

Accepted Manuscript

Differential internalin A levels in biofilms of *Listeria monocytogenes* grown on different surfaces and nutrient conditions

Niamh Gilmartin, Maria S. Gião, Charles W. Keevil, Richard O’Kennedy

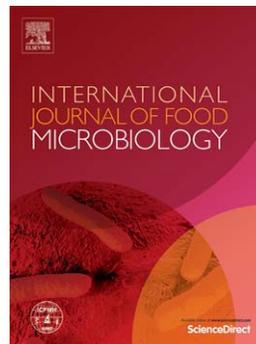
PII: S0168-1605(15)30184-7
DOI: doi: [10.1016/j.ijfoodmicro.2015.12.004](https://doi.org/10.1016/j.ijfoodmicro.2015.12.004)
Reference: FOOD 7102

To appear in: *International Journal of Food Microbiology*

Received date: 2 June 2015
Revised date: 30 November 2015
Accepted date: 11 December 2015

Please cite this article as: Gilmartin, Niamh, Gião, Maria S., Keevil, Charles W., O’Kennedy, Richard, Differential internalin A levels in biofilms of *Listeria monocytogenes* grown on different surfaces and nutrient conditions, *International Journal of Food Microbiology* (2015), doi: [10.1016/j.ijfoodmicro.2015.12.004](https://doi.org/10.1016/j.ijfoodmicro.2015.12.004)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



Differential internalin A levels in biofilms of *Listeria monocytogenes* grown on different surfaces and nutrient conditions

Niamh Gilmartin¹, Maria S. Gião², Charles W. Keevil², Richard O'Kennedy¹

1. School of Biotechnology and Biomedical Diagnostics Institute, Dublin City University, Dublin 9, Ireland

2. Centre for Biological Sciences, Life Sciences Building, Highfield Campus, University of Southampton, Southampton SO17 1BJ, U.K.

Corresponding author email: niamh.gilmartin@gmail.com

ACCEPTED MANUSCRIPT

Abstract

Listeria monocytogenes is a foodborne pathogen containing the surface protein, internalin A (InlA). The expression of this protein permits the invasion of *L. monocytogenes* into intestinal epithelial cells expressing the receptor E-cadherin, thus crossing the intestinal barrier and resulting in listeriosis. The main aim of this work was to investigate InlA levels in different *L. monocytogenes* strains in both planktonic and sessile states using an anti-InlA antibody. Biofilms were grown in high and low nutrient environments on glass, stainless steel and polytetrafluoroethylene (PTFE). This study demonstrated that InlA levels varied greatly between strains and serotypes of *L. monocytogenes*. However, the serotypes 1/2a, 1/2b and 4b, associated with the largest number of outbreaks of listeriosis consistently showed the highest InlA levels, regardless of nutrient content or planktonic or sessile state. Differences in InlA levels were also observed in biofilms grown on different surfaces such as glass, stainless steel and PTFE, with a significant reduction in InlA levels observed in biofilms on PTFE. Interestingly, although a large number of the total cells observed in biofilms formed in tap-water were non-cultivable, the virulence factor, InlA, was expressed at levels between 78-85%, thus indicating that these cells may still be virulent. A greater understanding of the factors that affect the levels of InlA on the surface of *L. monocytogenes*, is essential in the appreciation of the role of InlA in the persistence of biofilms containing *L. monocytogenes* and their potential to cause food borne disease.

Key words

Internalin A; *Listeria monocytogenes*; biofilms; planktonic cells

Highlights

- Reduction in InlA levels in biofilms of *L. monocytogenes* when compared to planktonic cells
- InlA levels highest in the *L. monocytogenes* serotypes 1/2a, 1/2b and 4b responsible for the majority of listeriosis outbreaks.
- Observed InlA levels between 78-85% indicate viable but non-cultivable cells may still be virulent.
- InlA levels in biofilms were not affected by low nutrient conditions but affected by growth on different surfaces such as glass, stainless steel and polytetrafluoroethylene.

1. Introduction

Listeria monocytogenes is a foodborne pathogen that causes gastroenteritis in healthy people and potentially cause life-threatening infections in high-risk individuals, such as the elderly, pregnant women and individuals suffering from immuno-compromising diseases, such as cancer or HIV. Due to the high incidence of listeriosis caused by the consumption of ready-to-eat (RTE) foods, the permitted limits in RTE foods not able to support growth of *L. monocytogenes* during their shelf life is 100 CFU/g. In foods that can support growth absence of *L. monocytogenes* in 5 x 25g is required, unless the manufacturer can demonstrate that the numbers will not exceed 100 CFU/g during shelf life. No *L. monocytogenes* is allowed in 5 x 25g samples of foods intended for infants or for special dietary purposes (European Commission, 2005). Reducing the contamination risk of this pathogen is challenging by virtue of its ability to grow at refrigeration temperatures, tolerate high salt concentrations and acidic media and form biofilms (Gandhi and Chikindas, 2007). The virulence of *L. monocytogenes* varies between strains but of the 13 known serotypes, three (1/2a, 1/2b, and 4b) account for the majority of the listeriosis outbreaks worldwide (Gianfranceschi et al., 2009; Kathariou, 2002; Mead et al., 2006). However, even when only low levels of contamination occur in food, major outbreaks of listeriosis may occur (Mead et al., 2006).

