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Short communication

Quantification of mature *Listeria monocytogenes* biofilm cells formed by an *in vitro* model: A comparison of different methods

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ABSTRACT

The presence of biofilms in food industrial environments is one of the main causes associated with food product contamination by *L. monocytogenes*. Biofilm control in the food industry is very relevant to public health and finding reliable and realistic quantification methods is essential. The aim of this study is to compare five *L. monocytogenes* biofilm quantification methods – conventional plate count, TEMPO, DEM, VIDAS and qPCR – and to examine a biodetector to visually detect biofilms in industrial settings. Results show that depending on the biofilm matrix production, the recovery of cells that conform the biofilm can be low and therefore, if it is an indirect method, microbial counts can be underestimated. At a species level, the methods that did not present significant differences were plate count, TEMPO ($P = 0.998$), DEM and qPCR ($P = 0.508$), so correlation studies were performed which established high correlation for plate count and TEMPO, but not for DEM and qPCR. The VIDAS method was adjusted so that it could quantify the biofilms, but the standard curve only allowed counts from 7 Log CFU cm^{-2}. Results also revealed that the different strains of *L. monocytogenes* possess different biofilm-forming abilities, although it was not possible to correlate the capacity to produce these structures with the distinct serotypes. Last, visually detecting biofilms on stainless steel coupons proved that in industrial environments nowadays they can be rapidly and qualitatively detected so that relevant decisions can immediately be taken.

1. Introduction

Listeria monocytogenes is a ubiquitous microorganism capable of contaminating a wide variety of foods when introduced into food processing environments due to its hardy growth characteristics (Borucki et al., 2003). The presence of *L. monocytogenes* on food products can be related to cross-contamination as the pathogen is able to form biofilms on food contact surfaces (Klančnik et al., 2015). Biofilms are microbial aggregations adhered to a surface and embedded by an extracellular matrix (ECM) consisting of proteins, polymeric substances and DNA, and able to function as both protective barrier and structural scaffold (Flemming and Wingender, 2010). The occurrence of these organized microbial communities in the food industry causes a constant microbial reservoir that constitutes a persistent source of contamination (Winkelströter and De Martinis, 2015).

The ability to rapidly detect the presence of biofilms on food contact surfaces is absolutely crucial for the food industry to be able to assess the hygienic state of the environment and take relevant decisions on cleaning and disinfection procedures (Ripolles-Avila et al., 2018c). However, when assessing the number of residential cells conforming biofilms, enumeration techniques, which are divided into direct and indirect methods, are employed. Direct methods are based on the direct observation of the microbial colonization and are highly relevant for evaluating the properties of the biofilm biomass, allowing one to observe the spatial organization of the biofilm and the heterogeneity and connection with the microbial communities in a more direct manner. Indirect methods, on the other hand, are based on detaching the microorganisms from the food contact surface prior to counting them. Different ways to dislodge the bacterial cells from the surface have been assessed by different researchers, including scraping, swabbing, sonicating and vortexing (Lourenço et al., 2012), the last two being the most indicated for detaching biofilms formed in vitro due to their capacity to destabilize the biofilm structure making it release the cells (Webber et al., 2015).

In the present study, the suitability of five different methods for quantifying the total cell amount in mature *L. monocytogenes* biofilms on stainless steel was evaluated. Seventeen strains of this pathogen of distinct origins were used to compare the different quantification methods. For the purpose of developing the assay both correlation curves, the first between relative units of fluorescence (RFU) and colony

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Table 1
Listeria monocytogenes isolates used in this study.

