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Examining the efficacy of mushroom industry biocides on *Listeria monocytogenes* biofilm

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Abstract

Aims: The aim of this study was to test the efficacy of new and currently used biocides in the mushroom industry for inactivating *L. monocytogenes* biofilm.

Methods and results: A lab-scale study was initially carried out to test the efficacy of eleven biocidal products against a cocktail of five *L. monocytogenes* strains that were grown to three-day biofilms on stainless steel coupons. Biocidal efficacy was then tested under clean and dirty conditions based on the EN 13697:2015 method. The results for the biocides tested ranged between 1.7-log to 6-log reduction of biofilm, with only the efficacy of the sodium hypochlorite-based biocide being significantly reduced in dirty conditions. A pilot-scale trial was then carried out on a subset of biocides against *L. monocytogenes* on concrete floors in a mushroom growing room and it was found that biocide efficacy in lab-scale did not translate well in pilot-scale.

Conclusions: Biocides that are used in the mushroom industry and potential alternative biocides were determined to be effective against *L. monocytogenes* biofilm in both lab-scale and pilot-scale experiments.

Significance and impact of the study: This study has direct impact for the industry as it provides information on the efficacy of currently used biocides and other biocidal products against *L. monocytogenes*, an added benefit to their primary use.

Keywords: *Listeria monocytogenes*; biofilm; biocides; mushroom industry

Introduction

Listeria monocytogenes is a major concern for the food industry. It is an opportunistic pathogen that causes listeriosis in humans, which has a high mortality rate in susceptible individuals. *L. monocytogenes* is a hardy organism that can grow under adverse conditions, including those encountered within the food production environment. It has been shown to persist for years within food industries and this persistence has been attributed to its ability to form biofilms (Møretrø and Langsrud, 2004; Bridier et al., 2015). It can form biofilms on food contact surfaces such as stainless steel and conveyor belts, and is also present on non-food contact surfaces such as floors and drains (Di Bonaventura et al., 2008; Silva et al., 2008; Chaturongkasumrit et al., 2011; Campdepadrós et al., 2012; Muhterem-Uyar et al., 2015). The

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primary route of transmission to humans is through contaminated food which highlights the importance of control strategies such as cleaning and disinfection within food production environments (Fagerlund et al., 2017).

In the food industry, quaternary ammonium compounds (QAC) such as benzalkonium chloride (BAC), sodium hypochlorite and peracetic acid are among the most commonly used biocides to control microbial load (Holah et al., 2003; Fagerlund et al., 2017). Their efficacy against *L. monocytogenes* is well documented in the literature, including studies that test the susceptibility of *L. monocytogenes* to biocides in its planktonic and biofilm forms (Ibusquiza et al., 2011; Cruz and Fletcher, 2012; Costa et al., 2016; Poimenidou et al., 2016; Skowron et al., 2018; Rodríguez-Melcón et al., 2018, 2019). These studies found that *L. monocytogenes* in biofilm form has decreased susceptibility to biocides, which may be attributed to factors including the slower growth of biofilm populations, limited diffusion of biocides through the biofilm matrix and the presence of subpopulations of "persister" cells (Bridier et al., 2011). Furthermore, other environmental factors such as temperature, concentration, surface type and influence of interfering substances such as hard water and organic material, are known to affect the efficacy of biocides (Russell, 2003; Skowron et al., 2018). Different food production environments have different potential influencing factors for biocide activity; therefore, it is ideal to test them in situ.

Fresh cultivated mushrooms*, Agaricus bisporus*, are Ireland's most valuable horticultural crop with a farm gate value of € 122 million in 2016 (DAFM, 2018) and to date, they have not been linked to any listeriosis cases. However, multiple studies have found that *L. monocytogenes* is present in the mushroom production environment and surfaces that are commonly found in this environment can support *L. monocytogenes* biofilm, which is of concern to the industry (Di Bonaventura et al., 2008; Silva et al., 2008; Viswanath et al., 2013; Murugesan et al., 2015; Pennone et al., 2018). Due to the nature of how mushrooms are grown, which is on organic substrates at temperatures between 18-25 °C with high relative humidity (> 85%), there are multiple factors that could interfere with biocidal activity that need to be considered.