Biofilms are populations of surface-associated microorganisms surrounded by an extracellular polymeric substance matrix primarily composed of polysaccharides (Donlan, 2002). Several studies (Bonsaglia et al., 2014; Chae et al., 2000; Gião and Keevil, 2014; Mai and Conner, 2007; Min et al., 2006) have demonstrated the ability of several strains of *L. monocytogenes* to form biofilms at different temperatures, nutrient conditions and on different surfaces. Strongly adherent *L. monocytogenes* strains were found to be more

invasive than those that are weakly adherent under certain conditions (Kushwaha and Muriana, 2010). The formation of biofilms protects *L. monocytogenes* from a variety of stress conditions, such as extreme temperatures, ultraviolet rays, biocides, desiccation and increased salt concentrations (see Carpentier and Cerf, 2011). In biofilms, *L. monocytogenes* also show greater tolerance for disinfectants and sanitizers thus hampering decontamination (Pan et al., 2006).

The expression of the 800 amino acid surface protein internalin A (InlA) on the surface of the cell allows *L. monocytogenes* to invade intestinal epithelial cells that express the receptor E-cadherin and thus cross the intestinal barrier (Gaillard et al., 1991; Mengaud et al., 1996). Mutations in the *inlA* gene resulting in premature stop codons and, therefore, a truncated form of InlA, were identified in numerous *L. monocytogenes* isolates from both clinical and food sources (Nightingale et al., 2005). Several studies (Jacquet et al., 2004; Nightingale et al., 2008, 2005) have demonstrated a reduction in the invasiveness of *L. monocytogenes* strains containing a truncated InlA with Chen *et al.* (2011) suggesting that *L. monocytogenes* isolates that encode a full-length InlA may be as much as 10,000-fold more virulent than isolates with a truncated InlA. InlA and InlB may also have a role in biofilm formation (Chen et al., 2008) with the expression of InlA and InlB shown to influence adherence to glass surfaces (Chen et al., 2009).

Hearty and co-workers (2006) developed a monoclonal antibody (mAb2B3) capable of binding exclusively to *L. monocytogenes* by targeting the virulence factor InlA. This antibody has a key advantage in terms of detection due to its ability to preferentially bind live cells. That study also reported differences in the levels of the surface marker InlA in *L. monocytogenes* under different conditions. Therefore, the aim of this work was to investigate

InlA levels in different *L. monocytogenes* strains in both planktonic and sessile states using the mAb2B3 antibody.

ACCEPTED MANUSCRIPT

2. Materials and Methods

2.1 Materials, culture maintenance and inocula preparation

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of reagent grade, unless otherwise indicated.

The bacterial strains used were shown in Table 1. All the cultures are maintained in protect vials at -80°C and recovered onto brain heart infusion (BHI) agar (Oxoid, UK) prior to each experiment. The BHI plates were then incubated at 37°C for 48 h, sub-cultured once and incubated at 37°C for 24 h before beginning the experiment. For the purpose of this study, planktonic cells refers to cells grown in broth while sessile cells refers to cells from biofilms.

2.2 Hybridoma culture, antibody purification and labelling

Hybridomas producing the monoclonal antibody 'mAb2B3' against the InIA protein on the surface of *L. monocytogenes*, [the production of which was described previously (Hearty et al., 2006)] were selectively cultured in Dulbecco's Modified Eagle Medium (DMEM) containing fetal calf serum (FCS) 5% (v/v). The mAb2B3 antibody was purified from the hybridoma supernatant by affinity chromatography using a Protein G-sepharose column. Labelled mAb2B3 was prepared by incubating a 1 mg/mL solution of a fluorescein isothiocyanate (FITC)-labelled goat anti-mouse antibody prepared in phosphate buffered saline (PBS; 10 mM PO_4^{3-} , 137 mM NaCl, and 2.7 mM KCl, pH 7.4) with a 1 mg/mL solution of mAb2B3 at 37°C for 30 min prior to use.

2.3 Planktonic cells growth and preparation

A loopful of the *L. monocytogenes* from a 24 h BHI agar plate was suspended in 10 mL BHI broth (Oxoid, UK) and incubated in a shaking incubator at 37°C overnight at 200 rpm. The

culture was centrifuged at 3500 g for 10 min and the pellet was washed twice with sterile PBS before the pellet was resuspended in 5 mL of sterile PBS. A 100 μ L volume of this suspension was then added to 10 mL BHI broth and incubated at 22 °C for 24 h. After incubation the culture was again centrifuged at 3,500 g for 10 min and the pellet was washed twice with sterile PBS before the pellet was resuspended in 5 mL of sterile PBS.

2.4 Surface preparation and biofilm formation

One cm² coupons of stainless steel AISI 304, polytetrafluoroethylene (PTFE) and glass, prepared as described previously (Gião and Keevil, 2014), were used as surfaces to grow biofilms. A loopful of the *L. monocytogenes* from a 24 h BHI agar plate was suspended in BHI broth (Oxoid, UK) or filter-sterilized dechlorinated tap water (TW) to a final concentration of 10⁷ cells/mL. The sterilized stainless steel, PTFE and glass coupons were placed in a 6-well microtitre plate (Greiner bio-one, UK) and 5 mL of each bacterial suspension added to an individual well. The plates were incubated at 22 °C for 24 h. After that time two coupons were removed from each bacterial suspension and gently rinsed in PBS to remove loosely attached cells. These coupons were transferred to a 15 mL centrifuge tube (Greiner bio-one, UK) containing 2 mL of sterile PBS and autoclaved glass beads of 2 mm diameter (Merck, UK) and vortexed for 1 min to remove biofilm from the coupons surface and ensure a homogeneous suspension of sessile cells.

