Food Research International 170 (2023) 112989

Contents lists available at ScienceDirect

Food Research International

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

Risk factor-based clustering of *Listeria monocytogenes* in food processing environments using principal component analysis

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ARTICLE INFO

Keywords:
Listeria monocytogenes
Foodborne pathogen
Biofilm
Principal component analysis
Transference
Smoked salmon
Benzalkonium chloride
Cell transfer
Transfer
Food processing environments
Ready-to-eat

ABSTRACT

Listeria monocytogenes has a range of strategies that allow it to persist as biofilms in food processing environments (FPE), making it a pathogen of concern to the food industry. The properties of these biofilms are highly variable among strains, and this significantly affects the risk of food contamination. The present study therefore aims to conduct a proof-of-concept study to cluster strains of *L. monocytogenes* by risk potential using principal component analysis, a multivariate approach.

A set of 22 strains, isolated from food processing environments, were typed by serogrouping and pulsed-field gel electrophoresis, showing a relatively high diversity. They were characterized in terms of several biofilm properties that might pose a potential risk of food contamination. The properties studied were tolerance to benzalkonium chloride (BAC), the structural parameters of biofilms (biomass, surface area, maximum and average thickness, surface to biovolume ratio and roughness coefficient) measured by confocal laser scanning microscopy and (3) transfer of biofilm cells to smoked salmon.

The PCA correlation circle revealed that the tolerance of biofilms to BAC was positively correlated with roughness, but negatively with biomass parameters. On the contrary, cell transfers were not related to three-dimensional structural parameters, which suggests the role of other variables yet unexplored. Additionally, hierarchical clustering grouped strains into three different clusters. One of them included the strains with high tolerance to BAC and roughness. Another one consisted of strains with enhanced transfer ability, whereas the third cluster contained those that stood out for the thickness of biofilms. The present study represents a novel and effective way to classify *L. monocytogenes* strains according to biofilm properties that condition the potential risk of reaching the consumer through food contamination. It would thus allow the selection of strains representative of different worst-case scenarios for future studies in support of QMRA and decision-making analysis.

1. Introduction

Listeria monocytogenes has an outstanding ability to survive and thrive in a broad range of environmental conditions. It thus exhibits a range of responses to environmental stresses potentially present in food processing environments (FPE), such as temperature (Ells et al., 2009; Phadtare & Inouye, 2008), pH (Nilsson et al., 2012; Wu et al., 2023) or low water activity (Farber et al., 1992; Nolan et al., 1992) among others. This ability makes it a pathogen of concern that has been consistently found in the food industry (Carpentier & Cerf, 2011; Ferreira et al., 2014; Rodríguez-López et al., 2015; Colagiorgi et al., 2017), which is thus continually faced with the challenge of preventing its presence in order to reduce the risk of listeriosis. The presence of *L. monocytogenes* in FPE is frequently linked to biofilms (Colagiorgi et al., 2017; Pérez-Baltar et al., 2021; Rodríguez-López, Bernárdez, et al., 2019). Biofilms provide increased resistance of *L. monocytogenes* to environmental stresses such as disinfectant tolerance (Ibusquiza et al., 2011; van der Veen & Abee, 2010; Yoon et al., 2015) and desiccation (Daneshvar Alavi & Truelstrup Hansen, 2013). Biofilm-cells can thus survive sanitation and eventually become persistent in FPE, causing recurrent cross-contamination and increasing the level of listeriosis (Abebe, 2020; Bai et al., 2021; Midelet & Carpentier, 2002).

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https://doi.org/10.1016/j.foodres.2023.112989
Received 20 February 2023; Received in revised form 11 May 2023; Accepted 15 May 2023
Available online 19 May 2023

The risk of contamination and – consequently – listeriosis, is additionally determined by the transfer of biofilm-cells to food matrices, which depends on biofilm properties and any factor that may condition them. Biofilm cells can be detached and transferred to food by direct contact with tools, gloves, conveyor belts, slicers, etc. (Keskinen et al., 2008; Qi et al., 2020, Herrera et al., 2023 – unpublished data). In spite of the fact that biofilms containing *L. monocytogenes* have been reported as a particularly hazardous source of contamination RTE food (Kurpas et al., 2018; Mazaheri et al., 2021; Ricci et al., 2018), the planktonic state is the current model for decision-making (Ismail et al., 2017; Keskinen et al., 2008; Lee & Wang, 2017; Qi et al., 2020).

The absence of thermal treatment before consumption makes RTE food high risk if contaminated. Accordingly, a number of quantitative microbial risk assessments (QMRA) have been conducted to guide mitigation strategies and focus regulatory actions for the control of *L. monocytogenes* in RTE food (Hadjicharalambous et al., 2019; Mataragas et al., 2010; Stefanou et al., 2022). Nevertheless, several cases and outbreaks are reported every year in the European Union, making listeriosis a disease of utmost concern, with a mortality rate of 13 % in 2020 (EFSA, 2021). Meanwhile, the CDC reported an incidence of 0.2 cases per 100,000 population per year in the U.S. (CDC, 2022). Incorporating the impact of biofilms on final contamination into QMRA would be an important achievement to food safety. This would allow new key management measures to be adopted that focus specifically on the true origin of risk events.

It is well known that almost any strain can form biofilms on any surface and any environmental conditions, but properties such as cell adhesion, growth, composition, architecture, final structure, etc., are highly variable amongst different strains. Therefore, biofilms formed in FPE must be examined to identify and select those strains that may pose a greater risk of contamination.

The large amount of data and variables required for this approach makes necessary to use a multivariate tool. Principal component analysis (PCA) is a multivariate exploratory data analysis technique used to group and sort a set of factors from a series of observations – individuals – by simplifying data as much as possible without losing important information (Abdi & Williams, 2010; Bro & Smilde, 2014). Large data sets can thus be visualized in a space of reduced dimensions, which allows the observation of trends and relationships amongst individuals and variables.

Though principal component analysis has been used in several scientific works on food microbiology (Costa et al., 2018; Szymczak et al., 2020; Valderrama et al., 2014), no study that relate strains and risk factors associated to contamination in food processing environments has been found. In particular, this approach to cluster or select *L. monocytogenes* according with their risk potential has no precedent. On the contrary, some works used PCA to select and prioritize lactic acid bacteria based on their probiotic potential and antimicrobial properties (Margalho et al., 2021; Sadeghi et al., 2022).

Consequently, the aim of the present study has been to conduct a proof-of-concept study to determine the potential use of PCA to cluster strains of *L. monocytogenes* from FPE into specific groups based on the characterization of biofilm properties. The strains of each cluster would stand out for certain properties that increase the risk of food contamination for different reasons (e.g. increased cell transfer to foods, increased resistance to biocides). PCA would thus allow the selection of strains representative of different worst-case scenarios for future studies in support of quantitative microbial risk assessments (QMRA).

2. Materials and methods

2.1. Bacterial strains and inoculum standardization

Twenty-two *L. monocytogenes* strains were used in this study. All of them were obtained from the culture collections of IIM-CSIC or the Food Science and Technology Department of the University of Cordoba. Strains had been isolated either from FPE – mainly from fish and meat industries – or food. A list of strains and their sources is shown in Table 1.

Stock cultures were preserved at –80 °C in sterile brain–heart infusion broth (BHI; Scharlab, Barcelona, Spain) with 50 % (v v^{-1}) glycerol. Working cultures were kept at –20 °C in same broth conditions. Reactivation of strains was done by adding 100 μL of working culture to 5 mL of tryptone soy broth (TSB, Scharlab, Barcelona, Spain) followed by overnight cultivation at 37 °C.

Inoculum standardization was done by adjusting reactivated cultures to $Abs_{700} = 0.1 \pm 0.01$, which corresponds to a cell concentration of approximately 10^8 CFU/mL. Inoculum concentration was always checked by plating in tryptone soy agar (TSA, Scharlab, Barcelona, Spain).

