International Journal of Food Microbiology 317 (2020) 108385

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

The ability of *Listeria monocytogenes* to form biofilm on surfaces relevant to the mushroom production environment

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ARTICLE INFO

Keywords:
Listeria monocytogenes
Biofilm
Mushroom industry

ABSTRACT

Due to its ubiquitous nature, *Listeria monocytogenes* is a threat to all fresh fruits and vegetables, including mushrooms, which are Ireland's largest horticultural crop. Although fresh cultivated mushrooms (*Agaricus bisporus*) have not been previously linked with listeriosis outbreaks, the pathogen still poses a threat to the industry, particularly due to its ability to form biofilms. This threat is highlighted by the multiple recalls of mushroom products caused by *L. monocytogenes* contamination and by previous studies demonstrating that *L. monocytogenes* is present in the mushroom production environment. In this study, the biofilm formation potential of *L. monocytogenes* strains isolated from the mushroom production environment was investigated on materials and at temperatures relevant to mushroom production. A preliminary assessment of biofilm formation of 73 mushroom industry isolates was undertaken using a crystal violet assay on polystyrene microtitre plates. The biofilm formation of a subset ($n = 7$) of these strains was then assessed on twelve different materials, including materials that are representative of the materials commonly found in the mushroom production environments, using the CDC biofilm reactor. Vertical scanning interferometry was used to determine the surface roughness of the chosen materials. All the strains tested using the CDC biofilm reactor were able to form biofilms on the different surfaces tested but material type was found to be a key determining factor on the levels of biofilm formed. Stainless steel, aluminium, rubber, polypropylene and polycarbonate were all able to support biofilm levels in the range of 4–4.9 $\log_{10}$ CFU/cm^2, for seven different *L. monocytogenes* strains. Mushroom industry-specific materials, including growing nets and tarpaulins, were found to support biofilms levels between 4.7 and 6.7 $\log_{10}$ CFU/cm^2. Concrete was found to be of concern as it supported 7.7 $\log_{10}$ CFU/cm^2 of biofilm for the same strains; however, sealing the concrete resulted in an approximately 2-log reduction in biofilm levels. The surface roughness of the materials varied greatly between the materials (0.7–3.5 $\log_{10}$ Ra) and was found to have a positive correlation with biofilm formation ($r_s = 0.573$) although marginally significant ($P = 0.051$). The results of this study indicate that *L. monocytogenes* can readily form biofilms on mushroom industry relevant surfaces, and additionally identifies surfaces of specific concern, where rigorous cleaning and disinfection is required.

1. Introduction

Listeria monocytogenes is a major bacterial foodborne pathogen and is ubiquitously found in nature, including in soil, water and plants (Sauders et al., 2012; Vivant et al., 2013; Weis and Seeliger, 1975). It is of particular importance as it causes listeriosis, an infectious disease with severe symptoms and very high hospitalisation and fatality rates (20–30%). Additionally, between 2013 and 2017, a significantly increasing trend of listeriosis cases has been observed in the European Union, with most cases associated with ready-to-eat food products (EFSA and ECDC, 2018). This increase is attributed to the increasing number of individuals above the age of 45 years, whom are more susceptible to listeriosis (Ricci et al., 2018). Due to its natural presence in the environment, *L. monocytogenes* poses a threat to all fresh fruit and vegetables, including mushrooms, which are Ireland's largest horticultural crop with a farm gate value of € 122 million in 2016 (DAFM, 2018). *L. monocytogenes* is a growing concern for the mushroom industry, as studies have shown that this pathogen can be found in

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https://doi.org/10.1016/j.ijfoodmicro.2019.108385
Received 6 June 2019; Received in revised form 8 September 2019; Accepted 8 October 2019
Available online 22 October 2019
0168-1605/

mushroom production facilities, which therefore poses a potential risk of product contamination (Chen et al., 2018; Murugesan et al., 2015; Pennone et al., 2018; Venturini et al., 2011; Viswanath et al., 2013). To the authors' knowledge, there have been no reports of listeriosis due to the consumption of fresh cultivated mushrooms (*Agaricus bisporus*). However, eleven recalls of mushroom products have occurred since 2013 based on the European Commission's Rapid Alert System for Food (RASFF) database, most of which were imported from Asia. Multiple recalls of sliced or stuffed mushroom products contaminated with *L. monocytogenes* have also occurred in USA and Canada in recent years (CFIA, 2019; FDA, 2019). None of these recalls were directly linked with causing listeriosis but still caused considerable economic burden for the mushroom industry.

L. monocytogenes can survive under adverse conditions in different types of environments, including food production environments, due to its ability to form biofilms. Being in a biofilm state gives *L. monocytogenes*, like other microorganisms, certain ecological advantages: increased chances of acquiring advantageous genes by horizontal gene transfer, better nutrient availability and metabolic cooperation through syntrophic relationships, and production of extracellular polymeric substances (EPS) matrix which enhances resistance to sanitizers, disinfectants, antimicrobial agents and other control procedures such as desiccation and ultraviolet light exposure (Aminov, 2011; Borucki et al., 2003; Daneshvar Alavi and Truelstrup Hansen, 2013; Davey and O'Toole, 2000; Ferreira et al., 2014; Gandhi and Chikindas, 2007; Zoz et al., 2017). Several factors have previously been shown to affect *L. monocytogenes* biofilm formation such as temperature, time, surface type, origin and nutrient availability (Cherifi et al., 2017; Govaert et al., 2018; Kadam et al., 2013; Nilsson et al., 2011). *L. monocytogenes* is divided into four phylogenetic lineages; lineage I (serotypes 1/2b, 3b, 4b, 4d, and 4e) and lineage II (serotypes 1/2a, 1/2c, 3a, and 3c) are of particular importance as they are mostly associated with human listeriosis cases (Liu et al., 2006; Orsi et al., 2011). The relationship between lineage/serotype and biofilm formation has been explored by previous studies, but with conflicting results. Borucki et al. (2003) and Combrouse et al. (2013) found lineage II strains to form more biofilms than lineage I, while Djordjevic et al. (2002) and Takahashi et al. (2009) observed the opposite.