In Europe, products that are intended to be used as biocides/disinfectants are managed under the Biocidal Products Regulation 528/2012, classifying them in different product types (PT) while products intended for use to protect plants from damaging influences are classified as plant protection products (PPP) under the European Commission Regulation No 1107/2009 (ECHA, 2019; European Commission, 2019). Biocides that are used within the mushroom production

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environment fall under PT2 (disinfectants and algaecides not intended for direct application to humans or animals), PT4 (food and feed area disinfectants) and PPP categories (O'Neill et al., 2015). Currently, the main biocides used in the mushroom industry are the PT4 category biocides sodium hypochlorite and QACs for any equipment, containers and surfaces that may come in contact with mushroom substrates or mushrooms (e.g. shelving, substrate nets, harvesting knives) and the PT2 category phenolic-based biocides for other non-food contact surfaces (e.g. concrete areas and foot dips). These agents are primarily used to clean and disinfect surfaces and eliminate unwanted microorganisms, including disease-causing bacteria, fungi and viruses that cause crop diseases (O'Neill et al., 2015). However, these products may also have efficacy against *L. monocytogenes* if present. Nyati et al. (2012) has shown that sodium hypochlorite's activity can be negatively influenced by high levels of organic matter, and indeed soiling has been shown to impact the efficacy of a range of biocidal agents (Maillard, 2013). There are concerns regarding the use of phenolic-based compounds due to the risk of taint and environmental safety (O'Neill et al., 2015). In addition, an increased tolerance of *L. monocytogenes* to biocides has been observed to occur within food production environments (Møretrø et al., 2017). To combat this, multiple authors have suggested the use of rotation schemes for biocides with different modes of action (Holah et al., 2003; Henriques and Fraqueza, 2017; Møretrø et al., 2017).

The aim of this study was to test the efficacy of currently used biocides against planktonic and biofilm states of *L. monocytogenes* under conditions found within the mushroom production environment. The efficacies of other biocides that are also approved for use within food production environments were also examined for efficacy.

Materials and Methods

Strains

This study was conducted with eight *L. monocytogenes* strains isolated from the mushroom production environment. The strains 2075, 2081 and 2355 were isolated and identified as persistent by Pulsed Field Gel Electrophoresis (PFGE) carried out by Pennone et al. (2018). The strains 3050, 3051, 3101, 3102 and 3104 were isolated from the production environment after steam cookout of commercial growing rooms (Pennone, personal communication). *L. monocytogenes* Scott A was used as a reference strain. All bacterial strains were stored on Protect beads (Technical Service Consultants Ltd., UK) and 50% glycerol at -80 °C. All the *L.*

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monocytogenes stored cultures were resuscitated by streaking a bead onto Tryptone Soya Agar (TSA; Oxoid, UK) and incubated at 37 °C overnight.

Biocides

The biocidal products, BP1-BP5, are currently used in the Irish mushroom industry while BP6-BP11 are not widely used but are approved for use (Table 1). Dilutions of each biocide were carried out according to the manufacturer's suggestions in hard water (sodium bicarbonate, magnesium chloride and calcium chloride, pH 7) as suggested in EN 13697 (European standard, 2015).

Minimum inhibitory concentration and minimum bactericidal concentration determination of biocides

The method used to test the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the biocides in this study was based on the method described by Sheridan et al. (2012), with modifications. In brief, overnight cultures of each *L. monocytogenes* strain were diluted to $\log_{10}$ 5 CFU ml^{-1} in double strength Mueller-Hinton broth (MHB; CM0405, Oxoid, UK) and 100 µl aliquots were dispensed into each well of a sterile 96-well microtitre plate (3799, Corning, US). The biocide solutions were prepared in hard water and diluted to manufacturer's recommended concentration (MRC), 50% of MRC, 10% of MRC and 1% of MRC. Three 100 µl aliquots of each diluted biocide solution were then added to the wells with liquid cultures and incubated at 37 °C for 24h. The optical density (OD) at 595 nm was taken before and after incubation using a Multiskan FC Microplate Photometer (ThermoFisher Scientific, UK) to monitor growth. The lowest concentration with no visible growth was determined as the MIC. The MBC was then determined by using 10 µl loops to transfer aliquots of the dilutions with no growth from the MIC microtitre plate and inoculating the wells of a new plate with 200 µl of Dey/Engley neutralising broth (DENB, Sigma-Aldrich, UK). The microtitre plates with DENB were then incubated at 37 °C for 24h and the MBC was determined as the lowest concentration which did not turn the purple DENB broth to yellow, representing no growth. This experiment was carried out twice and the results were expressed in percentage relative to the MRC (100%).

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Lab-scale testing of biocide efficacy

The method used to test the listericidal activity of the biocides was based on EN 13697 (European standard, 2015), with modifications. To simulate clean and dirty conditions, each biocide was prepared with either 0.3 g l^{-1} (clean) or 3.0 g l^{-1} (dirty) of bovine serum albumin (BSA; A7030, Sigma-Aldrich, UK) to act as an interfering substance.