2.2. Biofilm formation

AISI 316 stainless steel (SS) coupons (Comevisa, Vigo, Spain) were used for biofilm formation. Coupons were washed and sterilized before use. Coupons of two different sizes were used for different experiments (see below). Small size coupons (10 × 10 × 1 mm) were individually set into 24-well polystyrene microtiter plates, large size coupons (50 × 50 × 1 mm) were placed into petri plates. Each coupon was inoculated with 1 mL and 25 mL, respectively, of standardized inoculum diluted in TSB down to a final concentration of approximately 10^4 CFU/mL. The systems were cultured statically by at 25 °C, concentration was checked by plating.

2.3. Detection of benzalkonium chloride resistance genes

Genomic DNA was isolated from liquid 24 h-old culture of each strain using PureLink® Genomic DNA kit (Invitrogen, Thermo Fisher), following the manufacturer's instructions. The detection of the resistance cassette *bcr*ABC was performed according to Tamburro et al., (2015) PCR conditions, *L. monocytogenes* CDL69 was used as positive control. Presence of *qac*H was checked according to Chmielowska et al., (2021), using *L. monocytogenes* 6179 as positive control. Amplification products were resolved on 2 % agarose gel, images were taken by a GelDoc 2000 equipment and analysed with Quantity one software (BioRad, Hercules, CA).

2.4. Serogroup determination

The serovars of the strains were determined by multiplex-PCR following the method of Doumith et al., (2004). Briefly, 25 μL of DreamTac PCR Master Mix (Thermo Fisher Scientific, Massachusetts, USA) were mixed with 5 μL of *L. monocytogenes* DNA and 1 μM of primers for *lmo*0737, ORF2819 and ORF2110, 1.5 μM of primers for *lmo*1118 and 0.2 μM of primers for *prs*, to a final volume of 50 μL. PCR conditions were the following: an initial denaturing step of 3 min at 95 °C followed by 35 cycles of 25 s at 94 °C, 1:10 min at 53 °C and 1:10 min at 72 °C, and one final extension of 7 min at 72 °C. The amplicons were run on a 1.5 % agarose gel with RedSafe (iNtRON), Hyperladder 50 bp (Bioline) was included as molecular marker in all cases. Gel images were taken by a GelDoc 2000 equipment, as previously mentioned.

2.5. Pulse field gel electrophoresis (PFGE) subtyping

PFGE pulsotypes were obtained following the PulseNet procedure for *Listeria monocytogenes* (Graves & Swaminathan, 2001; PulseNet, 2017), using a CHEF-DR® III Electrophoresis apparatus (BioRad Laboratories, Hercules, CA). *L. monocytogenes* genomic DNA was obtained as previously described (Section 2.3) and digested with *AscI* and *ApaI*-restriction endonucleases (NewEngland, Biolabs). After electrophoresis, gels were dyed with GelRed™ 1X (Biotium, Hayward, CA) for subsequent visualization under UV light with a GelDoc equipment, as indicated in

Table 1
General information and characteristics of the 22 *Listeria monocytogenes* strains used in this study. Alongside the strain code, the collection source, food or environmental (Env.) and current mention in bibliography it is shown the serogroup identification and the surveyed presence of two main resistance to disinfectants genes, *bcr*ABC and *qac*H.

Strain code	Source	industry	Surface	Ref.	Serogroup	PCR group	*bcr*ABC	qacH
L1.A1	Env.	Fish	Globes	d	I.1. 1/2a–3a	IIa	–	+
L1.B1	Food	Fish	Food	a	I.1. 1/2a–3a	IIa	+	–
L1.C1	Food	Meat	Food	c	II.2. 1/2b–3b–7	IIb	–	–
L1.C5	Env.	Meat	Mincer	c	I.1. 1/2a–3a	IIa	–	–
L1.C6	Env.	Meat	Stuffer	c	I.1. 1/2a–3a	IIa	–	–
L1.C9	Food	Meat	Food	c	4b–4d–4e	IVb	–	–
L1.CH3	Env.	Fish	Hopper	a	I.1. 1/2a–3a	IIa	+	–
L1.CH6	Env.	Frozen food	Blade	a	I.1. 1/2a–3a	IIa	+	–
L1.D1	Env.	Fish	Conveyor	d	4b–4d–4e	IVb	–	–
L1.E1	Env.	Meat	Trolley	d	I.1. 1/2a–3a	IIa	–	+
L1.PM1	Env.	Meat	Clinic	a	4b–4d–4e	IVb	–	–
L1.34	Food	Grocery	Fish	e	4b–4d–4e	IVb	–	–
L1.38	Env.	Meat	Packing area	b	4b–4d–4e	IVb	–	–
L1.96	Env.	Meat	Drain	b	I.2. 1/2c–3c	IIc	–	–
L1.107	Env.	Meat	Floor	b	I.2. 1/2c–3c	IIc	–	–
L1.130	Env.	Meat	Drain	b	I.1. 1/2a–3a	IIa	–	–
L1.156	Env.	Fish	Drain	b	I.2. 1/2c–3c	IIc	–	–
L1.161	Env.	Fish	Drain	b	4b–4d–4e	IVb	–	–
L1.162	Env.	Fish	Drain	b	4b–4d–4e	IVb	–	–
L1.168	Env.	Fish	Drain	b	4b–4d–4e	IVb	–	–
L1.180	Env.	Fish	Drain	b	4b–4d–4e	IVb	–	–
L1.225	Env.	Meat	Drain	b	I.1. 1/2a–3a	IIa	–	–

References: a, this study; b, (Rodríguez-López, Bernárdez, et al., 2019), c, HIBRO-PAIDI AGR170, University of Cordoba; d, (Rodríguez-López et al., 2015); e, (Rodríguez-López, Barrenengoa, et al., 2019).

Section 2.3. Hierarchical clustering was subsequently performed by analyzing images with Bionumerics7 software (Applied Maths NV, Belgium). A dendrogram was then generated by using the unweighted pair group method with arithmetic mean (UPGMA) (Sneath & Sokal, 1973) clustering and Dice similarity index.

2.6. Tolerance to benzalkonium chloride

The tolerance to benzalkonium chloride (BAC) was tested in both planktonic and biofilm cells. The resistance of planktonic cells was assessed in terms of minimum inhibitory concentration (MIC) while biofilm resistance was tested by determining the minimum biofilm eradication concentration (MBEC). All assays were conducted at room temperature using 10 × 10 × 1 mm coupons.

MIC analysis was based on the method proposed by Wiegand et al., (2008), with some modifications. Thus, 100 μL of the inoculum (10^4 CFU/mL) and 100 μL of BAC at different concentrations – ranging from 0.28 to 4.5 μg/mL – were added onto each coupon placed into a 96 well-microtiter plate. The plate was cultured overnight at 37 °C inside a plastic container, in order to maintain moisture. Afterwards, absorbance readings were taken with a BioRad Microplate Reader (BioRad Laboratories, Hercules, CA). To confirm the absence of growth, undetectable growth was additionally checked by plating the culture medium on TSA (Scharlab, Barcelona, Spain) and searching for colonies after 24 h at 37 °C.

The determination of MBEC, which is the minimum concentration of a disinfectant able to kill all biofilm cells, was performed as previously described literature (Luppens et al., 2002; Vázquez-Sánchez et al., 2014), with slight variations. 24 h-biofilms formed on SS coupons (3 replicas) were collected and washed with phosphate-buffered saline (PBS) twice, in order to remove loosely attached cells and retain just biofilm cells. Then, 1.5 mL of each BAC concentration was added to a set of three coupons. BAC concentrations ranged from 750 to 5000 ug/mL, at 250 ug/mL intervals. After 10 min of exposure 1 mL of neutralizer was added (composition per liter: 34 g of KH_2PO_4 pH 7.2, 3 g of soy lecithin, 30 mL of Tween 80, 5 g $Na_2S_2O_3$, 1 g of L-histidine) and left to stand for 1 min at room temperature. Next, coupons were placed in a 24 well titer plate with 1 mL TSB, and incubated 24 h at 37 °C. Bacterial growth was monitored by reading absorbance at 655 nm in a Microplate reader. The absence of growth was monitored as for MIC.