L. monocytogenes can form biofilms on surfaces typically found in the food industry, including food contact surfaces, such as stainless steel, polystyrene, polypropylene, glass, silestone, marble and granite, many of which can also be found in the typical mushroom production environment (Beresford et al., 2001; Bridier et al., 2015; Di Bonaventura et al., 2008; Silva et al., 2008). Non-food contact surfaces that are generally wet and associated with food debris, such as floors and drains, are also a concern as they increase the likelihood of *L. monocytogenes* being present (Bolocan et al., 2015; Campdepadrós et al., 2012; Muhterem-Uyar et al., 2015). This is highlighted in studies finding *L. monocytogenes* on floors and drains in a mushroom production environment (Murugesan et al., 2015; Pennone et al., 2018; Viswanath et al., 2013). The presence of *L. monocytogenes* in the production environment is particularly important for the mushroom industry as mushrooms are categorised as ready-to-eat which means that they can be consumed without the application of a listericidal step such as cooking. *L. monocytogenes* can persist in different food production settings, with studies demonstrating its survival after multiple cleaning and disinfections steps in the dairy, ready-to-eat, meat processing and fish processing industries (Ferreira et al., 2014; In Lee et al., 2017; Jami et al., 2014; Møretrø and Langsrud, 2004). However, there has been limited focus on *L. monocytogenes* biofilm formation potential in commercial vegetable production, despite the potential risks. Such information is key to ensure that harbourage sites are identified, and that cleaning and disinfection regimes are designed and targeted for maximum efficacy. Additionally, there is a paucity of knowledge of the phenotypic behaviour of industry relevant strains, with many studies focusing on a limited strain set.

The crystal violet assay is the most frequently used biofilm quantification technique in microtitre plates (Azeredo et al., 2017). It has been used by multiple studies for testing the biofilm-forming potential of *L. monocytogenes* strains (Costa et al., 2016; Guo et al., 2019; Henriques and Fraqueza, 2017; Nowak et al., 2015). Its high-throughput capability allows for testing of multiple *L. monocytogenes* strains under different conditions simultaneously. However, it can have poor reproducibility due to the non-specific nature of the dye and the susceptibility of the biofilm to being washed away during aspiration of the reagents. In addition, there are a limited number of materials that can be tested for biofilm formation with this method. On the other hand, the CDC biofilm reactor (CBR) model developed by Donlan et al. (2002), has been demonstrated to be a reliable experimental tool for growing biofilms (Goeres et al., 2005). For this reason, it has been used in multiple studies to test the biofilm-forming potential of bacterial species on different materials (Almatroudi et al., 2015; Buse et al., 2014; Greene et al., 2016; Sánchez-Gómez et al., 2015). However, there has been limited focus on its use for testing *L. monocytogenes* biofilm formation on food industry relevant surfaces. The aim of this study therefore was to test the biofilm formation potential of a panel of mushroom industry derived isolates of *L. monocytogenes*, of different serogroups, on industry relevant surfaces using the CBR model system and examine the impact of surface roughness on biofilm formation.

2. Materials and methods

2.1. Strains

The *L. monocytogenes* strains ($n = 73$) that were used in this study (Supplementary Table 1) were serogrouped and characterised as persistent by Pennone et al. (2018) using Pulsed Field Gel Electrophoresis (PFGE); these authors defined persistence as the same pulsotype obtained from one company at least 6 months apart. *L. monocytogenes* Scott A and EGD-e strains were also used as reference strains (Supplementary Table 1). All bacterial strains were stored on Protect beads (Technical Service Consultants Ltd., UK) and 50% glycerol at −80 °C. All the stored cultures were resuscitated by streaking a bead onto Tryptone Soya Agar (TSA; Oxoid, UK) and incubating at 37 °C for 18–24 h.

2.2. Determination of biofilm-forming ability by crystal violet (CV) assay

Assessment of the biofilm-forming ability of all 73 *L. monocytogenes* strains was carried out based on the crystal violet assay described by Bolocan et al. (2017), with minor modifications. Briefly, liquid cultures of each strain in Brain Heart Infusion broth supplemented with 0.6% yeast extract (BHIYE; Oxoid, UK) were grown overnight at 37 °C. The overnight cultures were then centrifuged at 7000 × *g* for 7 min at 4 °C and were then resuspended in fresh BHIYE or BHIYE diluted 1:20 (dBHIYE), with a final cell concentration of 7–8 $\log_{10}$ CFU/ml. Two hundred microlitre aliquots of freshly prepared liquid cultures were inoculated into three wells of a sterile round-bottomed polystyrene tissue culture plate (Corning, NY, USA) and then covered with a sterile breathable rayon film (VWR, Ireland) to promote uniform gaseous exchange between all the wells. *L. monocytogenes* strain Scott A was included as a known biofilm-forming control for biofilm formation and sterile media (BHIYE and dBHIYE) as blanks or sterility control for each biofilm grown in the different media. The microtitre plates were then incubated statically in aerobic conditions for 72 h at 18 °C and 25 °C, chosen to reflect the temperatures at different growth stages of the mushroom crop. The biofilms were then washed with 200 µl aliquots of phosphate-buffered saline (PBS; Oxoid, UK) three times to remove unattached cells. The attached cells in each well were then fixed with 200 µl 95% methanol for 15 min, followed by emptying the contents and allowing to air dry for approximately 20 min. The fixed biofilms were then stained with 150 µl of 0.2% *w/v* crystal violet solution

Table 1
Strains used for the CDC biofilm reactor assay.

Strains	Biofilm formation (crystal violet assay)	Serogroup/ serotype	Isolated from[a]
2075	Moderate	4b-4d-4e	Drain
2076	Weak	1/2a-3a	Mushroom growth substrate
2081	Strong	1/2a-3a	Drain
2258	Weak	4b-4d-4e	Floor
2355	Strong	1/2a-3a	Floor
2357	Moderate	1/2b-3b-7	Picking trolley platform
Scott A	Moderate	4b	Clinical

[a] These strains were isolated from different surfaces within the mushroom production environment as part of the study by Pennone et al. (2018).

(Sigma-Aldrich, UK) for 15 min and then the excess stain was rinsed off under gently running tap water. After allowing biofilms to air dry, 200 µl of 35% acetic acid was added to each well. The crystal violet dye was allowed to resolubilise for 30 min on a shaking platform before measuring absorbance at 595 nm (Abs@595 nm) using a Multiskan FC Microplate Photometer (ThermoFisher Scientific, UK). Each experiment was then repeated three times. The results were interpreted based on the formula of Stepanović et al. (2000). The Abs@595 nm cut-off for the negative control was calculated by using the mean Abs@595 nm of all negative control wells plus three standard deviations (Abs_{NC}). The strains were then categorised as weak ($Abs_{NC} < Abs@595\ nm \leq 2 \times Abs_{NC}$), moderate ($2 \times Abs_{NC} < Abs@595\ nm \leq 4 \times Abs_{NC}$) or strong ($4 \times Abs_{NC} < Abs@595\ nm$) biofilm formers.