Liquid cultures of *L. monocytogenes* 2075, 2081, 2355, 3102 and 3104 were prepared in BHIYE and incubated overnight. The cells were then washed by centrifugation and resuspended in fresh BHIYE broth. Each liquid culture was then adjusted to a final cell concentration of $\log_{10}$ 5 CFU ml^{-1}. A bacterial cocktail solution was used to represent a heterogeneous *L. monocytogenes* biofilm and was prepared with equal amounts of each liquid culture mixed together. Then, 200 µl of *L. monocytogenes* cocktail was added to sterile stainless steel coupons (Type 304, measuring 3×0.8×0.1 cm) in triplicate for each test. The inoculated coupons were then incubated at 25 °C for 72h. Afterwards, the biofilms were washed with sterile PBS to remove unbound cells and air dried in a biosafety cabinet.

Air dried coupons with biofilms were placed in tubes with five millilitres of each biocide in either clean or dirty conditions, including hard water as a no treatment control. Exposure times recommended by the manufacturer (Table 1) were utilised where available. However, when exposure times were not mentioned, the treatment was carried out for five minutes. At the end of each contact time, the coupons were rinsed with two millilitres of DENB and transferred into tubes with five millilitres of DENB. Neutralisation and cell recovery were allowed for 10 minutes.

Enumeration of the surviving biofilms on the coupons was carried out based on the methods described by Dygico et al. (2019). The treated biofilms were then dislodged by sonication at 45 kHz for seven minutes (VWR, Ireland) and the coupons were vortexed for one minute. The disaggregated biofilms were serially diluted in Maximum Recovery Diluent (MRD; Oxoid, UK) and spread plated on TSAYE, in duplicates. The plates were incubated at 37 °C for 24 hours. The tubes with the released biofilms and coupons were also incubated to detect the presence of viable *L. monocytogenes* below direct detection enumeration limits, based on the pH indicator in DENB (bromocresol purple).

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Pilot-scale testing of biocide efficacy

A pilot-scale study was carried out in the experimental mushroom production facility at Teagasc Food Research Centre Ashtown (Dublin, Ireland), which has multiple growing rooms with aluminium shelving structures and sloped concrete floors leading to a drain at the centre of each room. Floor plots were marked with chalk within each growing room and were spaced apart by at least 50 cm. Each plot had a 56 x 39 cm (0.2 m^2) outer plot to avoid cross contamination and a 39 x 28 cm (0.1 m^2) inner plot where the inoculum was applied. All rooms were cleaned and disinfected after previous crops had been terminated, following standard practice. Before each experiment, a steam cookout procedure was carried out in each room, again following standard practice, whereby the room was heated up with steam to 60 °C and the air temperature maintained for three hours then allowed to cool down. During all experiments, the rooms were kept at 17-18 °C and >90% relative humidity, representing industrial mushroom growing conditions.

For this experiment, the same cocktail of *L. monocytogenes* strains as mentioned previously was used. To aid recovery the strains were marked with streptomycin resistance ($strep^R$) according to the method described by Blackburn and Davies (1994). The mutant strains that were most similar to the parent strains were then chosen as assessed by growth curves. The biocides used for the pilot-scale trial, BP3, BP5, BP6, BP10 and BP11, were chosen based on their use status, active ingredient and efficacy in the lab-scale experiment. The biocides were prepared according to the manufacturer's instructions in hard water.

Liquid cultures of each $strep^R$ *L. monocytogenes* strain were prepared in BHI and incubated at 20 °C for 48h. A cocktail was then prepared by pooling equal parts of each liquid culture. The inoculum was prepared by washing the cells by centrifugation, followed by resuspending in MRD with a final cell concentration of $\log_{10}$ 5 CFU ml^{-1}. High levels of *L. monocytogenes* were then established on each of the inner floor plots (0.1 m^2) over three days by inoculating each inner plot with 100 ml of the prepared cell suspension, repeated three times every 24h. After another 24h, the biocide treatments were carried out on duplicate plots by pouring 200 ml of each biocide solution over the inner and outer plot area to ensure sufficient coverage of the inoculated area, with contact times of five and 60 minutes. A five-minute contact time was tested to determine the efficacy of each biocide with short contact times, while 60 minutes was also tested to simulate prolonged contact times commonly used by the mushroom industry. Hard water was used similar to the biocides, to treat the inoculated floor plots acting as a no treatment control. At the end of each

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contact time, the inner plot was swabbed with a sterile sponge stick (SSL10NB; 3M, Ireland), which was then replaced in the bag and 10 ml of DENB was immediately added to neutralise the biocide. Following this, 80 ml of half-Fraser broth was added to each bag (CM0895; Oxoid, UK), homogenised and serially diluted in MRD. From each dilution, 100 µl aliquots were spread plated on to Agar *Listeria* Ottavani and Agosti (ALOA; CM1084, Oxoid, UK) that was supplemented with one milligram per millilitre of streptomycin (S9137, Sigma-Aldrich, UK). To detect the presence of viable *L. monocytogenes* below the limit of direct detection, the samples were then qualitatively analysed based on the enrichment methods described in ISO 11290-1:2017.