2.7. Confocal microscopy analysis

A number of architectural parameters were measured by confocal laser scanning microscopy (CLSM) in 120 h-biofilm formed on SS coupons. Prior to analysis, coupons were washed twice with 1 mL of PBS, for the removal of non-attached cells and then dyed with BacLight kit, (L7012 Invitrogen, Thermo Fisher Scientific, MA, USA).

Confocal stacks images were acquired to determine the tridimensional structural features. All images were taken using an upright confocal laser scanning microscope (CLSM) TSC-SPE Leica (Wetzlar, Germany), with an objective ACS APO 63.0 × 1.30 oil.

Image stacks were analyzed by the Comstat2 plugin (comstat.dk; (Heydorn et al., 2000; Vorregaard, 2008) of ImageJ software (imagej.nih.gov/ij, National Institutes of Health, USA). Biofilm architecture was characterized by the following quantitative parameters: (i) biomass, defined as the biovolume related to the sampled area ($\mu m^3/\mu m^2$); (ii) surface area (μm^2); (iii) average thickness (iv) maximum thickness (μm); (v) surface to biovolume ratio ($\mu m^2/\mu m^3$) and (vi) roughness coefficient (Ra).

2.8. Transfer to smoked salmon

Transfer experiments were performed using 120 h-old biofilms grown on 50 × 50 × 1 mm SS coupons. Salmon was purchased from a local grocery shop and cut into 4,5 cm diameter slices, all having similar weight. Fish microbiota was examined to discard the presence of *L. monocytogenes* in the product by plating on Agar Listeria Ottaviani and Agostini (ALOA; Scharlab, Barcelona, Spain).

Before transfer, biofilms were washed twice with PBS, to remove non-attached cells. Washed coupons (6 replicas) were the drained and three salmon slices were placed in contact with each coupon consecutively for 30 s, allowing the transfer of biofilm cells. Next, cells were recovered from salmon slices by sonication in 10 mL of peptone water, and then cultured overnight in ALOA plates at 37 °C. The transfer of L. monocytogenes to smoked salmon was calculated as follows:

$$Transfer\ rate(\%) = \frac{CFUslice}{CFUcoupon}$$

Where CFU slice represents the number of cells transferred from each coupon to each salmon slice, while CFU coupon represents the total number of cells attached to the surface of the coupon that comes in contact with each salmon slice (1590 mm^2). The latter was determined as the average of three biofilms formed under the same conditions on different coupons. Non-attached cells were first removed from coupons by washing twice with PBS. Coupons were then sonicated and subsequently pipetted to detach biofilm-cells. Once removed, biofilm cells were counted by plating on ALOA.

2.9. Data analysis

Statistical and multivariate analysis were done in R Studio (Posit team, 2022). ANOVA and Tukey HSD ($\alpha = 0.05$) post-hoc tests were used to set differences amongst structural parameters. Multivariate analysis of principal components and hierarchical clustering on principal components performed with *FactoMineR* package (Lê et al., 2008), for data analysis, and *factoextra* package (Kassambara & Mundt, 2020), for data visualization. Hierarchical clustering on principal components was done with Ward's minimum variance method.

3. Results and discussion

3.1. Genetic characterization of strains

Genetic diversity and phylogenetic relationships among the twenty-two strains were examined in terms of PCR serogrouping and PFGE subtyping. The presence of BAC-resistance genes (specifically, *bcr*ABC and *qac*H) was also explored (see Section 3.2).

Serogroups IIa (serovars 1/2a, and 3a) and IVb (serovars 4b, 4d and 4e) were the most represented (40.91 %, each) (Table 1). These serogroups have been predominantly found in environmental samples from meat and fish industries (Basha et al., 2019; Martín et al., 2014; Ochiai et al., 2010; Skowron et al., 2019; Wang et al., 2015). In contrast, serogroups IIc (1/2c and 3c, 13.64 %) and IIb (1/2b, 3b and 7, 4.55 %) were less representative. This agrees with previous studies in which serovar IIa was the most commonly found in food (Amajoud et al., 2018; Andritsos et al., 2021; Anwar et al., 2022) and food environments (Dunn et al., 2022; Lachtara et al., 2022; Lucchini et al., 2023).

Considering the inability of serogrouping to further discriminate amongst strains, PFGE subtyping was also conducted, this approach is commonly used in taxonomic and epidemiological studies due to its high discriminatory power (Tenover et al., 1995). From the analysis of endonuclease *Asc*I and *Apa*I fingerprints a dendrogram was developed. As shown in Fig. 1, such analysis divided the strains into two major

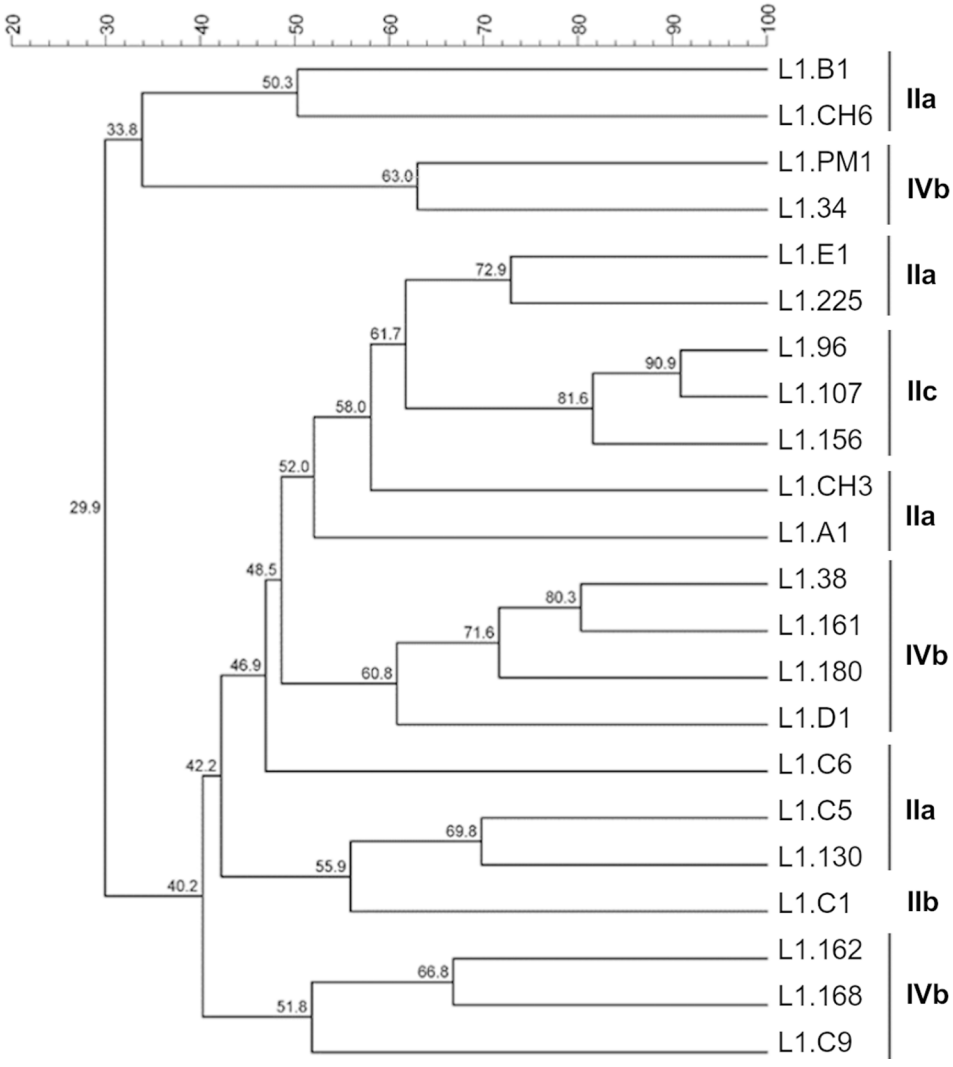

Fig. 1. Dendrogram corresponding to the UPGMA method from the pulsotype fingerprints of *Asc*I and *Apa*I endonucleases. On the right, serogroups are displayed according to the strain characterization.