2.3. Determination of biofilm-forming ability on different materials using the CDC biofilm reactor (CBR)

The biofilm-forming ability of seven selected strains (Table 1) on different materials was assessed by using the CDC Biofilm Reactor (model CBR 90; BioSurface Technologies, Bozeman, MT). Coupons with a diameter of 1.27 cm and thickness of 0.3 cm, made from different materials were also obtained from BioSurface Technologies: stainless steel type 304 (RD128-304), aluminium (RD128-AL), rubber (RD128-EPDM), polypropylene (RD128-PP), polycarbonate (RD128-PC), concrete (hollow polycarbonate cups filled with concrete, RD128-CC), borosilicate glass (RD128-GL), copper (RD128-Cu).

Materials that are specifically found in a mushroom production environment were also tested, including two types of tarpaulin that are used as inside and outside cover for mushroom growing rooms, Nicotarp and Nullatarp (J.F. McKenna Ltd., UK), and nylon growing net (J.F. McKenna Ltd., UK). Nicotarp is made out of high density/low density polyethylene with weaved structure while Nullatarp is a fibre reinforced rubber material with a lacquer finish. The tarpaulins and nets were cut into smaller pieces (1.8 × 1 cm) and were mounted inside the CBR using the CBR membrane holder (CBR-2203-MBM). Additionally, concrete coupons painted with an acrylic co-polymer elastic sealant (Rubcoat, Netherlands) were tested to simulate the areas of the growing rooms where sealant is commonly used, such as gaps between floors and walls. The CBR and the different components were cleaned and assembled according to the manufacturer's instructions.

The seven selected strains (Table 1) were chosen to represent the different serogroups, origin of isolation and biofilm-forming ability based on the CV assay. Testing the biofilm formation of the *L. monocytogenes* strains on surfaces inside the CBR was carried out based on the method described by Pérez-Conesa et al. (2011), with modifications. The different coupons were first installed inside the CBR in triplicate. The CBR unit was then sterilised and then filled with 350 ml of dBHIYE broth. An overnight liquid culture of each strain was then inoculated into the CBR at 1% of the CBR volume. The CBR was then operated in batch-phase for 24 h under shear, created by a baffled magnetic stir bar in conjunction with a magnetic stir plate running at 80 rpm. This was followed by fresh dBHIYE broth pumped into the CBR continuously (flow rate 0.8 ml/min) for another 48 h. The CBR runs were all performed at 25 °C. After each run, the CBR was dismantled and the coupons were taken out aseptically. For the concrete and sealed concrete coupons, only one side of these coupons were of relevance, thus, the biofilms from the polycarbonate cups were removed using a cotton swab soaked with 70% ethanol and were allowed to dry until all the ethanol had evaporated. The other coupons were washed with sterile PBS to remove loosely attached cells and were then placed into glass test tubes with 5 ml of PBS. The biofilms from all the coupons were then dislodged by sonication at 45 kHz for 7 min (VWR, Ireland) and were vortexed for 1 min. The disaggregated biofilms were serially diluted in Maximum Recovery Diluent (MRD; Oxoid, UK) and spread plated on TSAYE, in duplicate. The plates were incubated at 37 °C for 24 h. All the experiments were repeated three times and the results were expressed in $\log_{10}$ CFU/cm^2.

2.4. Surface roughness and imaging of coupons

The surface roughness measurement and imaging of the coupons without biofilms were determined using an optical profilometer (Veeco Wyko® NT1100, USA), operating in Vertical Scanning Interferometry (VSI) mode, with a magnification of 10× and 50×. Five measurements of average surface roughness (Ra) were obtained, with 10× magnification, across the span of each surface sample, except for the net coupon. The 50× magnification was used for the net surface to take Ra of the individual strands as it was not possible with the 10× magnification due to the gaps between each strand. Apart from stainless steel and copper, most samples required one layer of gold coating prior to examining the surface roughness as they had poor reflective surfaces. The concrete (unsealed) coupon had to be coated four times due to the porous nature of the material.

2.5. Confocal laser scanning microscopy (CLSM)

Biofilms on different surfaces were formed as described above using the CBR. Coupons with adhered biofilms were stained according to the LIVE/DEAD BacLight Bacterial Viability Kit instructions (L7012, Molecular Probes, USA). Individual stained coupons were then placed inside a 35 mm coverslip-bottomed µ-Dish (81158, ibidi GmbH, Germany) with a drop of mounting solution. To evaluate the biofilm structure on the different coupons, a confocal laser-scanning microscope was used (Leica SP8, Germany). Image analysis was then carried out using the LAS X software (Leica, Germany). Images were acquired using the 63× objective, using immersion oil.

2.6. Statistical analysis

The mean and standard deviation of the results were determined. Box plots were created to illustrate the distribution of data from the crystal violet assay and CBR experiments. A two-way analysis of variance (ANOVA) with Tukey's post hoc was conducted to examine the effects of coupon materials along with strains and serogroup, on biofilm formation. Estimates of effect size were expressed as partial eta squared (η_p^2). Pairwise comparisons were run for each simple main effect with reported *P*-values receiving Bonferroni adjustment within each simple main effect.

Ra data were log-transformed due to the large range and were analysed using a one-way ANOVA with Tukey's post hoc test to determine statistically significant differences between each coupon material. Spearman's rank correlation coefficient (r_s) was calculated to determine the correlation between the mean surface roughness (Ra) and biofilm formation while effect size was measured by partial eta squared (η_p^2). The confidence level for significance was 95% ($P < 0.05$). Statistical analyses were performed using the IBM SPSS

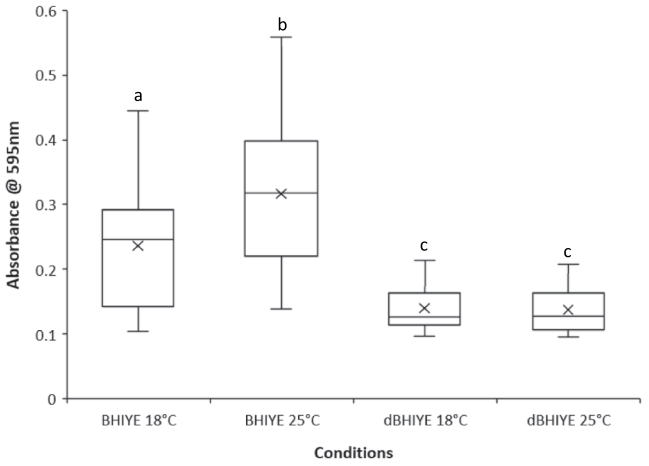

Fig. 1. Data distribution of average absorbance values from the crystal violet assay for the determination of biofilm formation by 73 *L. monocytogenes* strains from the mushroom production environment (plus Scott A and EGD-e) under different conditions. The line in the middle of the box represents the median while the X represents the mean. The whiskers represent the ranges for the bottom 25% and the top 25% of the data values, excluding outliers (represented by the circles). The means for each condition were then compared using ANOVA and the different letters (a–c) represent significant differences ($P < 0.05$) between each condition.

software (version 24.0, SPSS Inc., Chicago IL, USA).