2.5 Quantification of sessile cells

Total sessile cells were quantified by staining cells removed from the coupons with SYTO 9. Briefly, 1 mL of an appropriate dilution was mixed with 0.25 μ L of SYTO 9, incubated in the dark for 15 min, filtered through a 0.2 μ m pore size polycarbonate black Nucleopore[®] membrane (Whatman, UK) and air-dried. A drop of non-fluorescent immersion oil (Fluka,

UK) was placed on top of the membrane and a cover slip added. The membranes were observed under the episcopic differential interference contrast coupled with epi-fluorescence (EDIC/EF) microscope and 10 fields of view on each membrane were randomly chosen to count the number of cells (x100 objective lens). A correlation factor correlating the area of the field of view to the area of the membrane was calculated. This correlation factor was used to calculate the number of cells present on a membrane and, as each membrane contained the cells from 1 mL sample, the number of cells per mL. To quantify cultivable cells, 40 μ L of an appropriate dilution in sterile PBS was plated onto BHI agar and incubated at 37 °C for 24 h.

*2.6 Extraction of surface proteins from *L. monocytogenes* and Western blot analysis*

L. monocytogenes strains (Lm1-8) were grown to exponential phase in 10 mL of BHI broth. The cells were harvested and washed 3 times with 5 mL of sterile PBS. Surface proteins were solubilised by resuspending the washed pellet in 1 mL of 2% (w/v) sodium dodecyl sulfate (SDS) and incubating at 37 °C for 45 min while shaking at 125 rpm. SDS polyacrylamide gel electrophoresis and Western blotting were carried out as described previously (Hearty et al., 2006) using purified mAb2B3 as a probe at a concentration of 1 mg/mL.

2.7 Analysis of InlA levels in both planktonic and sessile cells

ELISA analysis

To measure the InlA levels per unit of bacteria, the concentration of both planktonic and sessile cells were adjusted using sterile PBS to the same optical density at 600nm value ($OD_{600} = 1.0$) prior to the ELISA. The wells of a Nunc Maxisorp™ plate were coated with 100 μ L aliquots of the adjusted cells to be tested. The plates were incubated at 37 °C for 1 h

and after washing three times with 20 mL of PBS and three times with 20 mL of PBS/T [PBS containing 0.05% (v/v) Tween 20] the wells were then blocked with a 200 μ L of a 5% (w/v) solution of dry skimmed milk powder (Premier International Foods Ltd., UK) in PBS at 37 °C for 1 h. After washing a 100 μ L sample of a 1 mg/mL solution of the unlabelled mAb2B3 was added to the wells and incubated at 37 °C for 1 h. The wells were washed and 100 μ L of a 1 mg/mL solution of the secondary antibody (horse radish peroxidase-labeled goat anti-mouse IgG) was added to the wells of the plate. After 1 h incubation at 37 °C the wells were washed and 100 μ L of 3,3',5,5'-Tetramethylbenzidine (TMB ELISA peroxidase substrate solution, cat no. OORA01684, Aviva Systems Biology, San Diego, USA) was added to each well and incubated for 15 min at room temperature. The reaction was stopped at this point by the addition of 50 μ L of 10% (v/v) HCl. The absorbance was read on a Sunrise plate reader (Tecan, Männedorf, Switzerland) at 450nm.

Microscope analysis

The ability of the antibody mAb2b3 to bind to the sessile cells of the different *L. monocytogenes* strains was investigated under a microscope after removal of the sessile cells from the surface into 1 mL of sterile PBS. Prior to microscopic investigation, the sessile cells were incubated with the FITC-labelled mAb2B3 solution (1 μ g/mL) for 1 h at 37 °C. After washing with PBS, 50 μ L was transferred to a glass slide and let to air dry. Cells were also stained with 4'6-diamidino-2-phenylindole (DAPI) and both FITC and DAPI stained cells were observed under a Leica TCS SP2 confocal system (Leica, Germany 20X). The % of *L. monocytogenes* cells expressing InIA on there surface were calculated from the images obtained using customised software developed in the Biomedical Diagnostic Institute. This software was written in C++ net and was developed for the calculation of the percentage levels when two images are compared. An image of the labelled cells in the DAPI channel

was compared to a corresponding image of the cells in the FITC channel (FITC-labelled anti-InlA antibody, mAb2B3) and thus the percentages of InlA were calculated.

3. Results and Discussion

3.1 Anti-InlA mAb2B3 can bind to both planktonic and sessile cells

The anti-InlA mAb2B3 successfully bound to all six *L. monocytogenes* serotypes tested in both planktonic and sessile states (Figure 1), however, the InlA levels in all strains were lower in sessile cells than in planktonic cells. This was consistent with the findings of Khemiri *et al.* (2014), who also observed a decrease in virulence factors during biofilm formation in *Legionella pneumophila*. The InlA levels shown varied greatly between strains and serotypes of *L. monocytogenes* which was also consistent with a previous study by Werbrouck *et al.* (2006). Although the InlA levels differed between the strains (Figure 1), interestingly the InlA levels were highest in strains Lm 2, 3, 4, 5 and 8 which account for the three serotypes (1/2a, 1/2b and 4b) associated with the majority of listeriosis outbreaks.

3.2 Truncated form of InlA does not affect % levels or biofilm forming ability

Western blot analysis (Figure 2) showed that strain Lm 3 had a truncated form of InlA compared to the other strains. Although isolated from different sources, the strains Lm 4 (CECT type strain), Lm 5 (isolated from cream cake) and Lm 3 (isolated from vegetables) share the same serotype 1/2b. As, previous studies (Chen *et al.*, 2008, 2009) showed that InlA and InlB proteins play an important role in adherence to surfaces, the ability of the Lm 3 strain containing a truncated form of InlA to form biofilms was compared to Lm4 and Lm5 strains containing a full length InlA.