Statistical analysis

Biofilm levels with no colony forming units from plates but positive after enrichment were assigned as $\log_{10}$ 0.1 CFU cm^{-2} (limit of detection). All the experiments were repeated three times and the biofilm reduction was calculated by subtracting the log biofilm levels after treatment from untreated biofilms formed on either stainless steel coupons or concrete floors. Average biofilm reduction results were calculated, including standard deviation. The biocide treatment results were expressed in $\log_{10}$ reduction (CFU cm^{-2}). Results were then analysed using analysis of variance (ANOVA) and significant differences ($P < 0.05$) were analysed using Tukey's HSD test.

Results

MIC and MBC determination

As shown in Table 2, the MIC values for the biocides for each of the *L. monocytogenes* strains in pure culture were either equal to or lower than the MBC values. Additionally, most of the MBC values were found to be lower than the MRC by at least 50% of the MRC, with the exception of BP1 and BP5. The MBC results for BP1, along with some MIC values, were found to be outside the range of the tested dilutions (more than MRC of BP1), while the MBC for BP5 were found to be equal to the MRC for strains 2075, 2355 and 3050. The biocides BP3, BP6 and BP7 (all QACs) were found to be the most potent biocides, with MIC and MBC values being determined at 1% dilution of the MRC. The phenolic-based biocide BP4 was also found to be potent, with MIC and MBC values at 10% of the MRC on average. The MIC and MBC values for each biocide were relatively consistent across all strains, except strain 2081, which was found to be more sensitive to BP9 and BP11. When grouped together, there were no notable differences between the biocide

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MIC and MBC values of the laboratory strain (Scott A), persistent mushroom industry isolates (2075, 2081 and 2355) and post-cookout isolates (3050, 3051, 3101, 3102 and 3104).

Inactivation of *L. monocytogenes* biofilm on stainless steel coupons

The *L. monocytogenes* strain cocktail used was able to form $\log_{10}$ 6 CFU cm^{-2} on average on the stainless steel coupons used. As shown in Table 3, all the commercial biocides tested were able to achieve at least a 3-log reduction of *L. monocytogenes* biofilm, using the dilutions and contact times suggested by the manufacturer (Table 1), except for BP1 and BP10 under dirty conditions. Both BP6 and BP11 were found to be the most effective biocides, with 6-log reductions of biofilms under both hygiene conditions tested (Table 3). The concentration of BSA added, simulating either clean or dirty conditions, was found to not affect the biofilm reduction by all biocides, except BP1. There was a significant 3-log difference in the results for BP1 under the clean and dirty conditions tested, while BP10 had a 1.6-log difference which was not statistically significant ($P > 0.05$). In the clean conditions, BP6 and BP11 were found to have a significantly higher reduction than BP9, which had the lowest reduction levels. In the dirty conditions, BP1 was found to have the lowest reduction ($P < 0.05$), followed by BP10, while the remaining biocides had more than a 4-log reduction of *L. monocytogenes* biofilms.

Inactivation of *L. monocytogenes* biofilm on concrete floor

A total of $\log_{10}$ 7.5 CFU of *L. monocytogenes* cocktail was inoculated on to each concrete plot and $\log_{10}$ 7.4 CFU ($\log_{10}$ 4.4 CFU cm^{-2}) was recovered on average from the control plots that were treated with hard water. There was no significant difference found between the inoculated levels of *L. monocytogenes* and recovered levels, or between *L. monocytogenes* levels from the plots treated with hard water for five minutes and 60 minutes. As shown in Table 4, after five minutes exposure, BP3 and BP10 were found to have the highest reduction ($P < 0.05$) with at least a 3-log reduction of *L. monocytogenes* biofilm greater than the other biocides. On other hand, after 60 minutes of exposure, BP3, BP6 and BP10 had a 4-log reduction of biofilm which was significantly higher than BP11 and BP5. The BP5 biocide was found to be the least effective, with a 1.5-log reduction of *L. monocytogenes* biofilm. A longer exposure time was found to have no effect on the biofilm reduction of BP3, BP5 and 10, with no significant difference between five minutes and 60 minutes exposure. On the other hand, BP6 and BP11 had a 3.3-log and 2.3-log increase in biofilm reduction, respectively, after longer exposure.

