 80 cm above floor.

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

L. monocytogenes is primarily a challenge for food producers that make ready-to-eat food that will be consumed without prior heat treatment at the consumer stage. Particularly, cooked food that is stored refrigerated for a long time in modified atmosphere such as deli meats/fish and soft cheeses, are often involved in food borne outbreaks. The main contamination source for such foods is the food production line after heat treatment and before packaging, where the pathogen can establish both on the equipment and the environment. *L. monocytogenes* is typically associated with and isolated from humid niches that are difficult to reach by ordinary manual C&D processes and it has been suggested that high survival can partly be explained by formation of resistant biofilms (Møretrø and Langsrud, 2004). To be superior to present manual disinfection processes, WRD should eliminate both *L. monocytogenes* present in small puddles and smaller droplets of rinsing water left on surfaces of equipment, floor and walls after cleaning, as well as those remaining and growing in humid and dirty niches that are difficult to reach. The technology should also be effective at low temperatures and against a wide variety of *Listeria* strains. The results from the present study suggest that WRD with hydrogen peroxide meet several of these criteria. The process appeared relatively robust to changes in temperature and could reduce bacteria within a timeframe that is consistent with daily disinfection processes. The experiments indicated that H_2O_2 WRD potentially target *L. monocytogenes* in humid niches by dissolving in the liquid phase. We also found that the method was robust against the presence of relevant organic material at the concentrations and exposure times tested. The strain variation with

regard to sensitivity to hydrogen peroxide was low, indicating that the results obtained most likely would be similar using other strains.

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

4. Conclusions

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

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Declaration of interest

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

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