clusters – with cut-offs values of 33.8 and 40.2 % –. Given a 50 % similarity as a cut-off threshold, strains – all but L1.C6 – were separated in six different clusters, three of which were further divided into smallest ones. Weighted the serogroups against the PFGE clustering, PFGE evidenced a thorough level of analysis, the majority of the strains were scarcely related, none but L1.96 and L1.107 showed a similarity higher than 90 %. These outcomes suggest considerable heterogeneity among the studied strains.

3.2. Resistance and tolerance to benzalkonium chloride (BAC)

As shown in Fig. 2A, MIC values ranged from 0.88 to 3.5 μg/ml, and strains were sorted into three categories according to such values, namely: "tolerant (MIC > 2.25 μg/mL)", "intermediate (MIC between 0.75 and 2.25 μg/mL)" and "susceptible (MIC < 0.75 μg/mL)".

The range obtained is narrow compared to those of previous studies, which have reported MIC values of up to 15–20 μg/mL (Rodríguez-Melcón et al., 2022; Soumet et al., 2005; Xu et al., 2019). BAC tolerance levels fluctuate amongst strains set, in this particular case, yet, such differences could be attributed to methodology differences. Methodological divergences would be solved by standardization, a broth microdilution method has been proposed by Wiegand et al., (2008) and more recently by Schug et al., (2020).

Thus, values of MBEC were up to >1000 times higher than MICs, ranging from 1500 to 5500 μg/mL (Fig. 2B). Four of the strains – L1.E1, L1.PM1, L1.C1 and L1.C6 –, showed the major MBEC values (4,250–5,500 μg/mL), and none of them was considered tolerant, according to MIC values. As expected, no correlation was found between MIC and MBEC (Pearson r = 0.20). However, some strains – L1.34, L1.CH6, L1.A1 and L1.B1–, showed high values of both MIC (3.5 μg/mL) and MBEC (4,000 μg/mL).

This lack of correlation had been previously observed, not only for *Listeria* (Barroso et al., 2019), but also for other bacterial pathogens, such as *staphylococci* (Brady et al., 2017). Several factors could explain the huge difference between MIC and MBEC. First, a physical barrier effect of biofilms hinders the diffusion of BAC, decreasing the concentration able to reach the cells. Secondly, the concept of MBEC requires complete removal of biofilm cells – instead of the inhibition at MIC –, therefore, wider ranges of BAC concentration are needed. As a result, *L. monocytogenes* classification in terms of MBEC tends to be imprecise, proving a conceptual difference between MIC and MBEC.

Also, it has been indicated that the decreased metabolic activity of biofilm cells increases their tolerance to biocides compared to cells in the planktonic state. A small population of biofilm cells may even enter a metabolically inactive state – dormancy – in which they show increased tolerance to antibiotics and biocides. In the clinical field, these cells are called persisters (Wood et al., 2013). This term, however, should not be confused with persistent, such as it is used in the food industry for PFGE types repeatedly found in a particular industry for longer than 6 months (Cherifi et al., 2020; D'Arrigo et al., 2020; Melero et al., 2019).

According to Martínez-Suárez et al., (2016) persistence is linked to genetic resistance mechanisms, which provide cells with the ability to avoid the effects of disinfectants. E.g. the inadequate rinsing of surfaces exposes cells to sub-lethal concentrations of biocides, leading to resistance overexpression (Tamburro et al., 2015).

The presence of *bcr*ABC and *qac*H, both BAC resistance genes, was examined in all strains. Only 5 out of 22 strains harbored either of these two genes. Thereby, L1.CH6, L1.B1 and L1.CH3 had the *bcr*ABC cassette, while L1.A1 and L1.E1 carried the *qac*H gene, but the two of them were not simultaneously present in any strain. A similar result was found by Møretrø et al., (2017), who did not detect both genes in any strains. Several previous studies aimed at detecting the presence of genetic determinants that could lead to BAC resistance. Thus, Ebner et al., (2015) found that 18 % of 142 strains isolated from different food matrices in Switzerland during 2011–2014, were resistant to BAC, being 80 % were positive for *qac*H and 12 % for *bcr*ABC. Similar results were reported in later works (Meier et al., 2017; Møretrø et al., 2017), in which the percentage of strains positive for *qac*H was slightly higher than for *bcr*ABC.

As shown in Fig. 2A and 2B, the *bcr*ABC positive strains L1.CH6 and L1.B1 showed high MIC and MBEC values. Similarly, the two strains positive for *qac*H (L1.A1 and L1.E1), also presented high values for MBEC (≥4250 μg/mL) and MIC (3.5 μg/mL). On the contrary, L1.CH3 showed lover values for both parameters.

Therefore, the presence of BAC-resistance determinants seemed to play a role in the phenotypic susceptibility response assessed by MIC and MBEC in 4 of 5 strains. In agreement with Noll et al., (2020) the phenotypic tolerance mechanism seems to be triggered by genetic factors rather than as a consequence of short-term adaptation. To this respect Müller et al., (2014) proved that *qac*H expression increased in the presence of BAC, and that strains with *qac*H had higher MIC values than mutants lacking it. However, this study reported that strains with the highest MIC values did not always have the *qac*H or *bcr*ABC genes, which, thus, did not seem to be required for them to have BAC

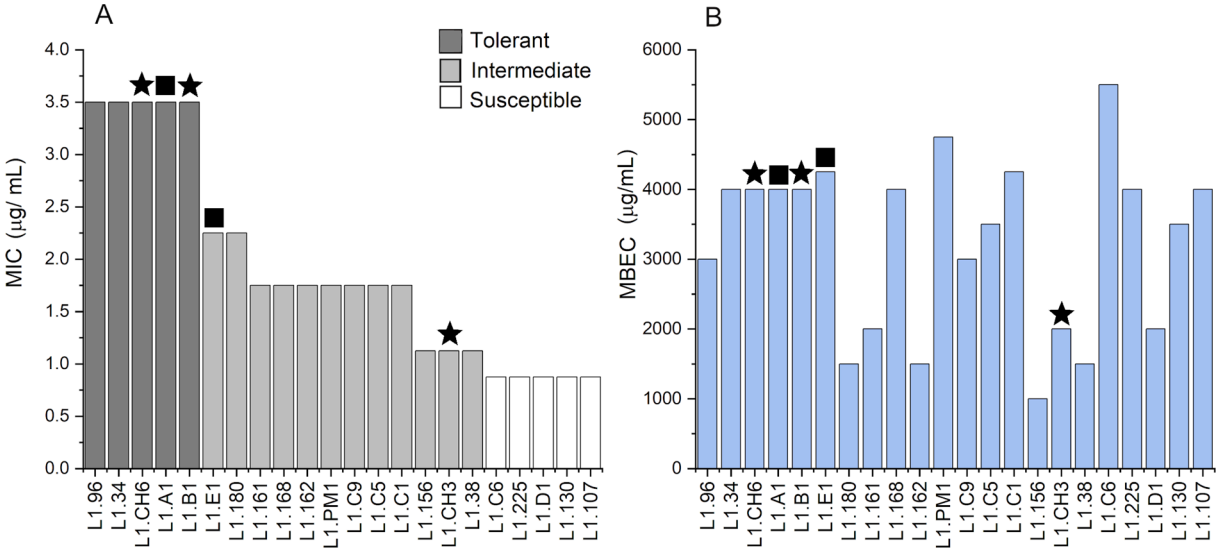

Fig. 2. Benzalkonium chloride (BAC) minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC). Star, isolates positive to *bcr*ABC; square, isolates positive to *qac*H.

resistance.