Isolates	Serovar	Origin[a]
5366	4b	CECT
5672	4b	CECT
5873	1/2a	CECT
911	1/2c	CECT
935	4b	CECT
A7	1/2a	López et al., 2008
P12	1/2a	López et al., 2007
R6	1/2a	López et al., 2013
S1(R)	1/2a	Ortiz et al., 2014
S1(S)	1/2a	Ortiz et al., 2014
S2–1	1/2a	Ortiz et al., 2014
S2-bac	1/2a	Ortiz et al., 2014
4423	1/2a	Ortiz et al., 2016
CDL69	1/2a	Ortiz et al., 2016
EGD-e	1/2a	Ortiz et al., 2016
S10-1	2a	Ortiz et al., 2016
S2-2	1/2a	Ortiz et al., 2016

[a] CECT (Spanish Type Culture Collection). From López et al., 2007, 2008, 2013, Ortiz et al., 2014 and Ortiz et al., 2016, the isolates were collected through different studies at an Iberian pork processing plant.

forming units (CFU) and the second between Ct values and CFU, were performed. Furthermore, the biofilms formed on stainless steel surfaces were also examined using a biofilm biodetector.

2. Materials and methods

2.1. Surfaces to test

AISI 316, grade 2B stainless steel coupons 2 cm in diameter and 1 mm thick were used. Just before use, coupons were cleaned with a non-bactericidal detergent (ADIS Higiene, Madrid, Spain), disinfected with 70% isopropanol (Panreac, Castellar del Vallès, Spain) and air-dried in a laminar flow cabinet according to UNE-EN 13697 regarding non-porous materials. To ensure their complete sterility, the stainless steel coupons were further autoclaved for 15 min at 121 °C before bacterial inoculation.

2.2. Bacterial strains

Seventeen *L. monocytogenes* strains were used in this study (Table 1). Isolates were obtained from (i) Spanish Type Culture Collection (CECT, Paterna, Spain) and (ii) different studies carried out at an Iberian pork processing plant (López et al., 2007, 2008, 2013; Ortiz et al., 2014, 2016). The strains were stored at 4 °C as freeze-dried cultures, then recovered on Tryptone Soya Broth (TSB, bioMérieux, Marcy l'Etoile, France) at 30 °C for 48 h, streaked onto Tryptone Soya Agar (TSA, Oxoid, Madrid, Spain) and cultivated at 30 °C for a further 48 h. Last, working cultures were kept on TSA slants at 4 °C to be used within 30 days.

2.3. Inoculum preparation and biofilm formation on the stainless steel coupons

Bacterial inoculum was prepared by culturing the *L. monocytogenes* strains on TSA slants overnight at 37 °C. The different colonies obtained on the TSA slants were inoculated into $TSYEB_{gluc1\%+NaCl2\%}$ [consisting of TSB supplemented with 0.3% w/v yeast extract (BD, Madrid, Spain), 1% w/v glucose (Biolife, Madrid, Spain) and 2% w/v sodium chloride (Panreac, Castellar del Vallès, Spain)] up to 0.2 McFarland Units. The bacterial suspension was decimally-diluted in Tryptone Saline Solution (TSS; 1 g of tryptone [BD, Madrid, Spain] and 8.5 g of sodium chloride per liter; pH 7.0 ± 0.2) and its culture concentration was determined using the TEMPO system (bioMérieux, Marcy l'Etoile, France).

To produce *L. monocytogenes* biofilms, 30 µL of the bacterial suspension was inoculated in the center of each stainless steel coupon, resulting in a concentration on the surface of 5-5.5 Log CFU cm^{-2}. The coupons were placed in sterile Petri dishes, which were subsequently inserted in a humidity chamber maintained at saturated relative humidity and incubated at 30 °C (Fuster-Valls et al., 2008) with the objective of promoting biofilm growth under moist conditions. Biofilms were formed in static conditions during a total incubation period of one week, which included a series of washing steps and the drawing of nutrients by adding more culture medium. The steps were carried out at 48 h + 24 h + 24 h + 72 h as these conditions have been previously observed to be optimal for *L. monocytogenes* biofilm formation (Ripolles-Avila et al., 2018a). The culture medium renewal consisted of washing the inoculated coupons twice with 3 mL of sterile distilled water and adding 30 µL of $TSYEB_{gluc1\%+NaCl2\%}$ to *L. monocytogenes* inoculated coupons to enhance the growth of the attached cells and promote biofilm formation. The stainless steel coupons were again placed under the established test conditions.