3. Results

3.1. Evaluation of biofilm-forming ability by crystal violet assay

In total, 73 strains of *L. monocytogenes* isolated from the mushroom production environment, plus the reference strains Scott A and EGD-e, were analysed for their biofilm-forming ability using the crystal violet assay. The conditions used (media and temperature) were observed to influence the biofilm forming ability of the strains, as illustrated in Fig. 1. Biofilm formation in BHIYE was significantly higher ($P < 0.05$) than the biofilms formed in dBHIYE. There was no significant difference ($P > 0.05$) between biofilms formed in dBHIYE at 18 °C and 25 °C while in BHIYE, significantly higher ($P < 0.05$) biofilm formation was observed at 25 °C than at 18 °C (Fig. 1).

The strains exhibited varying levels of biofilm formation, from weak to strong, under the different conditions tested, as shown in Fig. 2(A). Additionally, the majority of the mushroom industry isolates were able to form higher levels of biofilms than Scott A and EGD-e in all of the tested conditions. Strains 2910, 2081 and 2355 were all found to have high biofilm formation in BHIYE at both temperatures. In dBHIYE, at 18 °C and 25 °C, 40% and 44% (respectively) of strains were categorised as forming no biofilms (Abs@595 nm < 0.118) while weak biofilms (Abs@595 nm 0.118–0.236) were formed by 60% (18 °C) and 56% (25 °C) of strains. In BHIYE, at 25 °C, 25% of strains were classified as weak biofilm formers, 61.3% as moderate (Abs@595 nm 0.236–0.471) and 10% as strong (Abs@595 nm > 0.471) biofilm formers. At 18 °C, 8% of strains showed no biofilm, 40% demonstrated weak biofilm formation and 52% moderate biofilm formation.

The mushroom industry *L. monocytogenes* strains tested were from three serogroups [1/2a-3a ($n = 22$), 1/2b-3b-7 ($n = 30$) and 4b-4d-4e ($n = 21$)] and were found to form varying levels of biofilms under the different conditions tested. As illustrated in Fig. 2(B), there was little difference in biofilm formation between 18 °C and 25 °C in dBHIYE, following the trend shown in Fig. 1. In dBHIYE, serogroups 1/2a-3a, 1/2b-3b-7 and 4b-4d-4e had generally higher levels of biofilm than the controls (EGDe and Scott A) but no significant difference ($P > 0.05$) was found between the serogroups. Similarly, the three serogroups were found to predominantly form higher levels of biofilm at 18 °C and 25 °C in BHIYE however, no significant difference ($P > 0.05$) was found between the three serogroups and the controls. Only the 1/2a-3a serogroup was found to have significantly higher ($P < 0.05$) biofilm formation than EGDe in BHIYE at 18 °C, and also formed the most biofilm in BHIYE.

3.2. Evaluation of biofilm-forming ability using the CDC biofilm reactor

ANOVA analysis showed a significant ($P < 0.01$) effect of material type on biofilm formation. As illustrated in Fig. 3, all the *L. monocytogenes* strains were able to form biofilms on the 12 different materials tested, with cell numbers ranging between 2 Log_{10} CFU/cm^2 and 7.7 Log_{10} CFU/cm^2, on average. The highest levels of biofilm formation were found to be on concrete and Nicotarp with 7.7 Log_{10} CFU/cm^2 and 6.7 Log_{10} CFU/cm^2, respectively. Copper was found to support significantly less biofilm formation with 2 Log_{10} CFU/cm^2, with 23 out of 63 copper coupons tested having no counts. Aluminium, glass, net, Nullatarp, sealed concrete, polycarbonate, polypropylene, rubber and stainless steel had significant differences ($P < 0.05$) between them, and their average quantified biofilms ranged between 4.2 Log_{10} CFU/cm^2 to 5.3 Log_{10} CFU/cm^2. Interestingly, the concrete coupons painted with a concrete sealant had 5.3 Log_{10} CFU/cm^2 of biofilm, which was a significant decrease of 2.4 Log_{10} CFU/cm^2, when compared to the unsealed concrete coupons.

There was a statistically significant interaction effect between coupon material and strain on levels of biofilms formed ($P < 0.01$, $\eta_p^2 = 0.365$). This suggests that coupon material and strains account for 36.5% of the variance. Therefore, an analysis of simple main effects was performed and found that there was a statistically significant difference in biofilm formation between the different strains grown on the different coupon materials tested. A similar interaction was also found between coupon material and serogroup, albeit with lower effect ($P < 0.01$, $\eta_p^2 = 0.132$). Pairwise comparisons between coupon material and strains were carried out and the results are shown in Fig. 4. Homogenous subsets of the different strains with significant differences were observed for each material, excluding aluminium. The biofilms formed by the strains were generally similar on concrete, copper, glass, net, Nicotarp, rubber and sealed concrete. There was greater variability of the mean biofilm formation between each strain on Nullatarp, polycarbonate, polypropylene and stainless steel. Some strains showed higher biofilm formation on specific surfaces such 2076 on polycarbonate and net; 2081 on polypropylene and stainless steel; and Scott A on net, Nullatarp, Nicotarp and polypropylene.

Grouping the strain results by the respective serogroup showed significant differences ($P < 0.05$) between the strains within one serogroup on some surface materials: 2076 and 2355 (1/2a-3a) were significantly different on copper, polycarbonate and net; while 2075 and 2258 (4b-4d-4e) were significantly different on polycarbonate, polypropylene and stainless steel. Similarly, grouping the strain results by the respective CV assay results showed significant differences between the strains within each CV result category: 2076 and 2258 (both weak) were significantly different on glass, net polycarbonate and stainless steel; 2075 and 2357 (both moderate) were significantly different on Nullatarp and sealed concrete; while 2081 and 2355 (both strong) were significantly different on polycarbonate, polypropylene and stainless steel. Strain 2076, categorised as a weak biofilm former by CV assay, had the highest average biofilm on copper, glass, net and polycarbonate.