3.3. Biofilm architecture

In order to explore the tridimensional microstructure diversity of biofilms, formed by different strains, confocal microscopy analyses were performed. For each different strain, several stack images were acquired, covering the entire height of the biofilms. Fig. 3 displays images of different strains, highlighting the structural diversity amongst them.

To further study and quantify the microstructural characteristics of

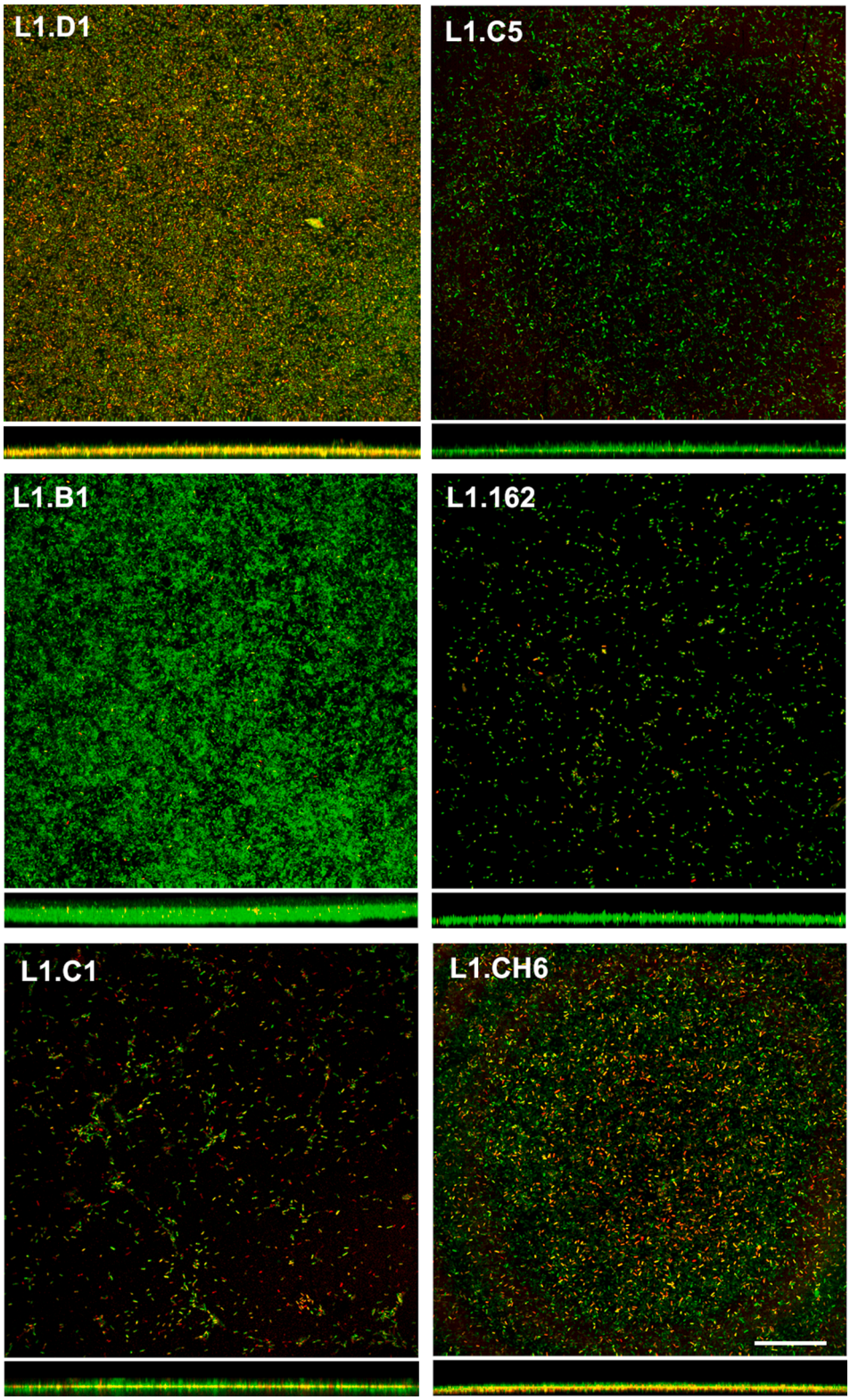

Fig. 3. Biofilm images by confocal laser scanning microscopy, stained with SYTO9 (green) and propidium iodine (red). Maximum projections of each strain are shown for XY axis (scale bar 30 μm) and XZ axis (total height 15 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

biofilms, the parameters of biomass, surface area, average and maximum thickness, surface to biovolume ration and the roughness coefficient (Ra) were analyzed. As shown in Fig. 4, significant differences ($p < 0.05$) were found in all parameters. Within the surveilled parameters, the reported values of each strain displayed similar organization in certain cases (Pearson's r 0.98), e.g. biomass and surface area (Fig. 4A and 4D, respectively). For both parameters, a group of strains clearly stood out from the others, biofilms of L1.D1, L1.34 and L1.38 were significantly different from those other strains, being the strongest biofilms formers of the study.

From the biomass definition, the biovolume can be determined by considering the substratum field (30,544.55 ± 0.1 μm^2). Biovolume results were lower than those of previous studies (Bridier et al., 2010; Darsonval et al., 2021; Guilbaud et al., 2015; Mosquera-Fernández et al., 2016). Such differences could be explained by the use of polystyrene microtiter plates as support matrix for biofilms. Thus, it has been showed that the number of attached cells on polystyrene (5.6 log CFU/cm^2) was significantly higher ($p = 0.002$) than on staintless steel (4.7 log CFU/cm^2) (Poimenidou et al., 2016).

A remarkable similarity was also observed between the results for maximum thickness and average thickness (Fig. 4B and 4E, respectively). Three strains– L1.B1, L1.34 and L1.96 – clearly stood out in terms of the average thickness of their biofilms. Two of them also showed the highest maximum thickness values. These parameters describe the height distribution of the biofilm. Maximum thickness represents the highest point, paying no attention to pores and voids inside the biofilm, whereas average thickness gives a measure of the spatial size of the biofilm (Heydorn et al., 2000).

Given the similitude between both thickness, maximum thickness has been regularly used in literature for depicting the one-dimension structure of the biofilm. The maximum thickness values obtained are similar to those previously reported (Mosquera-Fernández et al., 2016). Accordingly, *L. monocytogenes* strains did not form thick biofilms after 120 h under the conditions of study.

Thickness also allows the calculation of the dimensionless roughness coefficient (Ra). Ra is the average deviation of thickness data and therefore measures the variability of heights across the biofilm, showing how much the thickness varies (Heydorn et al., 2000; Murga et al., 1995). It is, thus, an indicator of the heterogeneity of the structure. Ra showed significant differences ($p < 0.05$) between strains, with L1.C1, L1.C5 and L1.PM1 biofilms being the most heterogeneous (Fig. 4C). Previous works have stated $Ra < 1$ values for biofilms of several (4 to 20) strains of *L. monocytogenes* (Alonso-Calleja et al., 2019; Bridier et al., 2010; Rodríguez-Melcón et al., 2019), which contrast with those in this study where more than half of the strains showed values > 1. According to the definition of (Murga et al., 1995), $Ra < 1$ corresponds to rough biofilms and $Ra > 1$ to patchy ones. Such differences could be attributed to the heterogeneity of the studied strains, which has been previously acquainted, also the differences in methodology could play a certain role in it, as well as some unknown factor, thus the system needs further investigation.