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Discussion

The main goal of this study was to determine the activity of mushroom industry relevant biocides against *L. monocytogenes* in both lab conditions and in conditions relevant to those found in the mushroom production environment. All the biocides were found to be bactericidal against the planktonic *L. monocytogenes* strains tested at both MRC and half of recommended concentrations, except BP1 (Table 2). However, when comparing their efficacy in lab scale conditions on stainless steel coupons (Table 3) or on concrete floors in commercial like conditions (Table 4) differences could be observed for a number of biocide classes.

Biocides with QACs are among the most commonly used biocides in the food industry due to their low toxicity and effectiveness at low concentrations (Holah et al., 2003; Gerba, 2015). Previous studies have shown that QACs can leave residues on surfaces and that exposure to these low levels of QACs can result in increased resistance of *L. monocytogenes* (Carpentier and Cerf, 1993; Kastbjerg and Gram, 2012; Mousavi et al., 2013; Rodríguez-Melcón et al., 2018). In addition, Rodríguez-Melcón et al. (2018) found that exposure to below MIC levels of BAC can increase biofilm formation of resistant *L. monocytogenes* strains. However, Kastbjerg and Gram (2012) highlighted that highly tolerant strains are rare, while Gerba (2015) suggested that as long as proper use and handling of QACs are observed, there is no reason for its use to be restricted. In this study, three QAC-based biocides (BP3, BP6 and BP7) were included and were found to be the most potent among the biocides tested based on the lab-scale tests, as they were active down to 1% of MRC against planktonic *L. monocytogenes* while achieving more than a 5-log reduction of *L. monocytogenes* biofilm on stainless steel under both clean and dirty conditions. Nyati et al. (2012) also observed that QACs were not affected by high levels of organic compounds. Despite having different formulations and treatment times, with BP6 having an extra QAC active ingredient (Didecyldimethylammonium chloride; DDAC) and longer treatment time, the ANOVA analysis found no significant difference between the efficacy of the three QAC-based biocides. However, in the pilot-scale tests, BP3 was found to be more effective than BP6 against *L. monocytogenes* on concrete floors as it resulted in a 4-log reduction regardless of the exposure times tested, while BP6 needed a longer exposure time to obtain the same reduction (Table 4). This could be due to the lower concentration of gluteraldehyde in BP6 (537 ppm) compared to BP3 (1430 ppm), despite the inclusion of DDAC in the formulation. Interestingly, Cruz and Fletcher (2012) found that different commercial products utilising similar concentrations of BAC, significantly differed

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in concentrations needed to achieve a 5-log reduction of *L. monocytogenes* in suspension. This highlights the importance of considering both the final concentrations of the active ingredients of the biocides applied, as well as other ingredients and formulations can crucially impact their efficacy. Another important aspect to consider is that biocides can degrade during storage, particularly if not stored as recommended by manufacturers which may result in the active concentration being lower than specified on the label.

The peroxide-based biocides (BP10 and BP11) used in this study were found to be effective against planktonic *L. monocytogenes* and biofilm on stainless steel. This is similar to the findings of other studies (Knowles and Roller, 2001; Cruz and Fletcher, 2012; Skowron et al., 2018, 2019). BP10 had 1.6-log less reduction of *L. monocytogenes* biofilm in dirty conditions but this was not statistically significant ($p>0.05$). Previous studies have found that the efficacy of hydrogen peroxide and peracetic acid (BP10) are not hindered by the presence of organic materials, including soiled surfaces (Aarnisalo et al., 2000; Briñez et al., 2006). Both active ingredients of BP11, troclosene sodium and potassium peroxymonosulphate, have been found previously to be effective against *L. monocytogenes* in both planktonic and biofilm forms (Chaitiemwong et al., 2014; Skowron et al., 2019); Simpson Beauchamp et al. (2012) found potassium peroxymonosulphate to be effective against *E. coli* biofilms. This study found that BP11 was not as potent as the other biocides against *L. monocytogenes* on concrete floors when used for a short period of time, but was more effective with a longer exposure time. In contrast, despite having lower log reductions than BP11 against biofilms on stainless steel, BP10 was found to be very effective in reducing *L. monocytogenes* on concrete floors. Previous studies have found peracetic acid and hydrogen peroxide based biocides to be more effective than QAC-based biocides (Ibusquiza et al., 2011; Skowron et al., 2019); however, this was not the case in this study. Based on the results from the pilot-scale study, a peroxide-based biocide would be a potential alternative for the mushroom industry. Compared to other biocides such as QACs, peracetic acid and hydrogen peroxide are considered safer for the environment and leave no residues, while also having a lower risk of inducing bacterial resistance in other foodborne pathogens (Briñez et al., 2006; Ikai et al., 2013; Soumet et al., 2016). Furthermore, O'Neill et al. (2015) found that a commercially available peroxide-based product, utilising peracetic acid and hydrogen peroxide, was effective against the basidiospore and mycelium of *Agaricus bisporus* and green mould-causing *Trichoderma aggresivum*. However, due to their corrosive nature against metals, a targeted approach would be needed for application in the mushroom production environment. The