No significant difference was observed between the total number of cells observed between the strains Lm 4 and 5 and the reference strain NCTC 13372 (with a full length InIA) compared to Lm 3 in either culture medium or tap water (Figure 3). This indicates that the truncated InIA observed in Lm 3 had no effect on the ability of the strain to form biofilms in either a high (medium) or low (water) nutrient rich environment. Wang and colleagues (2015) demonstrated that strains with full-length InIA isolated from retail deli environments had also enhanced adhesion and therefore biofilm forming ability when compared to a truncated form. Contrary to these findings, Françoisa *et al.* (2009) demonstrated that a truncated form of InIA showed enhanced biofilm formation in clinical isolates when compared to full-length InIA suggesting that more evidence may be required to demonstrate the effect of truncated InIA on a strains ability to form biofilms.

The percentage of cells expressing InIA on the surface (Table 2) was also unaffected by the truncated form of InIA (Lm 3) or the high or low nutrient environment (percentage of cells expressing InIA were 78-85% in tap water versus 84-85% in medium). Not all of the cells shown in Figure 4A and 4B showed binding of the anti-InIA mAb2B3 to the surface, likely due to the fact that mAb2B3 will only bind to live cells (Hearty *et al.*, 2006) whereas DAPI can stain both live and dead cells. The binding site in InIA for the mAb2B3 is effected by conformational changes when the cells are dead and therefore no binding of the mAb2B3 will occur however the dead cell membrane can still be stained with the DAPI stain.

As shown in Figure 3, the total number of cells observed in biofilms formed by the three strains Lm 3, 4, 5 on glass, stainless steel and PTFE surfaces in medium were significantly less than biofilms formed in tap water, thus confirming previous work also demonstrating lower cell numbers observed in biofilms formed in tap water (Gião and Keevil, 2014). Also,

the number of cultivable cells decreased significantly for biofilms formed in tap water, possibly due to low nutrient availability thus resulting in a large number of the total cells observed in the tap water biofilms being viable but non-cultivable (VBNC). The virulence factor InlA was expressed at levels between 78-85% in sessile cells grown in tap water thus suggesting that the VNBC cells may still be virulent, as shown in Table 2. In contrast to the number of cells observed, the % InlA observed in the biofilms grown on tap water, did not differ significantly from biofilms grown in medium.

3.3 Biofilms formed on different substrates can differ in InlA levels

Differences in InlA levels were observed between *L. monocytogenes* strains in formed biofilms (Figure 5). The strains Lm 2, 3, 4, 5 and 8, which represent the three serotypes (1/2a, 1/2b and 4b) most common in listeriosis outbreaks, again showed the highest InlA levels. Although the InlA levels differed between strains there was no significant difference in InlA levels between biofilms of the same strain grown in tap water when compared to medium (Figures 5 A and B) regardless of the surface used. Similarly, only small differences were observed between InlA levels in biofilms grown on the hydrophilic surfaces glass and stainless steel, but, in contrast, the InlA levels in biofilms grown on the hydrophobic surface PTFE was significantly reduced. The detrimental effect of hydrophobic surfaces on *L. monocytogenes* biofilms was also demonstrated by Bonsaglia *et al.* (2014) in which the hydrophobic surfaces effected biofilm formation

4. Conclusion

Environmental factors that affect the InlA levels in *L. monocytogenes* may also have the potential to effect the virulence of the strain. The fact that this study has shown that InlA levels were unaffected by low-nutrient conditions and that InlA was present in cells of a

viable but non-cultivable state particularly in a biofilms is of great concern for food safety. Recognising that InlA is a major virulence factor in *L. monocytogenes*, this study highlighted the potential of hydrophobic surfaces in the reduction of InlA levels on the surface of *L. monocytogenes*. With further investigations this may have implications in the control of *L. monocytogenes* in the food production environment.

Acknowledgements

The authors would like to thank to Ms. Sonia Porta and Mr. Jose Belenguer from Ainia – Centro Tecnológico (Spain) and to Mrs. Nuria de la Fuente and Mr. Enrique Orihuel from Betelgeux, S. L. (Spain) for providing the *L. monocytogenes* strains. The authors would also like to thank Dr. Hansjürgen Schuppe from the Centre for Biological Sciences (University of Southampton) for all his support during the confocal observations and Mr. Peter McCluskey from Biomedical Diagnostics Institute for use of customised software. This work was supported by the European Commission within the Seventh Framework Programme, “BioliSME – Speedy system for sampling and detecting *Listeria monocytogenes* in agri-food and related European industries”, no. FP7-SME-232037 and BioliSME II, no. FP7-SME-286713.

5. References

- Bonsaglia, E.C.R., Silva, N.C.C., Fernandes Júnior, a., Araújo Júnior, J.P., Tsunemi, M.H., Rall, V.L.M., 2014. Production of biofilm by *Listeria monocytogenes* in different materials and temperatures. *Food Control* 35, 386–391.
- Carpentier, B., Cerf, O., 2011. Review - Persistence of *Listeria monocytogenes* in food industry equipment and premises. *Int. J. Food Microbiol.* 145, 1–8.
- Chae, M.S., Chae, M.S., Schraft, H., Schraft, H., 2000. Comparative evaluation of adhesion and biofilm formation of different *Listeria monocytogenes* strains. *Int. J. Food Microbiol.* 62, 103–111.
- Chen, B.Y., Kim, T.J., Jung, Y.S., Silva, J.L., 2008. Attachment strength of *Listeria monocytogenes* and its internalin-negative mutants. *Food Biophys.* 3, 329–332.