3.3. Coupon surface roughness and surface visualisation

The Ra for the surface of each material tested using the optical profilometer is shown in Fig. 5(A), where a large variability was found between the different materials, ranging from 0.7 log_{10} nm up to

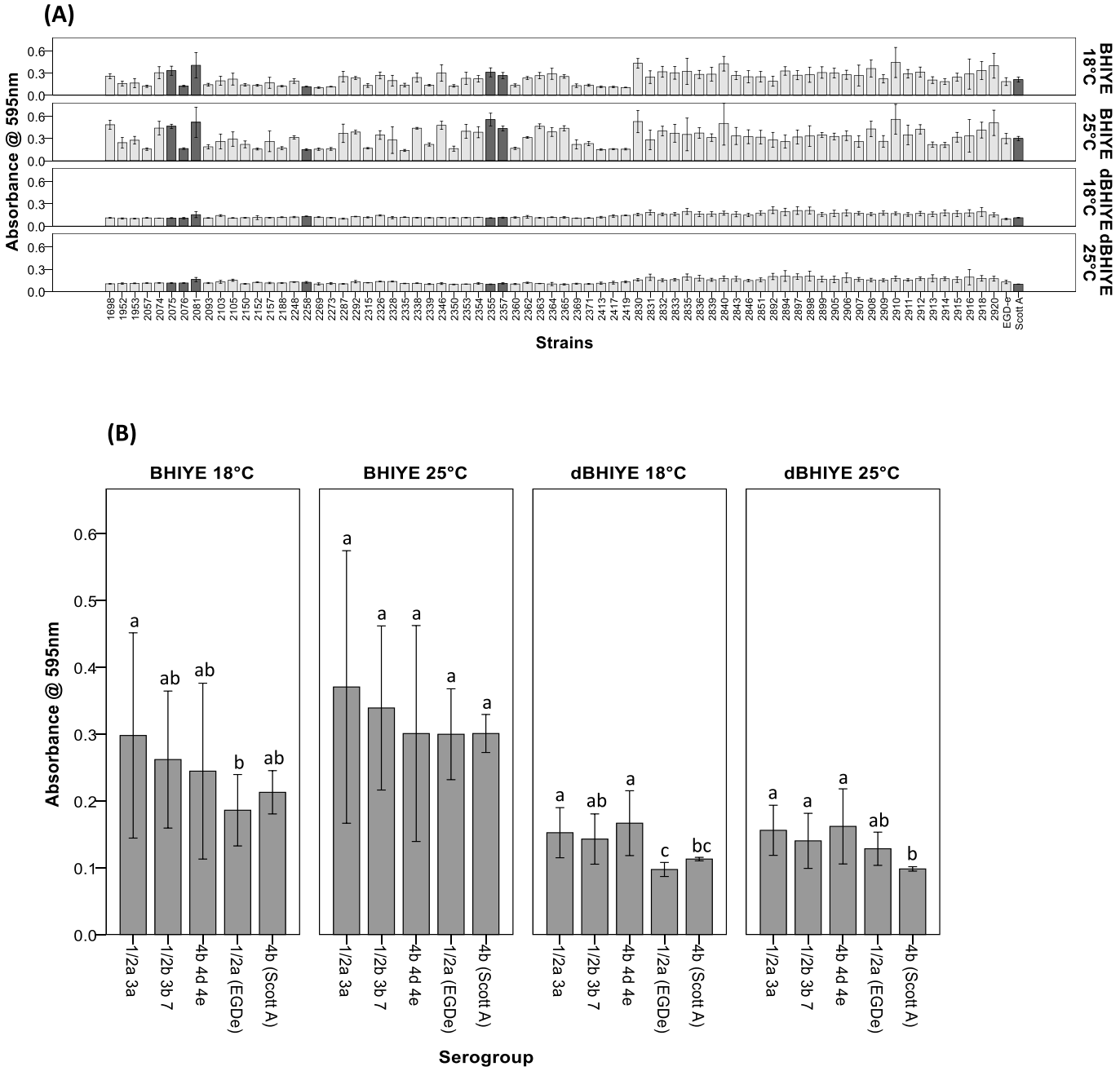

Fig. 2. (A): Mean biofilm formation, including standard deviations, of each *L. monocytogenes* strain under different conditions (dark grey columns represent the strains chosen for the CBR experiment); (B): Mean biofilm formation, including standard deviations, of *L. monocytogenes* strains of different serogroups [1/2a-3a (n = 22), 1/2b-3b-7 (n = 30), 4b-4d-4e (n = 21)] under different conditions, assessed by crystal violet assay. The different letters (a–c) represent significant differences ($P < 0.05$) between the different serogroups in each condition.

3.5 $\log_{10}$ nm. Glass had the lowest Ra at 0.7 $\log_{10}$ nm and was significantly lower ($P < 0.05$) than the other materials. Both concrete and Nicotarp had the same Ra after transformation with 3.5 $\log_{10}$ nm. However, the concrete measurement is likely to underestimate roughness due to the four layers of gold coating. Despite this, concrete still had a significantly higher ($P < 0.05$) Ra than the other surfaces, excluding Nicotarp and polypropylene. The sealant used on concrete was found to be effective at reducing the surface roughness of concrete as it has a significantly lower measurement (3.2 $\log_{10}$ nm) compared to unsealed concrete.

The biofilm formation of the seven *L. monocytogenes* strains (Table 1) on the 12 different surfaces tested were found to have a moderately positive relationship to the Ra of each surface type with a Spearman's rank correlation coefficient $r_s = 0.573$ and was marginally significant with $P = 0.051$ (Fig. 5(B)). Exclusion of the outliers (copper, Nicotarp and concrete) due to their other properties influencing biofilm formation such as porosity and antimicrobial activity, resulted in a weakly positive relationship between biofilm formation and Ra, with no significance (Fig. 5(B)).