2.4. Biofilm quantification by DEM

After the incubation time, the biofilms formed on the surfaces were washed twice with 3 mL of sterile distilled water to remove the unattached cells. The stainless steel surfaces were stained with 5 µL of Live/Dead BacLight (Molecular Probes, Oregon, USA), and incubated in darkness at 20–22 °C for 15 min according to the manufacturer's instructions. Readings were performed with an epifluorescent microscope BX51/BX52 (Olympus, Tokyo, Japan) equipped with a mercury lamp of 100 W (USH-103OL, Olympus), a double pass filter (U-M51004 F/R – V2, Olympus) and a digital camera (DP50-CU, Olympus). The stained samples were observed with 20× objective and the images were analyzed using the analySIS Auto 3.2 software (Soft Imaging System, Münster, Germany).

2.5. Biofilm quantification by conventional culture plating, TEMPO, VIDAS and qPCR

After the incubation time, biofilms formed on the surfaces were washed twice with 3 mL of sterile distilled water to remove unattached cells and then placed on a sterile flask containing glass beads and a neutralizer solution (TSS with 30 g Tween 80 [Scharlab, Barcelona, Spain] for every 1000 mL of deionized water (pH 7.0 ± 0.2)). The samples were then vortexed at 40 Hz for 90 s to dislodge the cells attached to the surface to quantify them.

In the case of conventional culture plating and the TEMPO system (bioMérieux, Marcy l'Etoile, France), the resulting suspension was decimally-diluted in TSS and transferred to TSA slants in the case of conventional culture plating and to a vial previously hydrated with 3 mL of sterile distilled water for the TEMPO system. The vial was then vortexed to homogenize its content and transferred into an enumeration card with 48 wells of 3 different volumes through the TEMPO Filler Unit. During incubation, the growth of the microorganism modifies the fluorescent signal of the medium, which is detected by the TEMPO Reader Unit. Depending on the number and size of the positive wells, the system calculates the number of microorganisms present in the sample.

In the case of VIDAS (bioMérieux, Marcy l'Etoile, France), 225 µL of the non-diluted sample was transferred into the sample well of the mini VIDAS LMX reagent strip (bioMérieux, Marcy l'Etoile, France) and introduced into the equipment. The results were obtained automatically after 80 min.

Last, for qPCR, the IQ-Check *Listeria monocytogenes* II PCR Detection Kit (Bioser, Barcelona, Spain) was employed. 1.5 mL of the non-diluted sample was transferred to an Eppendorf tube and centrifuged by the Heraeus Pico 17 Centrifuge (Thermo Scientific, Madrid, Spain) for 5 min at 12,000 ×*g*. The obtained supernatant was then discarded and

250 μL of the lysis reagent was added to the pellet, vortexed for 3 min and placed in the heat block at 95 °C for 20 min. The Eppendorf tubes containing the samples were subsequently centrifuged for 5 min at 12,000 ×*g*. The PCR mix containing the amplification solution and the fluorescent probes was then prepared following the kit's PCR mix calculation guide. From there, 45 μL of the PCR mix and 5 μL of the supernatant of the processed sample were introduced into the different wells of the plate, which were then sealed with optical caps. The samples were introduced into the MiniOpticon system (Bioser, Barcelona, Spain) and the results were automatically obtained after 70 min. The obtained Ct values of each sample were analyzed using the CFX Manager (Bioser, Barcelona, Spain).

2.6. Detection of the biofilms produced by the different strains of L. monocytogenes

After the incubation period, stainless steel coupons were washed twice with 3 mL of distilled water to eliminate the unattached cells. Once the washing steps were finished, one drop of a peroxide biodetector called BioFinder (iTram Higiene, Vic, Spain), which was developed for directly detecting biofilms produced by catalase-positive bacteria on food contact surfaces, was withdrawn into the coupons containing the 17 *L. monocytogenes* strains and the reaction was observed after 10 s.