- Chen, B.-Y., Kim, T.-J., Silva, J.L., Jung, Y.-S., 2009. Positive correlation between the expression of *inlA* and *inlB* genes of *Listeria monocytogenes* and its attachment strength on glass surface. *Food Biophys.* 4, 304–311.
- Chen, Y., Ross, W.H., Whiting, R.C., van Stelten, A., Nightingale, K.K., Wiedmann, M., Scott, V.N., 2011. Variation in *Listeria monocytogenes* dose responses in relation to subtypes encoding a full-length or truncated internalin A. *Appl. Environ. Microbiol.* 77, 1171–1180.
- Donlan, R.M., 2002. Biofilms: Microbial life on surfaces. *Emerg. Infect. Dis.* 8, 881–890.
- European Commission, 2005. Commission regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Off. J. Eur. Union L.*
- Franciosa, G., Maugliani, A., Scalfaro, C., Floridi, F., Aureli, P., 2009. Expression of internalin A and biofilm formation among *Listeria monocytogenes* clinical isolates. *Int. J. Immunopath. Ph.* 22, 183–193.
- Gaillard, J.-L., Berche, P., Frehel, C., Gouln, E., Cossart, P., 1991. Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. *Cell* 65, 1127–1141.
- Gandhi, M., Chikindas, M.L., 2007. *Listeria*: A foodborne pathogen that knows how to survive. *Int. J. Food Microbiol.* 113, 1–15.
- Gianfranceschi, M.V., D'Ottavio, M.C., Gattuso, A., Bella, A., Aureli, P., 2009. Distribution of serotypes and pulsotypes of *Listeria monocytogenes* from human, food and environmental isolates (Italy 2002-2005). *Food Microbiol.* 26, 520–526.
- Gião, M.S., Keevil, C.W., 2014. *Listeria monocytogenes* can form biofilms in tap water and enter into the viable but non-cultivable state. *Microb. Ecol.* 67, 603–11.
- Hearty, S., Leonard, P., Quinn, J., O'Kennedy, R., 2006. Production, characterisation and potential application of a novel monoclonal antibody for rapid identification of virulent *Listeria monocytogenes*. *J. Microbiol. Methods* 66, 294–312.
- Jacquet, C., Doumith, M., Gordon, J.I., Martin, P.M. V, Cossart, P., Lecuit, M., 2004. A molecular marker for evaluating the pathogenic potential of foodborne *Listeria monocytogenes*. *J. Infect. Dis.* 189, 2094–2100.
- Kathariou, S., 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *J. Food Prot.* 65, 1811–1829.
- Khemiri, A., Lecheheb, S.A., Chi Song, P.C., Jouenne, T., Cosette, P., 2014. Proteomic regulation during *Legionella pneumophila* biofilm development: decrease of virulence factors and enhancement of response to oxidative stress. *J. Water Heal.* 12, 242–53.
- Kushwaha, K., Muriana, P.M., 2010. Comparison of invasiveness among surface-adherent variants of *Listeria monocytogenes* in Caco-2 cell culture assays. *Int. J. Food Microbiol.* 138, 166–171.

- Mai, T.L., Conner, D.E., 2007. Effect of temperature and growth media on the attachment of *Listeria monocytogenes* to stainless steel. *Int. J. Food Microbiol.* 120, 282–286.
- Mead, P.S., Dunne, E.F., Graves, L., Wiedmann, M., Patrick, M., Hunter, S., Salehi, E., Mostashari, F., Craig, a, Mshar, P., Bannerman, T., Sauders, B.D., Hayes, P., Dewitt, W., Sparling, P., Griffin, P., Morse, D., Slutsker, L., Swaminathan, B., 2006. Nationwide outbreak of listeriosis due to contaminated meat. *Epidemiol. Infect.* 134, 744–751.
- Mengaud, J., Ohayon, H., Gounon, P., Mege, R.M., Cossart, P., 1996. E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. *Cell* 84, 923–932.
- Min, S.C., Schraft, H., Hansen, L.T., Mackereth, R., 2006. Effects of physicochemical surface characteristics of *Listeria monocytogenes* strains on attachment to glass. *Food Microbiol.* 23, 250–259.
- Nightingale, K.K., Ivy, R. a., Ho, a. J., Fortes, E.D., Njaa, B.L., Peters, R.M., Wiedmann, M., 2008. inlA premature stop codons are common among *Listeria monocytogenes* isolates from foods and yield virulence-attenuated strains that confer protection against fully virulent strains. *Appl. Environ. Microbiol.* 74, 6570–6583.
- Nightingale, K.K., Windham, K., Martin, K.E., Yeung, M., Wiedmann, M., 2005. Select *Listeria monocytogenes* subtypes commonly found in foods carry distinct Nonsense Mutations in inlA, Leading to Expression of Truncated and Secreted Internalin A, and are associated with a reduced invasion phenotype for human intestinal epithelial cells. *Appl. Environ. Microbiol.* 71, 8764–8772.
- Pan, Y., Breidt, F., Kathariou, S., 2006. Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. *Appl. Environ. Microbiol.* 72, 7711–7717.
- Wang, J., Ray, A.J., Hammons, S.R., Oliver, H.F., 2015. Persistent and transient *Listeria monocytogenes* strains from retail deli environments vary in their ability to adhere and form biofilms and rarely have inlA premature stop codons. *Foodborne Pathog. Dis.* 12, 151–8.
- Werbrouck, H., Grijspeerdt, K., Botteldoorn, N., Van Pamel, E., Rijpens, N., Van Damme, J., Uyttendaele, M., Herman, L., Van Coillie, E., 2006. Differential inlA and inlB expression and interaction with human intestinal and liver cells by *Listeria monocytogenes* strains of different origins. *Appl. Environ. Microbiol.* 72, 3862–3871.