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cost of the implementation of a new biocide regime also needs to be considered by growers, as such biocides may be more expensive than traditionally used biocides.

Phenolic-based biocides have been commonly used in the mushroom industry and were found in this study to be effective against *L. monocytogenes* in planktonic and biofilm form, regardless of clean or dirty conditions. However, their use is now restricted to the disinfection of non-food contact surfaces such as concrete surfaces and foot-dips. Furthermore, there is a trend away from utilising phenolic-based biocides in the mushroom industry (e.g. for disinfecting concrete floors in empty growing rooms) due to their tainting potential within a food production environment and their negative impact on the environment due to their toxicity to aquatic life (Holah, 1995; Davoren and Fogarty, 2005; O'Neill et al., 2015).

The mode of action of chlorine-based biocides is based on its reaction with proteins and enzymes but the presence of organic materials results in a reaction between chlorine and extraneous proteins which reduces the levels of free chlorine which can exert antimicrobial activity (Nyati et al., 2012; Waters and Hung, 2014). Similar to the findings of Nyati et al. (2012), BSA negatively affected BP1, with a 3-log difference in biofilm reduction between clean and dirty conditions (Table 3). Ibusquiza et al. (2011) concluded that organic substances in the media used are also likely to interfere with biocide activity. This highlights the importance of the cleaning step prior to disinfection in commercial facilities and ensuring that there is a sufficient level of active ingredient in the biocide when used in either clean or dirty conditions.

In addition to organic materials, previous studies have found that temperature, surface type and surface roughness also affect biocide efficacy (Neglia et al., 2008; Chaturongkasumrit et al., 2011; Skowron et al., 2018). The biocides tested in this study, especially the ones used in the pilot-scale trial, were found to be effective against *L. monocytogenes* at temperatures that are typically encountered within a mushroom production environment. Other studies have tested biocides with similar active ingredients at similar temperatures and found that temperature had no effect on biocide efficacy (Aarnisalo et al., 2000; Lourenço et al., 2009; Neglia et al., 2008). Nonetheless, it is relevant to note that decreasing the environmental temperature can result in a decrease in biocide efficacy (Maillard, 2013). Chaturongkasumrit et al. (2011) found that an increase in surface roughness correlated with a decrease in biocide efficacy against *L. monocytogenes* while Chaitiemwong et al. (2014) found that *L. monocytogenes* that is positioned deep within a groove on stainless steel, is less susceptible to disinfectants. Additionally, Chaitiemwong et al. (2014)

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found that increasing exposure time from five minutes to 20 minutes had no significant effect on reduction while in this study, two tested biocides (BP6 and BP11) had a significant increase in *L. monocytogenes* reduction following a longer exposure time. In this study, a decrease in efficacy of BP5, BP6 and BP11 against *L. monocytogenes* biofilm between stainless steel and concrete floors was observed, and although this may be attributed to the differences of the methodologies used for each test, the surface roughness of stainless steel and concrete floors could also be a contributing factor. In addition to the porosity of concrete, Dygico et al. (2019) also previously reported that concrete had a significantly higher surface roughness than stainless steel, >3100 nm and 470 nm respectively. Furthermore, Frank and Chmielewski (2001) found that surface roughness correlates with the cleanability of a surface; it affects the retention of soil or other organic substances which may act as interfering substances for biocides applied. These highlight the importance of the cleaning step prior to disinfection to remove organic materials, as well as adherence to recommended exposure times, which may need to be extended depending on the surface and organic material residues.