2.7. Statistical analysis

Experiments with DEM were performed in duplicate on three different days (n = 6) and the rest of experiments were carried out in quadruplicate on three different days (n = 12). Bacterial counts were converted into decimal logarithmic values to nearly match the assumption of a normal distribution. The Shapiro-Wilk test was employed for contrasting normality and the data from the experiments were analyzed using One Way Anova and Tukey's Test with the SPSS statistical software package, and a regression study was also performed. A $P < 0.05$ was considered as statistically significant. The global differences were examined followed by an analysis of the individual data sets.

3. Results and discussion

3.1. Comparison of different methods for L. monocytogenes biofilm quantification

Different methods to quantify mature and well-established biofilms were assessed to compare their effectiveness regarding quantifying the cells present in the biofilms (Table 2). The comparison between the counting methods was carried out by strain and subsets of values with statistical differences ($P < 0.05$) were generated, which allowed them to be grouped. The results showed that for the 911, R6, S1(R), S1 (S), EDG-e, CDL69 and S2-1 *L. monocytogenes* strains the methods could be classified into two different subsets. The first included the four methods employed (DEM, plate count, TEMPO and qPCR), which did not present significant differences ($P = 0.173$; $P = 0.432$; $P = 0.358$; $P = 0.285$; $P = 0.189$; $P = 0.149$, $P = 0.144$, respectively), and the second consisted of only the VIDAS method, indicating that for these strains any of the methods included in the first subset could be chosen indistinctly as a method of microbial counting since there are no significant differences between them. Branck et al. (2017) stated that if the biofilm is strong dislodging its cells for analysis can cause incomplete disintegration of the clumps, resulting in cell numbers being underestimated. However, the fact that there were no significant differences among the direct observations of the biofilms formed on the stainless steel coupons (i.e., DEM) and the plate count, TEMPO and qPCR methods indicates that for these seven strains biofilm cell recovery was appropriate, which in turn makes sense since they are strains that have generally not reached the highest counts, indicating that they are not as strong biofilm-producers as others. The results show that the strains possess different biofilm-forming abilities and so have distinct bacterial counts, as previously reported (Borucki et al., 2003; Ripolles-Avila et al., 2018b).

Three subgroups were established for the A7, P12, S2-bac and 4423 strains with no significant differences among the methods within each of them. The first two strains, A7 and P12, were included in the first group (plate count, TEMPO and DEM), the second group (DEM and qPCR) and the third group (VIDAS). The other two strains, S2-bac and 4423, were classified similarly but with two difference: first, the S2-bac strain was also included in the second subset because plate count did not present significant differences either from DEM or qPCR ($P = 0.966$; $P = 0.058$, respectively); and second, the 4423 strain was likewise included in the second subset because TEMPO did not present significant differences either from DEM or qPCR ($P = 0.917$; $P = 0.088$, respectively). The fact that the quantification methods of a subset did not show significant differences among them implies that these counting methodologies could be used indistinctly. Something different did occur, however, with strain 5672, which presented the highest viable cell count obtained by DEM. It was the only strain that did not give significant differences ($P = 0.278$) between DEM and VIDAS as quantification methods, indicating that for this strain the recovery of adhered cells was low, which could be due to the fact that it has a high production rate of substances forming the matrix embedding the biofilm, conferring protection and resistance against the detachment of cells (Wilson et al., 2004).

The food industry needs fast techniques for the routine microbiological analysis of food and food contact surfaces to ensure food safety standards (Nemati et al., 2016). VIDAS satisfies this requirement while being easy to handle and allowing a large number of samples to be analyzed simultaneously. Nevertheless, VIDAS is not a quantification technique but a detection technique. For this reason, a standard curve between the RFU, obtained after evaluating a sample by this method, and the CFU was performed in the present study. The standard curve proved to be unable to quantify up to 7 log CFU cm^{-2}, which is when the bacterial load is very high. Thus, the only strain that did not show significant differences between VIDAS and DEM was the strain with the greatest biofilm-forming ability (i.e., 5672). The other subset found for this strain included plate count, TEMPO and qPCR, which also showed no significant differences ($P = 0.900$). This could be attributed to the fact that as *L. monocytogenes* 5672 was the highest biofilm producer, it may have secreted more matrix and the cell recovery for the indirect methods could have been insufficient. Nevertheless, VIDAS, which is also an indirect method, showed no significant differences ($P = 0.278$) with the direct quantification method (i.e., DEM), although it presented ($P < 0.05$) with the rest of the quantification methods, indicating that this method overestimated the result. This could be the reason why the results of the two methods coincide, while VIDAS would not be a real result. Future efforts in searching for methodologies to adequately recover microbial cells that form biofilms, especially those that have a very abundant protective matrix, which include applying certain enzymes to break down the biofilms and recovering the cells for further quantification, could be an interesting line of research.