Figures and Tables

Table 1. Bacterial strains used

Strain Name	Serotype	Source
<i>L. monocytogenes</i> (Lm 1)	1a	CECT4031
<i>L. monocytogenes</i> (Lm 2)	1/2a	Vegetable
<i>L. monocytogenes</i> (Lm 3)	1/2b	Vegetable
<i>L. monocytogenes</i> (Lm 4)	1/2b	CECT 936
<i>L. monocytogenes</i> (Lm 5)	1/2b	Cream cake
<i>L. monocytogenes</i> (Lm 6)	1/2c	CECT 911
<i>L. monocytogenes</i> (Lm 7)	3a	CECT 933
<i>L. monocytogenes</i> (Lm 8)	4b	CECT 4032
<i>L. monocytogenes</i> NCTC 13372	1/2c	NCTC® 13372
<i>L. innocua</i>		ATCC 11288

NCTC® = National Collection of Type Cultures

CECT= Colección Española de Cultivos Tipo (Spanish type culture collection)

ATCC= American Type Culture Collection

Table 2. Percentage of *L. monocytogenes* cells expressing InlA in biofilms formed in BHI medium and tapwater

		Percentage* of <i>L. monocytogenes</i> cells in biofilms expressing InlA on their surface (Average\pm SD**)	
		Biofilms formed in BHI medium	Biofilms formed in Tap water
<i>L. monocytogenes</i> strains	Lm 3	82 \pm 6	82 \pm 10
	Lm 4	85 \pm 13	80 \pm 6
	Lm 5	85 \pm 2	85 \pm 2
	NCTC 13372	85 \pm 6	84 \pm 8

* Percentage calculated from the microscope images obtained using customised software

**SD = the standard deviation from the mean for 3 experiments

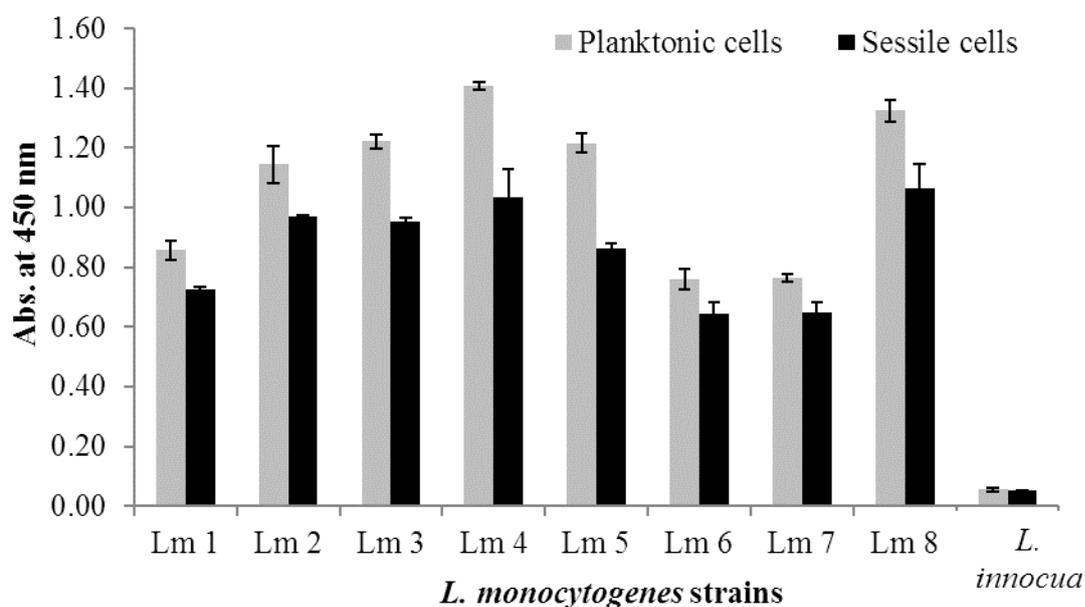


Figure 1. Analysis of InlA levels in planktonic and sessile cells of different *L. monocytogenes* strains. The InlA levels were measured using an anti-InlA specific antibody in an ELISA format and the error bars represent standard deviation of 3 independent experiments. Prior to ELISA analysis the planktonic cells were grown in BHI medium overnight at 22 °C and the sessile cells were recovered from biofilms formed on stainless steel in BHI medium for 24 h at 22 °C. To ensure the InlA levels measured were per unit of bacteria, the concentration of both planktonic and sessile cells was adjusted using sterile PBS to the same optical density at 600nm value ($OD_{600} = 1.0$) prior to the ELISA.

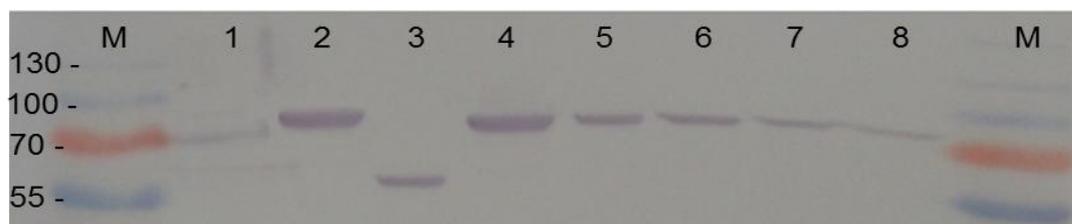


Figure 2. Western blot analysis of InlA isolated from the different *L. monocytogenes* strains as follows: Lm1 (1), Lm2 (2), Lm3 (3), Lm4 (4), Lm5 (5), Lm6 (6), Lm7 (7) and Lm8 (8) and was representative of three determinations. The marker (M) used was PageRuler plus from ThermoFischer. The amount of material loaded corresponds to 10 μ g of protein from bacteria harvested in exponential phase ($OD_{600} = 0.5$). The blot was probed with anti-InlA mAb2B3 followed by an HRP labelled rabbit anti-mouse antibody. Bands representing the full length InlA (approx. 80 kDa) are observed in lanes 1,2, 4-8 while a band representing a truncated form of InlA (approx. 60 kDa) is observed in lane 3.