In this study, a biocide (BP5) that is currently used in the mushroom industry that utilises animal and plant derived products was found to be effective against *L. monocytogenes*. Potassium salts of fatty acid (Cas no: 67701-09-1), commonly referred to as soap salts, are derived from animal fats or plant oils and are generally regarded as safe (GRAS) by the US Food and Drug Administration (21CFR172.863). They are commonly used as insecticides, herbicides, fungicides, and algaecides (EFSA, 2013). Rosin (CAS no: 8050-09-7), on the other hand, is a naturally occurring compound derived from wood resin that has been previously shown to have bactericidal effect (Sipponen and Laitinen, 2011; Söderberg et al., 1990). Santovito et al. (2018) found Rosin to be effective against *L. monocytogenes* and other Gram-positive bacteria; however, it was only tested against the planktonic form of the different bacteria. The results of that study were in accordance with the results of this study for BP5. In addition, this study also found that Rosin with soap salts performed as well as other biocides against *L. monocytogenes* biofilms on stainless steel, regardless of the levels of BSA, but did not maintain this performance on concrete floors, where it achieved a 1-log reduction of *L. monocytogenes* on average. It is worth noting though that the instructed use for this biocide was to allow for the surface to dry and not to be washed down, allowing for it to build up over time, which may improve its results if tested for a longer exposure time. These results suggest that environmentally benign alternatives to traditional biocides can be

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of use within the mushroom industry, although further optimisation would be needed to improve their efficacy.

The present work shows that the biocides used as plant protection agents with different active ingredients are effective against *L. monocytogenes*, including biocides that are not currently used in the mushroom industry. The results also show that the MRC for all the biocides were found to be sufficient to reduce *L. monocytogenes* numbers, but crucially, efficacies differed depending on the levels of interfering substances and the test method, more specifically, lab-scale tests and pilot-scale tests. In addition, this study identified other potential biocides for use in the mushroom industry, including potentially more environmentally friendly alternatives. This study highlights the importance of the upscale testing of biocides that are proven to be efficacious in the lab, to testing in a food production environment setting, as the efficacy might differ depending on multiple factors that are unique to each environment. Additionally, it demonstrates that biocide usage regimes in mushroom production can be effective at reducing *L. monocytogenes* biofilm levels, as well as their primary focus of eliminating any pest and disease-causing organisms that may be present.

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Conflict of interest

No conflict of interest declared.

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Table 1: List of biocides used in this study

			Manufacturer's instructions		
Biocidal products	Product Classification	Active ingredients (%)	Dilution	Active ingredients final concentration (ppm)	Treatment duration (drying time)
BP1[a]	PT4	Sodium hypochlorite (14%)	0.1%	150	To dry (~20 minutes)
BP2[a]	PT2, PT3	Biphenyl-2-ol (18.02%) Chlorophene (4.32%)	1.0%	1802 432	5 minutes*
BP3[a]	PT3, PT4	Benzalkonium chloride (9.6%) Glutaraldehyde (14.3%)	1.0%	960 1430	5 minutes*
BP4[a]	PT2	Chlorophene (5%) Chlorocresol (10%)	0.4%	200 400	To dry (~20 minutes)
BP5[a]	PPP	Potassium salts of fatty acids (47%) Rosin (9%)	2.0%	9400 1800	To dry (~20 minutes)
BP6[b]	PT2, PT3	Benzalkonium chloride (17.06%) Glutaraldehyde (10.73%) Didecyldimethylammonium chloride (7.8%)	0.5%	853 537 390	15 minutes
BP7[b]	PT3[†]	Benzalkonium chloride (10%) Glutaraldehyde (15%)	1.0%	1000 1500	5 minutes*
BP8[b]	PT3[†]	Iodine (3%)	2.0%	600	5 minutes*
BP9[b]	Under review[‡]	N-(3-aminopropyl)-n-dodecylpropane-1, 3-diamine (1-10%) Alcohols, C9-11, Ethoxylated (1-10%) Tetrasodium Ethylene Diamine Tetraacetate (1-10%) Propan-2-ol (1-10%)	1.0%	500 500 500 500	15 minutes

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BP10[b]	PT3, PT4, PT5	Peracetic acid (4.9%) Hydrogen peroxide (10-30%)	0.5%	245 1000	5 minutes*
BP11[b]	PT3, PT4, PT5	Troclosene sodium (5%) Potassium peroxymonosulfate (23%)	1.0%	500 2300	5 minutes*

[a] Biocides that are currently used in the Irish mushroom industry

[b] Biocides that are currently approved for use but not currently utilised in the mushroom industry

* Treatment times were not specified on manufacturer's label

† Other biocidal products with the same active ingredients are approved for use as PT2 and PT4.

‡ The active ingredient N-(3-aminopropyl)-n-dodecylpropane-1, 3-diamine is currently under review by the European Chemicals Agency, while the rest are accepted and widely used.