Biofilm images depicting the influence of surface topography on biofilms formed are shown in Supplementary Fig. 1A and B. On polypropylene (Supp. Fig. 1A), a micro colony can be observed, which was situated on a crevice on the surface of the material which was mostly composed of living cells (green). The concrete surface (Supp. Fig. 1b) can be seen to have high levels of living cells in a biofilm with the roughness of the surface being highlighted by the higher concentrations

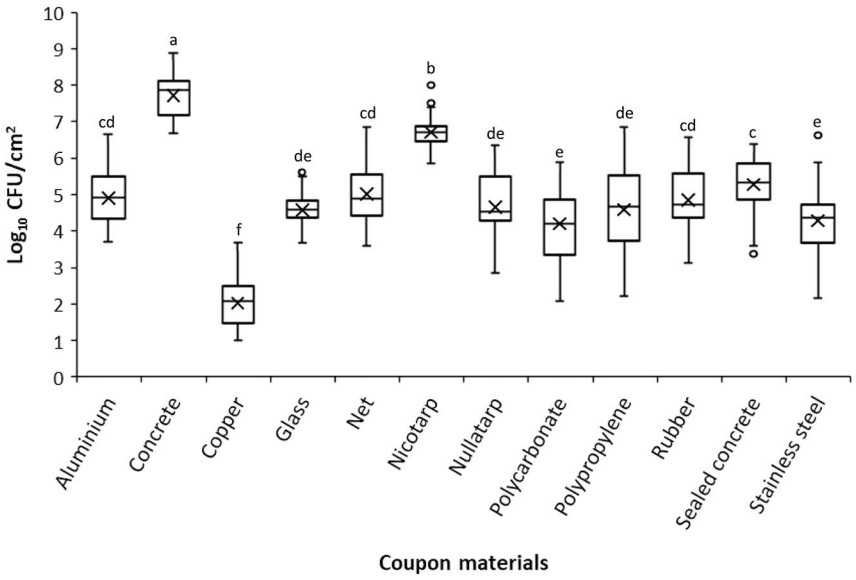

Fig. 3. Boxplots showing data distribution of biofilm formation of *L. monocytogenes* strains on different coupon materials. The line in the middle of the box represents the median while the X represents the mean. The whiskers represent the ranges for the bottom 25% and the top 25% of the data values, excluding outliers (represented by the circles). The different letters from each coupon material represent significant differences ($P < 0.05$) between the different strains/materials tested.

of cells in deeper sections, creating a wave-like pattern.

4. Discussion

In this study, 73 mushroom industry isolates of *L. monocytogenes*, along with reference strains EDG-e and Scott A, were initially tested for their biofilm-forming ability using the well-established crystal violet assay method, followed by testing biofilm formation of selected isolates on the surfaces of different materials used in the mushroom industry using the CBR. Despite the previously outlined limitations of the crystal violet assay (Azeredo et al., 2017; Da Silva and De Martinis, 2013), it was found to be useful in this study to narrow down the list of strains of interest for testing on the CBR.

In this study, it was found that nutrient availability heavily influenced biofilm formation of *L. monocytogenes* on polystyrene microtitre plates, as the 1:20 diluted BHIYE broth was only able to support weak biofilm formation at best, while BHIYE supported moderate to strong biofilm formation. This was similar to other crystal violet assay based findings; Govaert et al. (2018) and Poimenidou et al. (2016) observed higher biofilm formation in nutrient-rich media compared to nutrient-poor media. In contrast, higher biofilm formation from nutrient-poor media has been observed by other studies (Djordjevic et al., 2002; Harvey et al., 2007; Kadam et al., 2013). Interestingly, Galvão et al. (2012) found no significant difference between biofilm formation from BHI and TSB, and 1:10 BHI and 1:10 TSB. Overney et al. (2016) have previously demonstrated that the use of modified conventional culture media is not always representative of the conditions faced in situ and that the use of food soils is more relevant. However, it was found to be sufficient for the purposes of this study as biofilms were able to form in dBHIYE in the CBR model. In addition, multiple studies have also used diluted media to mimic the low levels of nutrient in soil or surfaces within food processing environments (Cherifi et al., 2017; Kadam et al., 2013; Zetzmann et al., 2015).

The temperatures encountered in mushroom production (18 °C and 25 °C) were found to support *L. monocytogenes* biofilm formation when tested in polystyrene microtitre plates, with significantly higher biofilm formation at 25 °C (Fig. 1). The results of this study are in accordance with previously reported findings, in which increased biofilm formation on microtitre plates is observed with increasing temperature (ranging from 4 °C to 37 °C), irrespective of incubation time (Di Bonaventura et al., 2008; Kadam et al., 2013; Nilsson et al., 2011).

Characterisation of the biofilm forming potential of industry isolates is vital due to the significance of *L. monocytogenes* biofilm on food safety. This study found that biofilm formation on microtitre plates was not affected by lineage or serogroup of the strains tested. There was no significant difference observed between the three serogroups (i.e. 1/2a-3a, 1/2b-3b-7 and 4b-4d-4e) under the different conditions (media and temperature) tested, which is in agreement with other crystal violet assay based studies (Di Bonaventura et al., 2008; Doijad et al., 2015; Lourenço et al., 2012). Nowak et al. (2017) associated strain persistence with higher biofilm formation when compared to sporadic strains but, while only persistent strains (Pennone et al., 2018) from the mushroom industry were used in this study, reference strains were also tested, and no significant difference was found between the reference strains and the industry strains (Fig. 2(B)). The influence of lineage or serogroup/serotype on biofilm formation of *L. monocytogenes* is an issue of some debate (Borucki et al., 2003; Combrouse et al., 2013; Djordjevic et al., 2002; Takahashi et al., 2009). The different conditions, such as media and temperature, used for these aforementioned studies may be the inherent cause for differences in results (Cherifi et al., 2017; Combrouse et al., 2013).

In contrast to the divergent findings for lineage/serogroup effect on *L. monocytogenes* biofilm formation, multiple studies with consistent findings, found that biofilm formation can be strain dependent and that there was high variability between strains (Di Bonaventura et al., 2008; Henriques and Fraqueza, 2017; Kadam et al., 2013; Nowak et al., 2017). This was echoed by the results in this study as shown in Fig. 2(A). The large standard deviations observed in both Figs. 1 and 2(B) could be attributed to this variability. Moreover, this strain variability in biofilm formation was further highlighted by Folsom et al. (2006), where different isolates of the same Scott A strain obtained from different labs had significantly different levels of biofilm formation. Thus, the influence of lineage/serogroup on biofilm formation may not be significant enough to overcome the inter-strain variability of *L. monocytogenes* strains.