As regards the surface to biovolume ratio, the biofilms of three strains – L1.C5, L1.PM1 and L1.225 – showed the highest values, following the predominant tendency of a few strains standing out amongst the others (Fig. 4F). The ratio depicts how biofilm adapts to the environment, showing the area exposed to nutrient flow (Heydorn et al., 2000; Vorregaard, 2008). Surface area or biomass alone do not give an idea of the tridimensional disposition of the biofilm, but the surface to biovolume ratio provides a three-dimensional image of how the biomass lays out. Hence this ratio shows the real distribution of biomass, giving an idea of the biofilm architecture.

Lastly, no correspondence was found between any structural parameters of the biofilms and the serotype or PFGE profiles, which agrees with previous studies that also reported a complete lack of relationship

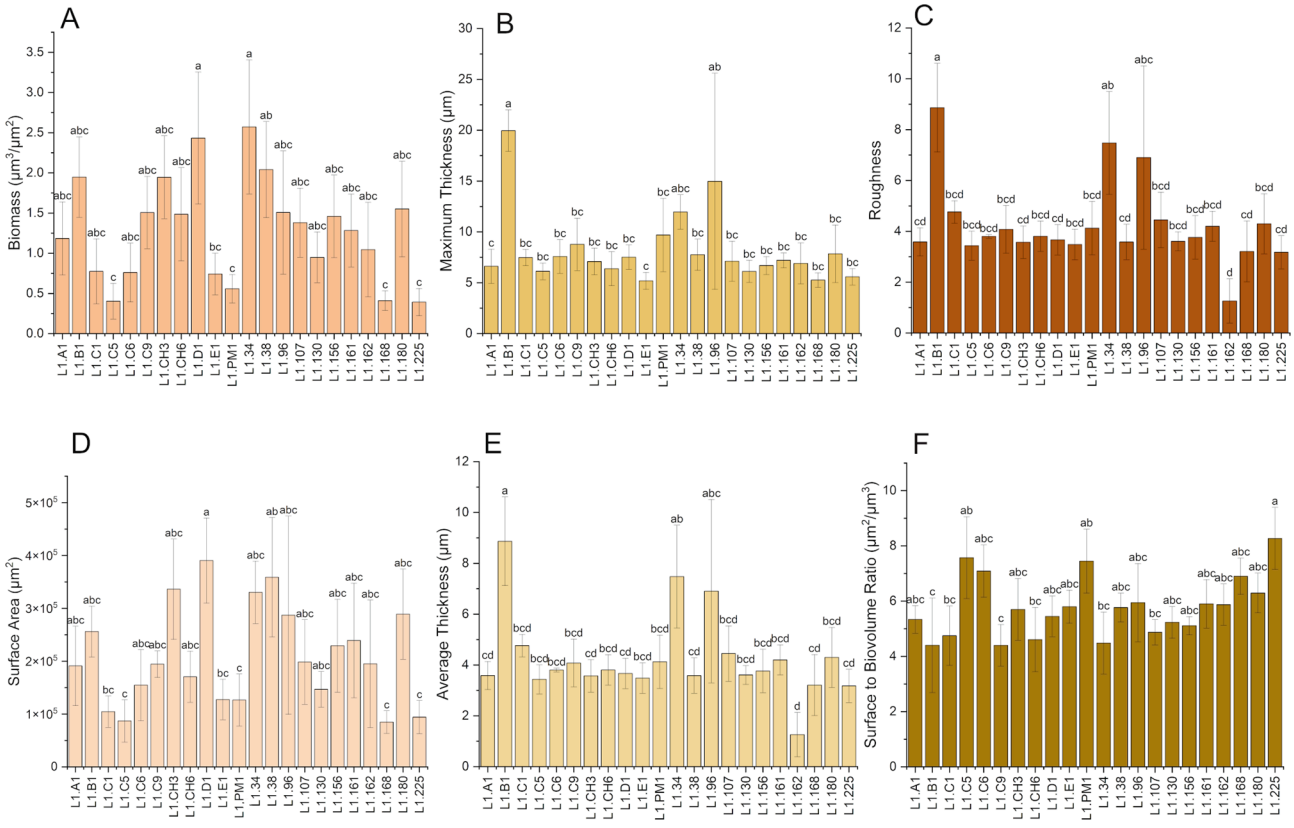

Fig. 4. Structural parameters for the *L. monocytogenes* isolates surveilled, (A) Biomass ($\mu m^3/\mu m^2$); (B) surface area (μm^2); (C) average and (D) maximum thickness (μm); (E) surface to biovolume ratio ($\mu m^2/\mu m^3$) and (F) roughness coefficient (Ra). Means that do not share a letter are significantly different ($p < 0.05$).

or either a weak one (Borucki et al., 2003; Guilbaud et al., 2015). Although (Borucki et al., 2003) reported serotypes 1/2a and 1/2c, belonging to serogroups IIa and IIc respectively, were associated with an increased biofilm formation, such a relationship was not found under the conditions of this study.

To recapitulate, it can be said that the majority of the biofilms were quite – significantly – different between each other in terms of microstructure. In addition, the amount of information given by the microstructure analysis – a mix of three-dimensional and two-dimensional parameters – claims for the integrative approach followed in this study, in order to provide a solid classification of the strains that can be used in QMRA.

3.4. Transfer to smoked salmon

As shown in Fig. 5, most of the strains displayed transfer between 45 and 70 % in the first contact with smoked salmon. Biofilms of L1.A1 and L1.C6 stood out having transfer rate values over 70 %, whereas five strains– L1.C1, L1. 96, L1.130, L1.162, L1.225 – presented the lowest rates (<45 %). For each and each isolate, these values decrease throughout successive contacts.

For most strains, a positive correlation was found between the initial number of attached cells – biofilm cells –, and the number of transferred cells – contaminating cells – (Pearson's r, 0.85). These results support that cell transfer is basically a physical phenomenon. Indeed, bacterial transmission to food, or other surfaces, has been proved to occur through cohesive failure, a physical phenomenon that results from compression or shear forces between donor and the receiver surfaces (Gusnaniar et al., 2017; Wang et al., 2022). However, not all the strains with the highest initial numbers of attached cells had the highest transfer rates, as illustrated in Fig. 6 (e.g. L1.C1). It seems clear, thus, that the concentration of initially attached cells is not the only determining factor of cell transfer. Therefore, it is necessary to find out which other properties explain the differences in transfer rate. Such properties could be some structural parameters of biofilms (see Section 3.3), biofilm hydrophobicity or the extracellular polymeric substances (EPS) content. Thus, Wang et al., (2022) found recently a positive correlation between cell surface hydrophobicity and transfer rates.