DEM technique is a direct counting method, since it allows the cells adhered to the surface to be observed. However, plate count, TEMPO and qPCR, are considered as indirect methods as they require the cells that form the biofilm to detach from the surface before they can be quantified (Costerton et al., 1995). For indirect methods, the adequate extraction of the microorganisms from the different surfaces before microbiological analysis is crucial (Ismaïl et al., 2013). In the present study, the recovery method employed for indirectly quantifying the cells forming the biofilms was the use of glass beads and shaken rinses, as this has previously been reported to effectively dislodge attached bacteria from stainless steel (González-Rivas et al., 2018).

Results obtained by DEM were expressed as viable cells, although non-viable cells can also be observed by this technique. qPCR, however,

Table 2
Listeria monocytogenes biofilm cell counts (Log CFU cm^{-2}) by five quantification methods (Direct Epifluorescence Microscopy; Conventional plate count; TEMPO; VIDAS and qPCR). Standard error of the mean was calculated.

L. monocytogenes strains	DEM	Plate count	TEMPO	VIDAS	qPCR
911	5.87 ± 0.24^{a}	5.58 ± 0.12^{a}	5.62 ± 0.11^{a}	7.29 ± 0.01^{b}	5.99 ± 0.15^{a}
A7	5.99 ± 0.17^{ab}	5.75 ± 0.08^{a}	5.74 ± 0.11^{a}	7.32 ± 0.01^{c}	6.32 ± 0.13^{b}
R6	6.31 ± 0.22^{a}	6.44 ± 0.11^{a}	6.26 ± 0.07^{a}	7.33 ± 0.01^{b}	6.49 ± 0.07^{a}
P12	6.32 ± 0.25^{ab}	6.05 ± 0.13^{a}	6.05 ± 0.11^{a}	7.34 ± 0.01^{c}	6.53 ± 0.06^{b}
S1(S)	6.40 ± 0.11^{a}	6.15 ± 0.10^{a}	6.10 ± 0.10^{a}	7.33 ± 0.01^{b}	6.28 ± 0.14^{a}
S1(R)	6.41 ± 0.07^{a}	6.09 ± 0.14^{a}	6.17 ± 0.14^{a}	7.33 ± 0.01^{b}	6.39 ± 0.13^{a}
EDG-e	6.42 ± 0.18^{a}	6.15 ± 0.06^{a}	6.17 ± 0.08^{a}	7.33 ± 0.01^{b}	6.08 ± 0.16^{a}
S10–1	6.42 ± 0.34^{ab}	6.17 ± 0.07^{a}	6.23 ± 0.08^{ab}	7.34 ± 0.01^{d}	6.64 ± 0.08^{c}
S2-bac	6.46 ± 0.26^{ab}	6.36 ± 0.05^{ab}	6.29 ± 0.07^{a}	7.33 ± 0.01^{c}	6.67 ± 0.07^{b}
5366	6.48 ± 0.14^{b}	6.06 ± 0.06^{a}	6.10 ± 0.06^{a}	7.30 ± 0.01^{c}	6.14 ± 0.03^{a}
5873	6.49 ± 0.19^{b}	5.65 ± 0.18^{a}	5.67 ± 0.18^{a}	7.29 ± 0.01^{c}	5.87 ± 0.10^{a}
CDL69	6.52 ± 0.10^{a}	6.24 ± 0.06^{a}	6.20 ± 0.09^{a}	7.31 ± 0.01^{b}	6.46 ± 0.15^{a}
S2–2	6.54 ± 0.19^{bc}	6.23 ± 0.06^{a}	6.28 ± 0.07^{ab}	7.32 ± 0.01^{d}	6.69 ± 0.08^{c}
935	6.66 ± 0.20^{c}	6.06 ± 0.07^{a}	6.08 ± 0.10^{ab}	7.32 ± 0.01^{d}	6.45 ± 0.1^{bc}
S2–1	6.70 ± 0.13^{a}	6.43 ± 0.06^{a}	6.43 ± 0.09^{a}	7.35 ± 0.02^{b}	6.55 ± 0.09^{a}
4423	6.72 ± 0.12^{ab}	6.53 ± 0.08^{a}	6.61 ± 0.07^{ab}	7.36 ± 0.02^{c}	6.88 ± 0.1^{b}
5672	6.94 ± 0.20^{b}	5.93 ± 0.14^{a}	6.09 ± 0.12^{a}	7.30 ± 0.01^{b}	5.95 ± 0.12^{a}