To identify the mushroom industry materials of particular concern for supporting *L. monocytogenes* biofilm, a quantitative biofilm formation assay on different materials were tested using the CDC biofilm reactor using minimal media (dBHIYE) for simulation of nutrient-poor conditions within the production environment. Biofilm formation by *L. monocytogenes* was found to be significantly influenced by material type and to a lesser extent by strain, with each strain behaving differently on the different materials. However, despite the significant interaction effect between coupon material and strains, there was no feature of interest observed in Fig. 4. Therefore, the main effects of material type on biofilm formation were focused on instead, which suggests that

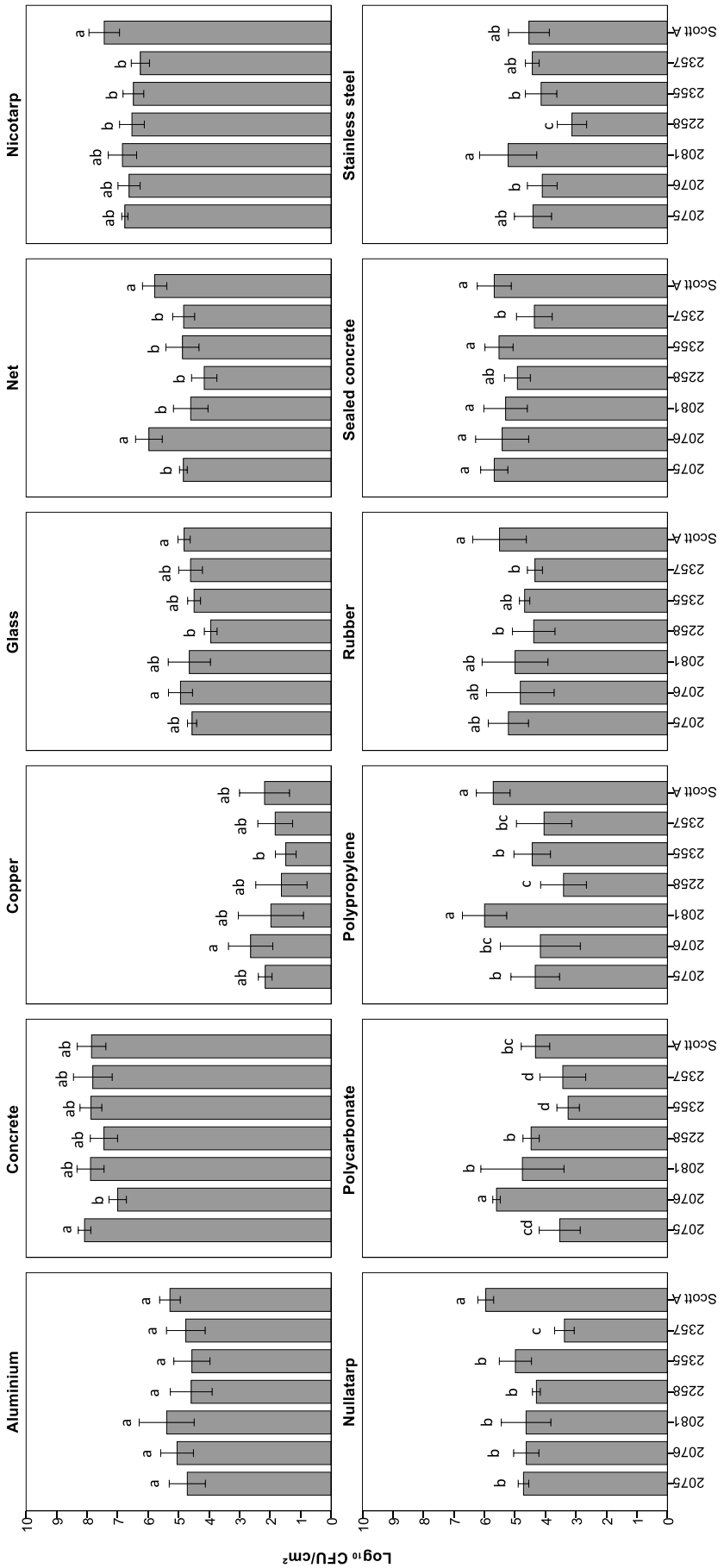

Fig. 4. Mean biofilm formation, including standard deviations, of *L. monocytogenes* strains on the different coupon materials from the CBR experiment. The different letters (a–d) from each coupon material represent significant differences ($P < 0.05$) between the different strains tested.

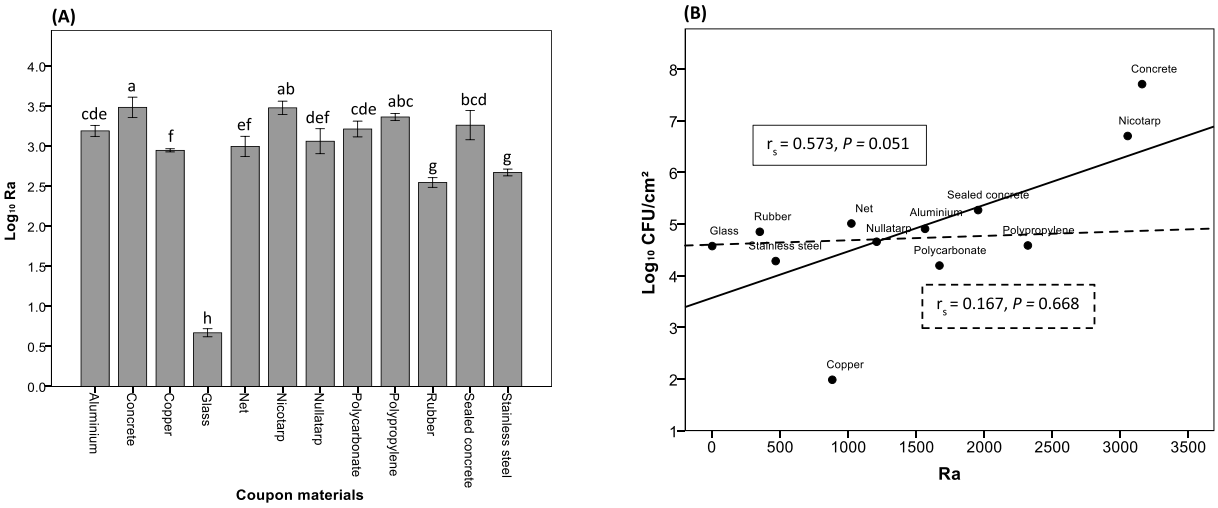

Fig. 5. (A): Mean surface roughness (Log_{10} Ra), including standard deviations, of the different materials without biofilms, determined using an optical profilometer. The different letters (a–h) represent significant differences ($P < 0.05$) between the different surface types; (B): Spearman's rank correlation (r_s) between mean surface roughness (Ra) measurements from the coupon materials and biofilms formed (Log_{10} CFU/cm^2) using the CBR system. The solid line represents the correlation between the biofilm formation and surface roughness for all the coupons while the dotted line excludes the outliers (copper, concrete and Nicotarp).