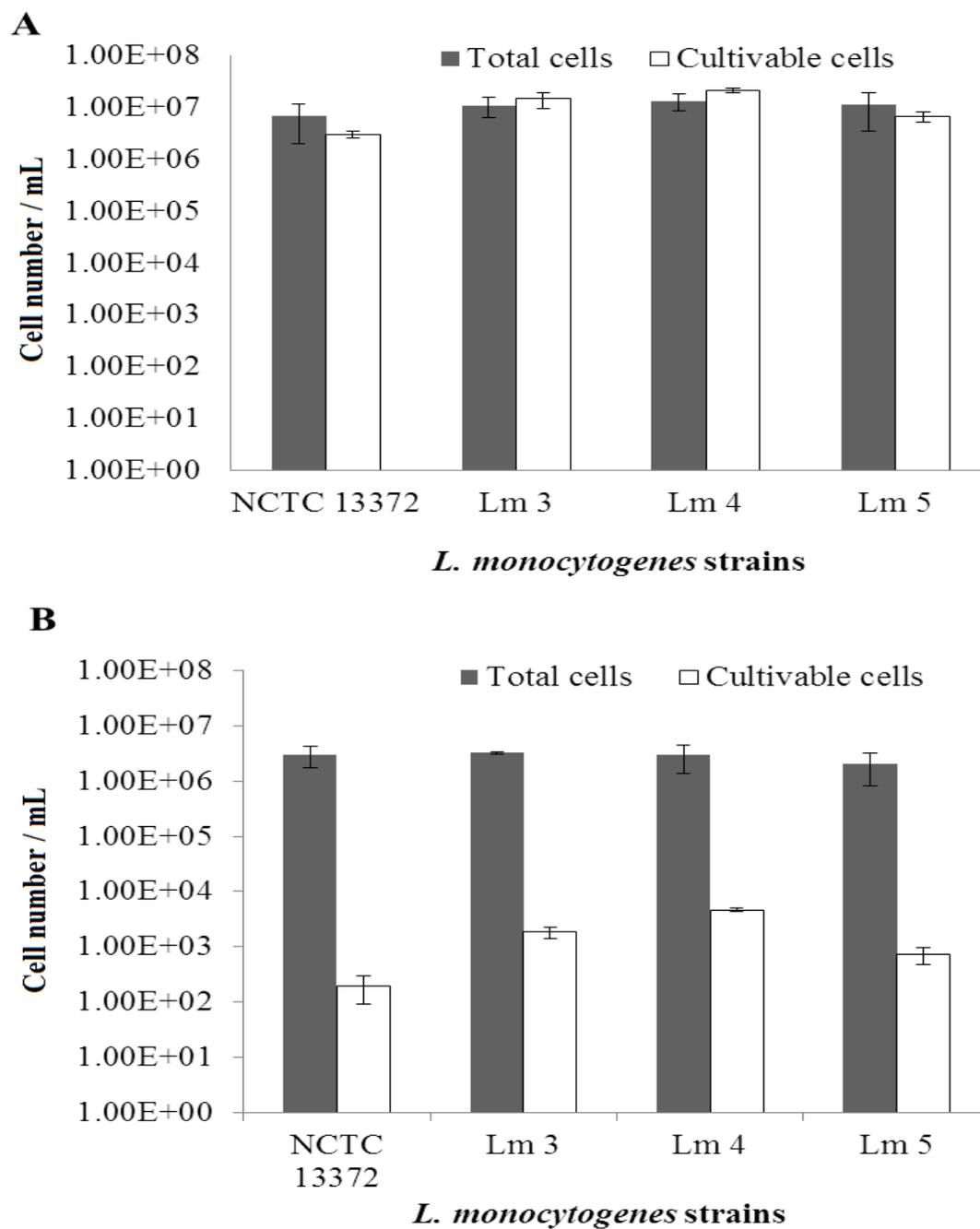


Figure 3. The number of total and cultivable cells of *L. monocytogenes* biofilms formed on stainless steel surfaces in BHI medium (**A**) or tap water (**B**) after 24 h at 22 °C. Error bars represent standard deviation of 3 independent experiments.