$^{a–d}$ Means within a row lacking a common superscript differ significantly ($P < 0.05$).

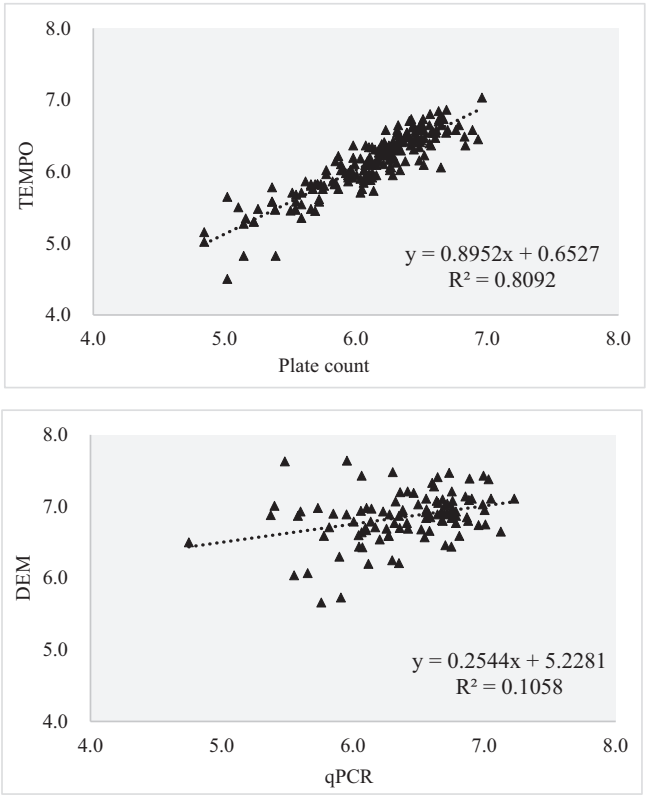

Fig. 1. Correlation of the counts (Log CFU cm^{-2}) between (a) TEMPO and plate count ($R^2 = 0.809$); (b) Direct Epifluorescence Microscopy (DEM) and qPCR ($R^2 = 0.1058$).

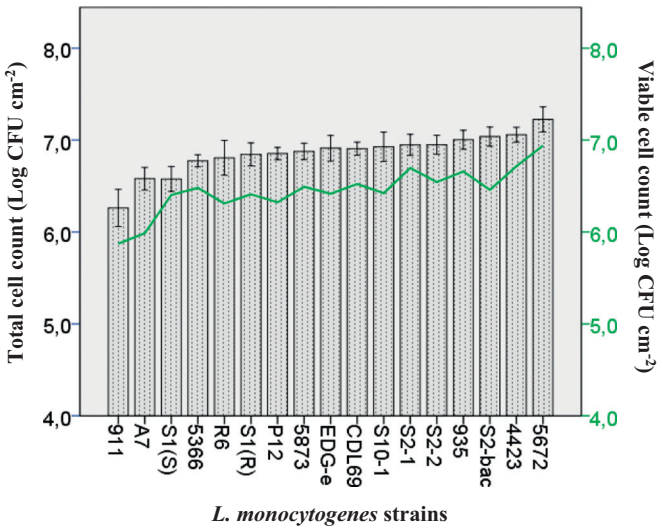

Fig. 2. Microbial counts (Log CFU cm^{-2}) of total cells, represented in bars, and viable cells, represented with a green line, after the DEM analysis of biofilms produced by different strains of *Listeria monocytogenes*. The error bars represent the standard error of the mean. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