PPP: plant protection products; PT: product type; BP: biocidal product.

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Table 2: The MIC and MBC values relative to the manufacturer's recommended concentration (100%) for *L. monocytogenes* strains exposed to biocides for 24h.

Biocides	MIC of biocide (% of MRC)*	MBC of biocide (% of MRC)*	Variations observed
BP1	50 to >100	>100	MIC: 50% for 3050 and 3102, 75% for 2355, 100% for 2081, >100% for all other strains
BP2	10	50	
BP3	1	1	
BP4	10	10	
BP5	1 to 10	10 to 100	MIC:1% for 2081, 10% for all others MBC: 10% for 2081, 3051 and 3102, 50% for Scott A,3101 and 3104, 100% for all other strains
BP6	1	1	
BP7	1	1	
BP8	50	50	
BP9	1 to 10	4 to 50	MIC: 1% for 2081, 3050, 3051 and 3102, 4% for Scott A, 7% for 2355 and 10% for other strains MBC: 4% for 2081, 7% for 3051, 10% for 2075, 2355, 3050 and 3102, 37% for 3104 and 50% for 3101
BP10	50	50	

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BP11	10 to 50	23 to 67	MIC: 10% for 3101, 23% for 2075, 2081 and 3050, 50% for all others MBC: 23% for 2081, 37% for 2075, 67% for 3101 and 50% for all other strains

Scott A, laboratory strain; 2075, 2081 and 2355, persistent mushroom industry isolates; 3050, 3051, 3101, 3102 and 3104, mushroom industry post-cookout isolates

Manufacturer's recommended concentrations are provided in Table 1.

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Table 3: Reduction of *L. monocytogenes* biofilms formed on stainless steel coupons by biocides

Biocidal products[b]	Mean biofilm reduction (Log_{10} CFU cm^{-2} ± SD)[a]	
	Clean conditions[c]	Dirty conditions[c]
BP1*	4.7 ± 2.0^{AB}	1.7 ± 0.8^{C}
BP2	5.5 ± 0.9^{AB}	5.6 ± 0.5^{A}
BP3	$\mathbf{4.9 \pm 1.3^{AB}}$	$\mathbf{5.0 \pm 1.4^{A}}$
BP4	4.8 ± 1.5^{AB}	4.9 ± 1.5^{A}
BP5	$\mathbf{5.4 \pm 0.8^{AB}}$	$\mathbf{5.4 \pm 1.2^{A}}$
BP6	$\mathbf{6.0 \pm 0.0^{A}}$	$\mathbf{6.0 \pm 0.0^{A}}$
BP7	5.1 ± 1.4^{AB}	5.1 ± 1.3^{A}
BP8	5.5 ± 1.1^{AB}	5.8 ± 0.6^{A}
BP9	3.7 ± 1.5^{B}	4.3 ± 1.3^{AB}
BP10	$\mathbf{4.4 \pm 2.0^{AB}}$	$\mathbf{2.8 \pm 2.5^{BC}}$
BP11	$\mathbf{5.9 \pm 0.1^{A}}$	$\mathbf{6.0 \pm 0.0^{A}}$

In bold, biocides selected for pilot study

[a] The initial level of *L. monocytogenes* biofilm on the stainless steel coupon was log_{10} 6 CFU cms^{-2} on average.

[b] Biocidal products with an asterisk (*) have significantly different (P <0.05) results between clean and dirty conditions

[c] Values in the same column with different capital letters (A-C) are significantly different (P <0.05).

Table 4: Reduction of *L. monocytogenes* on concrete floors

Biocidal products[b]	Mean biofilm reduction (Log_{10} CFU cm^{-2} ± SD)[a]	
	5 minutes[c]	60 minutes[c]
BP3	4.0 ± 0.6^{A}	4.5 ± 0.0^{A}
BP5	1.1 ± 0.4^{B}	1.5 ± 0.5^{C}
BP6*	1.0 ± 0.5^{B}	4.3 ± 0.2^{A}
BP10	4.1 ± 0.2^{A}	4.4 ± 0.1^{A}
BP11*	0.6 ± 0.8^{B}	2.9 ± 0.2^{B}

[a] The initial level of *L. monocytogenes* on the concrete floor plots

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was $\log_{10}$ 4.4 CFU cm^{-2} on average.

[b] Biocidal products with an asterisk (*) have significantly different ($P < 0.05$) results between 5 minutes and 60 minutes

[c] Values in the same column with different capital letters (A-C) are significantly different ($P < 0.05$).

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