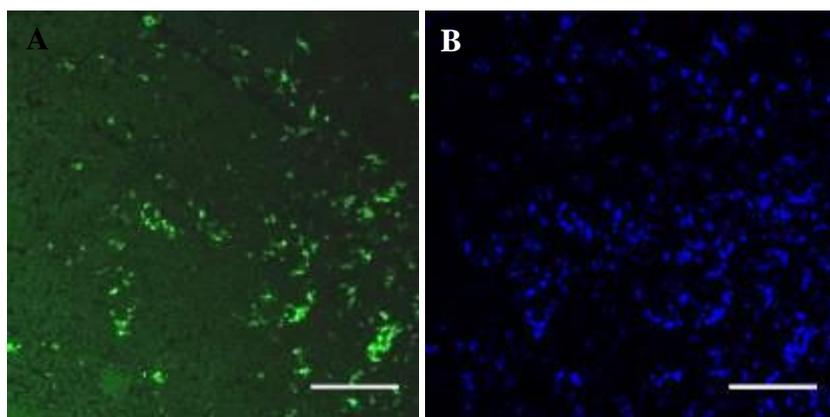


Figure 4. InlA levels on surface of *L. monocytogenes* strains grown in biofilms on stainless steel at 22 °C for 24 h. **(A)** Confocal microscope image (20X magnification) of sessile cells stained with FITC-labelled anti-InlA mAb2B3 from biofilm of Lm 4 formed in tap water. **(B)** Confocal microscope image (20X magnification) of sessile cells stained with DAPI from biofilm of Lm 4 formed in tap water. The size bars represent 50 µm.

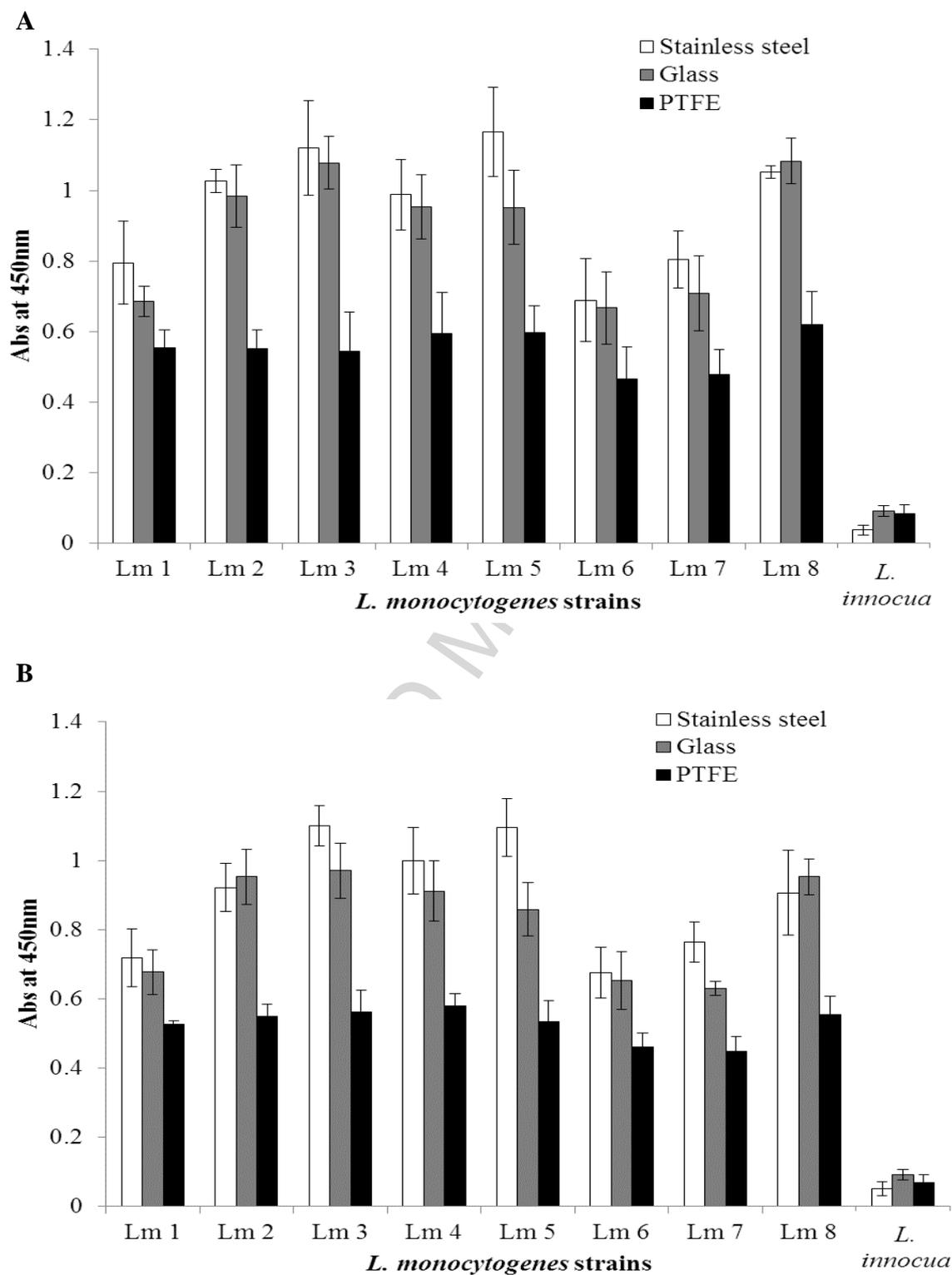


Figure 5. InA levels in biofilms of different *Listeria* strains formed in (A) BHI medium and (B) tap water using different surfaces. The cell concentration for all strains was adjusted to $OD_{600} = 1.0$ before the ELISA was performed. The error bars represent +/- the standard deviation from the mean for 3 experiments.

Highlights

- Reduction in InlA levels in biofilms of *L. monocytogenes* when compared to planktonic cells
- InlA levels highest in the *L. monocytogenes* serotypes 1/2a, 1/2b and 4b responsible for the majority of listeriosis outbreaks.
- Observed InlA levels between 78-85% indicate viable but non-cultivable cells may still be virulent.
- InlA levels in biofilms were not affected by low nutrient conditions but affected by growth on different surfaces such as glass, stainless steel and polytetrafluoroethylene.