detects DNA strands without discriminating between viable and non-viable cells. It is therefore possible that quantification by qPCR was higher than quantification by DEM, since in the biofilms formed by these strains a slightly higher percentage of non-viable cells was found compared to the rest of the strains, as reported by other authors (Guilbaud et al., 2005; Winkelströter and De Martinis, 2015). Nevertheless, the quantification of *L. monocytogenes* cells present in biofilms by qPCR has been shown to provide accurate measures (Klančnik et al., 2015). The correlation curve obtained for qPCR between the Ct values and CFU mL^{-1} showed a linear correlation ($R^2 = 0{,}9917$) with a detection limit of 10^2 CFU mL^{-1}, as other authors have reported (Elizaquível et al., 2011).

The comparison among counting methods was also carried out at a species level. The statistical study determined that there were three remarkable subsets. The first included plate count and TEMPO ($P = 0.998$), the second included DEM and qPCR ($P = 0.508$), and the third included only VIDAS, which had no correlation with the rest of the methods. Consequently, correlation studies were also performed to assess the similarities between plate count and TEMPO and between DEM counts and qPCR (Fig. 1). Results obtained show a high correlation between plate count and TEMPO ($R^2 = 0.8092$), but they do not show a direct correlation between DEM and qPCR ($R^2 = 0.1058$). Although there were no statistically significant differences at the species level ($P > 0.05$), in the correlation study it was shown that DEM and qPCR could not be used interchangeably to quantify the cells that conform *L. monocytogenes* biofilms, but that this would depend on the specific species and its recovery. This study also found that by means of plate count and TEMPO, the counts obtained could be differentiated from those obtained with DEM and qPCR, which could be attributed to non-

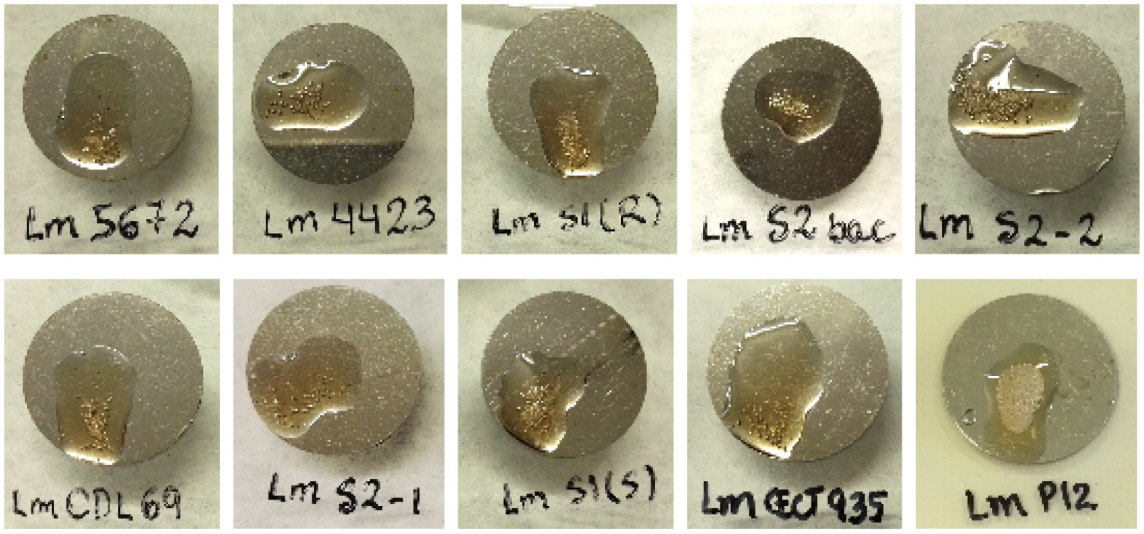

Fig. 3. Reactions after applying BioFinder to detect the biofilms of different strains of *Listeria monocytogenes*. The presence of biofilms is related to bubbles appearing on the surface.

viable cells.