Further, EPS affects the cohesion and retention of water – forming a hydrated polymer – (Flemming, 2016), which could hamper transfer events. Firstly, as cell transfer takes place by cohesive failure, EPS would impede the process, hence fewer cells would be able to reach the food matrix. Secondly, EPS organization in channels and voids establish the water retention (Quan et al., 2022). Dried biofilms have been proved to have better transfer rates than wet ones (Rodríguez et al., 2007), hence highly EPS-producing strains may have low transfer rates. A study in *Staphylococcus epidermidis*, using EPS-producing and non-EPS-producing strains reported a slightly higher number of transferred cells for non-EPS-producers (Gusnaniar et al., 2018). Biofilms of non-EPS-producing

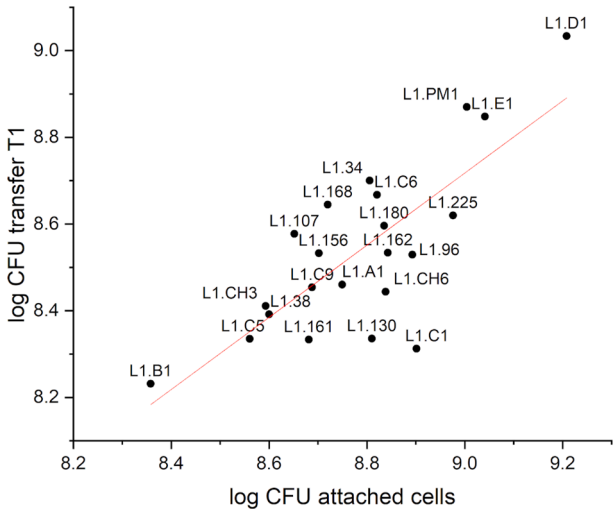

Fig. 6. Correspondence of CFU attached to the biofilm matrix and CFU/g transferred to salmon slices (Pearson's r 0.76).

strains remain compressed after the transfer, without relaxation, while the viscoelastic properties of EPS allow EPS-producing biofilms to recover their initial morphology. In fact, further research is needed to unravel the role of EPS in the transfer process and search for strain-specific differences due to EPS production.

3.5. Principal component analysis

PCA was used to reduce the dimensional space from 10 – the number of variables under study – to only 2–3 dimensions or principal components. The first two dimensions explained most of the variance (62.70 %) of the original data, which increased up to over 79 % when the third dimension was also considered. The contributions of each original variable to the dimensions are displayed in Fig. 7A. Accordingly, the first dimension is depicted majorly by structural parameters, whereas the transfer of cells at first and second contact are the variables that most contribute to the second dimension, and the third dimension – bearing the least percentage of variance explained – is described mainly by MBEC and cell transfer at the first contact.

Also, PCA reveals the relations amongst original variables, as depicted in the correlation circle shown in Fig. 7B, where additionally the cos2 heatmap shows the goodness of the representation of each variable on the loading plot. Accordingly, the structural parameters were split into three groups. Firstly, biomass and surface area, which contributed most to the first dimension as well as being the best represented variables in the plot, were positively correlated with each other. They also had a negative relationship with both roughness and surface to

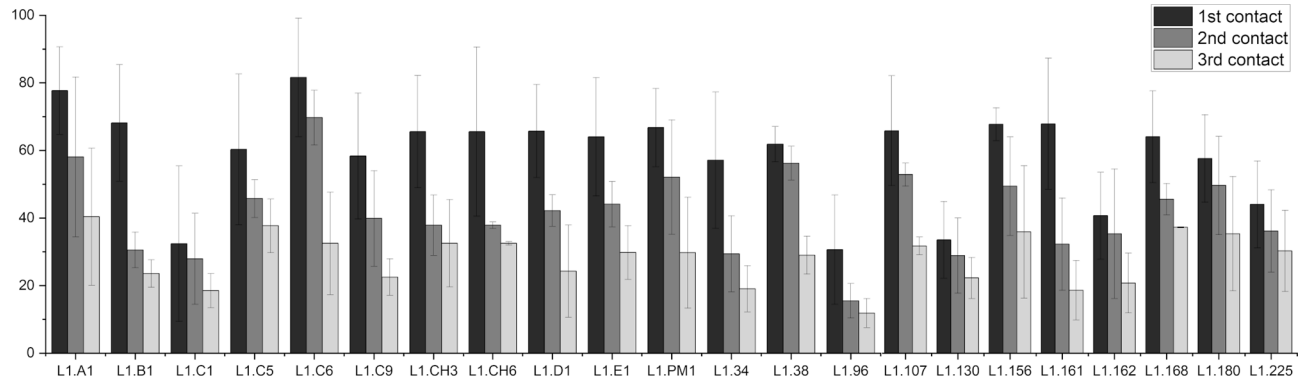

Fig. 5. Percentage of CFU/g transferred to smoked salmon. Error bars represent the standard error of the mean (n = 6).

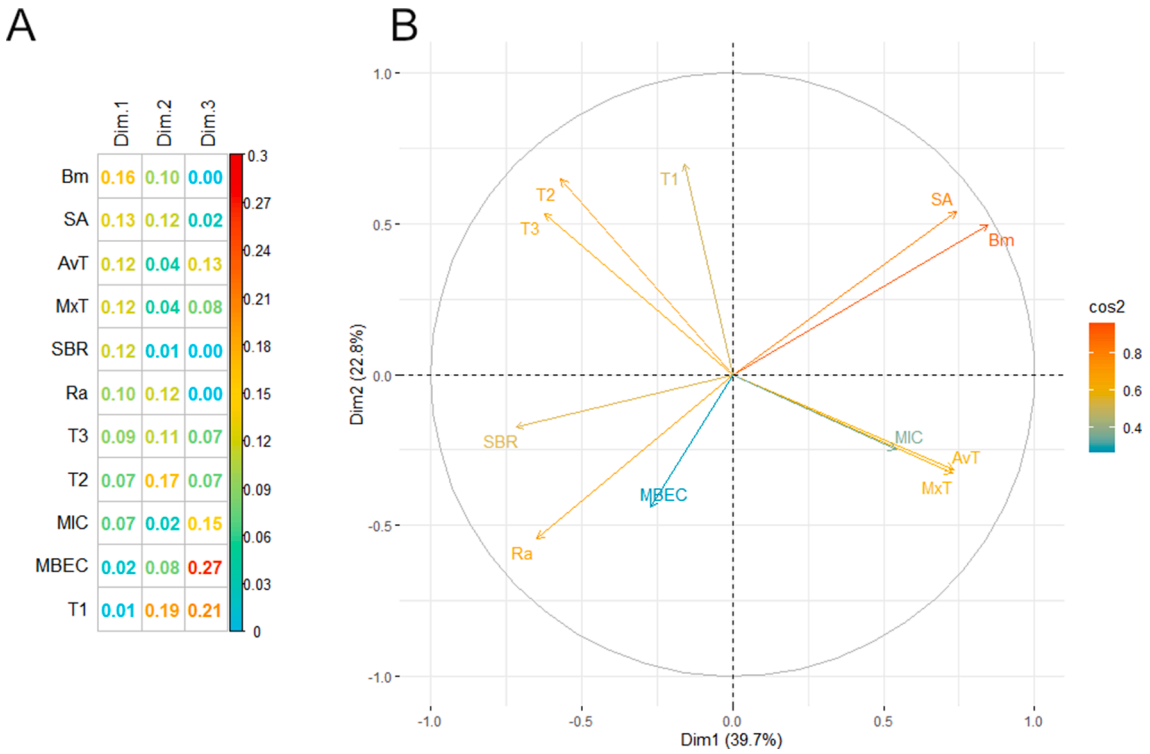

Fig. 7. A, variable contributions to each dimension (dim.). B, graph of variables, arrows remark relationship among variables, positive correlated variables point at the same side of the plot, negative correlated ones point to opposite sides. Color bar represent cos2 values of each variable. Bm, biomass; SA, surface area; AvT, average thickness; MxT, maximum thickness; Ra, roughness coefficient; SBR, surface to biovolume ratio; T1, transfer in first contact; T2, transfer in second contact; T3 transfer in third contact.

biovolume ratio. Secondly, roughness and surface to biovolume ratio shared a positive correlation, and lastly, average and maximum thickness were grouped together – highly correlated – but lacked relationship with other structural parameters.