coupon material have a stronger influence on the biofilm formation of *L. monocytogenes* (Fig. 3). Corcoran et al. (2013) and Greene et al. (2016) also found varying levels of biofilm formation of *Salmonella* spp. and *Acinetobacter baumannii* on different materials using the CBR. In this study, all the surfaces tested, excluding copper, were found to support biofilm levels of > 4.2 Log_{10} CFU/cm^2 (Fig. 3). This was similar to the biofilm levels formed in other studies assessing biofilm formation on stainless steel, polymers, rubber and glass (Abeysundara et al., 2017; Silva et al., 2008). Both Nicotarp and concrete materials were found to have significantly higher levels of biofilm (6.7 Log_{10} CFU/cm^2 and 7.7 Log_{10} CFU/cm^2, respectively). This could be attributed to the overlapping layers of the Nicotarp and the porous surface of the concrete where cells could be entrapped, creating harbourage sites. Surface roughness (Ra) results, shown in Fig. 5(A), support this, as both concrete and Nicotarp had significantly higher Ra values than the other surfaces, except when compared to polypropylene and sealed concrete.

The weakly positive (r_s = 0.167) and insignificant correlation observed between the Ra of the different surfaces without the outliers suggests that surface topography is not a key determining factor on biofilm formation on industry-relevant surfaces. However, biofilm formation may be more likely at very high Ra levels, such as for concrete and Nicotarp (both > 3500 nm) as they both also supported the highest levels of biofilms. Rodriguez et al. (2008) and Hilbert et al. (2003) found that biofilm formation on stainless steel was independent of Ra, which varied between 10 nm and 900 nm. The materials in this study that fall into this range, namely stainless steel, glass, rubber and net (copper excluded), had very similar levels of biofilm formation. Other studies have also demonstrated a weak positive correlation between Ra and biofilm formation which were not statistically significant (Greene et al., 2016; Medilanski et al., 2002). The potential for harbourage sites is demonstrated clearly on the CLSM images of the surfaces, shown in Supplementary Fig. 1A and B. Despite the low cell surface coverage observed across the polypropylene surface (Supp. Fig. 1A), a micro colony structure was able to form on a crevice while, the image of the concrete (Supp. Fig. 1B) with the biofilm, highlights the issue faced in the industry of biofilm formation on porous surfaces. As concrete was identified to be of most concern in terms of supporting biofilm formation, an acrylic co-polymer elastic sealant, which reduces the porosity of the surface, was tested and found to significantly reduce the Ra of concrete and reduce biofilm formation by > 99%. Despite the lack of correlation between Ra and biofilms formed, this suggests that surface roughness may still play a role in biofilm formation and be a potential intervention target.

In the case of copper, it was found to support the least amount of biofilm, despite a significantly higher Ra than stainless steel and rubber. These results for both copper and glass suggest that there are other factors that influence biofilm formation. Greene et al. (2016) found that ionic charge and hydrophobicity of surface material also plays a role on biofilm formation. Copper has been shown, in previous studies, to have an antimicrobial effect against gram positive and gram negative bacteria, including *L. monocytogenes* (Latorre et al., 2015; Lu et al., 2014).

Similar to the crystal violet assay results, the effect of lineage/serogroup and strain on biofilm formation using the CBR were also analysed. Quantitative data distribution of biofilm for each strain did not show differences between the different strains, apart from Scott A having higher biofilm formed compared to the industry isolates (data not shown). Grouping the strains by their serogroups also achieved similar data distributions. Other studies have associated Scott A and its 4b serotype as a strong biofilm-former (Borucki et al., 2003; Harvey et al., 2007). Vázquez-Sánchez et al. (2017) found no correlation between serogroup and biofilm formation on polystyrene at 25 °C but, interestingly, observed higher biofilm formation from lineage II than lineage I when grown on stainless steel. This study also found that serogroup 1/2a-3a (lineage II; strains 2076, 2081 and 2355) had higher biofilm than serogroup 1/2b-3b-7 (lineage I; strain 2357) but not to serogroup 4b-4d-4e (lineage I; strains 2075 and 2258). However, it should be noted that only one strain was tested that represents the 1/2b-3b-7 serogroup.

In this study, the importance of utilising different methodologies for assessing biofilm formation was highlighted as there was no clear relationship observed between biofilm forming ability assessed by CV assay and CBR. Interestingly, Doijad et al. (2015) found matching results between crystal violet results and quantitative biofilm results, while Henriques and Fraqueza (2017) found no correlation. Lourenço et al. (2012) also echoes the results from this study, in which they found no correlation between crystal violet assay results and biofilm formation on stainless steel coupons but explains that, these discrepancies can be attributed to strain variation and differences in methodologies. This has also been reported for other bacterial species (Sadiq et al., 2017).

5. Conclusions

The results of this study show that temperature, nutrient availability and inter-strain variability are important factors for determining the

biofilm formation of *L. monocytogenes* on polystyrene microtitre plates. Additionally, the type of material making up the surface is a significant factor on the biofilm-forming potential of *L. monocytogenes* strains and thus, their ability to persist in the environment. This study has shown that, in accordance to previous work on food industry relevant surfaces, mushroom industry isolates of *L. monocytogenes* can form high levels of biofilm on the surfaces of different materials commonly found in the mushroom industry, in particular unsealed concrete and Nicotarp. This identification of the high-risk surfaces will allow a targeted approach for the industry to control *L. monocytogenes*, especially during cleaning and disinfection processes.

Acknowledgements

The authors would like to thank Dr. Kieran Jordan and Mr. Vincenzo Pennone for providing the *L. monocytogenes* strains used in this study; Dr. Sarah Brady, of University College Dublin, for her assistance with the surface roughness measurements of the surfaces; Dr. Gavin McManus, of Trinity College Dublin, for his assistance with the CLSM analysis; and Dr. Jim Grant for his statistical assistance. The authors would also like to give special thanks to Ms. Lisa Purk for her assistance with the lab work. This study was supported by the Department of Agriculture, Food and the Marine, Ireland under the Food Institutional Research Measure (Project 14F881).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.108385.

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