3.2. Comparison of biofilm viable and total cell counts among strains

Fig. 2 shows the microbial counts obtained by DEM in ascending order from the least (911) to the most (5672) biofilm-producing *L. monocytogenes* strains. The total amount of cells (i.e., viable and non-viable cells) ranged from 6.26 Log CFU cm^{-2} to 7.23 Log CFU cm^{-2}. The number of viable cells ranged from 5.87 Log CFU cm^{-2} to 6.94 Log CFU cm^{-2}. Four significantly different ($P < 0.05$) groups (A to D) were found, with no significant differences ($P = 0.091$, $P = 0.082$, $P = 0.192$ and $P = 0.061$; for groups A, B, C and D, respectively) between strains belonging to a same group. It must also be pointed out that, in general, the proportion of non-viable cells was quite consistently maintained among *L. monocytogenes* strains, although for some of them, such as A7, R6, P12, EDG-e, S10-1, S2-2 and S2-bac, the proportion of non-viable cells was higher.

Several researchers have associated serotypes with diverse attributes of *L. monocytogenes*, with biofilm formation among them (Orsi et al., 2011; Wang et al., 2017; Zoz et al., 2017). Nevertheless, the relationship between serotype and *L. monocytogenes* biofilm formation remains unsolved (Kadam et al., 2013). Results revealed that serotype 4b presented significant differences ($P = 0.003$) from 1/2c serotype. In fact, two of the strains considered as strong biofilm-producers are included in this group (5672 and 935) from a total of 3 strains with this serotype. Regarding the rest of the serotypes, the other comparison that showed significant differences ($P = 0.032$) was serotype 1/2a, which included most of the strains used in the study, vs. serotype 1/2c, which only included the lowest biofilm-producer strain (i.e., 911). The results obtained do not allow us to conclude whether there exists a relationship between the serotype and biofilm forming capacity. This may be due to the fact that the distribution of serotypes analyzed was not homogeneous, since 12 of them came from the food industry, in which there was a dominance of serotype 1/2a.

3.3. Visual detection of biofilm production by BioFinder

Detecting biofilm production by *L. monocytogenes* strains was also made using BioFinder. This product showed a marked positive reaction with abundant bubble formation (Fig. 3). These results concur with those described in the study for quantifying biofilms, so BioFinder can be used as a screening tool to identify stronger and weaker biofilm-forming strains. Furthermore, the results complement those previously reported when testing this product for detecting biofilms formed by microorganisms, one of which was *L. monocytogenes*, on stainless steel and polystyrene surfaces (Ripolles-Avila et al., 2018c). In this case, *L. monocytogenes* were shown to have a slightly positive reaction, but the biofilms were formed during a maximum incubation period of 72 h and therefore the structures were not as mature as in the present study, which could explain the more marked reaction observed here.

4. Conclusions

Results obtained in this study indicate that the counting method to be selected for maximum realistic *L. monocytogenes* biofilm quantification depends on how productive the strain is in generating the matrix embedding the biofilm. This is because the robustness of the biofilm depends on the production of ECM, so the recovery of the cells that form the biofilm can be lower in those whose cells do not produce ECM abundantly. *L. monocytogenes* strains possess different biofilm-forming abilities, although it could not be proven a direct correlation to serotype differences, which implies that it is an individual characteristic of each strain. Last, the biodetector used to rapidly detect biofilm growth on stainless steel coupons can be an important tool for the food industry. The election of a standard model for the formation and quantification of mature biofilms generated with the same conditions will allow the study to be of reference and comparable.

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