This multivariate approach has thus been able to decipher some relations amongst the structural parameters under study. Then, biofilms with higher roughness would have more surface exposed to nutrient flow and tend to be low biomass. At the same time, high-biomass biofilms would tend to have more uniform surface profiles, maintaining a larger surface. Also, thickness would vary independently of biomass and roughness. On the contrary, a positive association between thickness and roughness had been previously described for *Listeria* – *Lactobacillus* mixed biofilms (Olszewska & Diez-Gonzalez, 2021). The apparent contradiction is likely accounted for the major role of *Lactobacillus* in such biofilms.

With regard to BAC tolerance, a clear lack of correlation was observed between MIC and MBEC, as previously mentioned in Section 3.2. On the contrary, MBEC correlated closely with the roughness and surface to biovolume ratio, which seems to indicate that biofilms with high roughness hampers the diffusion of the disinfectant through the matrix, decreasing its effectiveness. This effect had been previously observed in *Pseudomonas aeruginosa* biofilms, on which the biofilm composition and density explains the difference in BAC penetration (Bridier, Dubois-Brissonnet, et al., 2011). The extracellular matrix has also been considered to play a major role to delay the diffusion of biocide.

Interestingly, transfer rates were not associated with any other parameter but thickness. This suggest a lack of relevance of structural parameters in transfer phenomena, as well as the existence of other factors – not included in the analysis – that could affect biofilm-cells transfer. The negative correlation of transfer rates with thickness points out that the thicker the biofilm, the lower the transfer initially, and it could be related to a higher EPS content. As mentioned in Section 3.4, previous studies have reported the importance of wettability and EPS – content and composition – in the transfer of biofilm cells. However, none of the parameters used in the study were unable to capture the variation in EPS content and composition, hence further studies would be needed in order to explore this issue.

Accessory to PCA, a hierarchical clustering on principal components (HCPC) was performed. The HCPC algorithm splits individuals – the strains – into several groups of similar variable patterns. As a result, the strains were sorted in three different groups or clusters (Fig. 8). Clusters can be described in terms of the original variables (data not shown), by their location in the score plot. Thus, strains belonging to each cluster were particularly characterized by those properties with which they were correlated positively. These properties can be easily identified by overlaying the circle of correlation – variables factor map – and the individuals factor map. Accordingly, cluster 1 scored high for roughness, surface to biovolume ratio, and MBEC, while biomass and surface area scores were quite low. Cluster 2 strains were represented by high scores in transfer at the first contact, biomass and surface area. Cluster 3 – the smallest one, with only three strains – was represented by high scores in both average and maximum thickness and tolerance to BAC in the planktonic state (MIC).

These characteristics and, therefore, each cluster – and strains thereof – are associated with particular potential risk factors. Cluster 1 presented a greater tolerance to BAC and surface area exposed to nutrient flow, which makes them form resistant biofilms persistence potential. Cluster 2 strains stood out for forming biofilms with a great transfer ability in the first contacts and a high biomass which entails a higher risk of high level-contamination. Cluster 3 strains, showed a high BAC resistance in the planktonic state and formed biofilms with lower transfer ability in the first contacts.

Thus, hierarchical clustering groups strains with similar biofilm properties, which are associated to different risk factors. The strains belonging to each cluster would be most suitable for the study of the risk

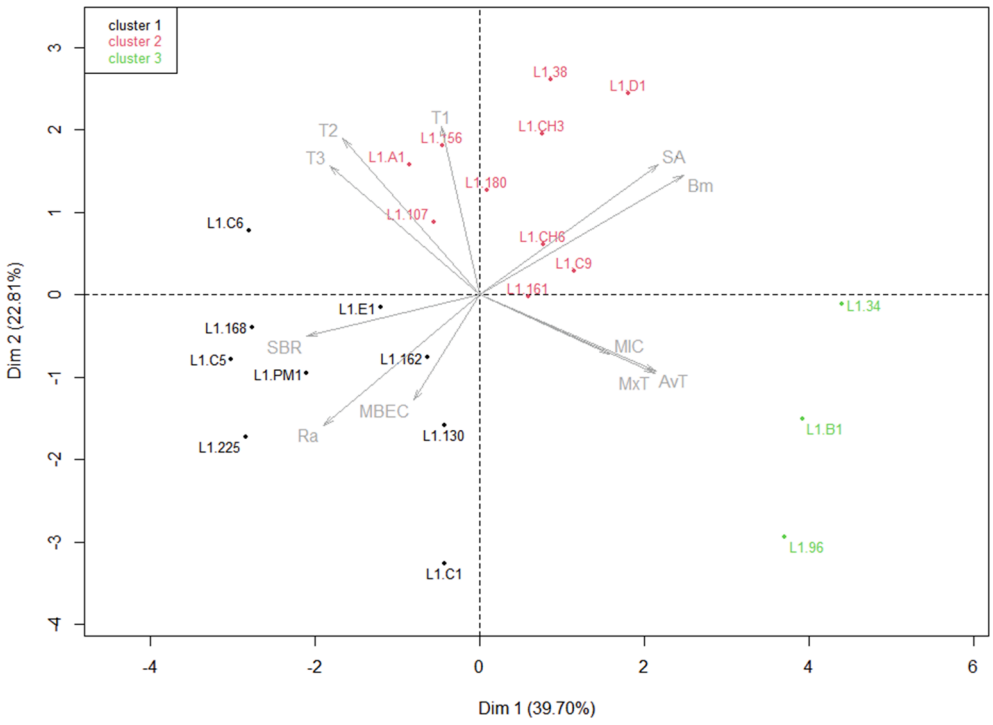

Fig. 8. Factor map of isolates, *L. monocytogenes* strains are sorted in three clusters according to the hierarchical clustering of principal components (HCPC). Clusters are described by variable distribution in the principal components.

factors with which they correlate best. Furthermore, it selects the most representative strains as the ones closest to the centroid of each cluster– e.g. L1.E1, L1.C5, L1.168, L1.225, L1.PM1 in cluster 1 –. Hence, the approach allows a well-argued selection of representative strains for further studies which would support quantitative microbial risk assessments.

This study has been conducted under a particular experimental scenario. However, this approach has as a great advantage that it is open to further inputs that were not covered here, making it thus possible to increase the number of strains under study, address new scenarios involving a broader range of conditions (e.g. temperatures, nutrients), other food products or further risk factors that may be encountered or appear in different food processing environments, or to determine further biofilm properties (i.e. chemical composition). Similarly, inputs from polymicrobial biofilms consisting of *L. monocytogenes* and accompanying bacteria could be incorporated or even addressed independently. In fact, this approach is also open to remove inputs (of any kind) that have not relevance in particular scenarios or to pre-select strains of interest in specific food sectors. In other words, it could be adapted to different cases –even allowing case-by-case studies-, from specific conditions of a particular industry to a much larger scenario.

3.6. Conclusions

Being well-known the great importance of biofilms as a primary source of food contamination by *L. monocytogenes*, incorporating the impact of biofilms on food contamination into QMRA would be an important achievement to food safety, and it would additionally allow new key management measures to be adopted that focus specifically on the true origin of risk events.

In this line, the present work has demonstrated the potential of PCA to select strains of *L. monocytogenes* forming biofilms with specific environmental contamination risk factors in food processing industries, which can thus be representative of different worst-case scenarios of biofilm-associated environmental contamination (e.g. a high transfer of bacteria to foods, a high resistance to biocides), all of which affect differently but significantly the risk of food contamination. Using strains representative of such scenarios would support the integration of the impact of biofilms on food contamination into QMRA.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This research was financially supported by the Spanish Ministry of Science and Innovation (ASEQURA, PID2019-10 8420RB-C31). The authors would like to thank S. Rodríguez Carrera, T. Blanco, A. Gallego, M. Bernárdez and P. Rodríguez for the helpful support in all the work and the priceless advices.

Author contributions

MLC and JJRH conceived the study and revised the manuscript. RN drafted and wrote the manuscript. LGS contributed extensively with the initial screening. LSR was in charge of the microscopy analysis and methodology.

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