BIOFILM-FORMING ABILITY ASSESSMENT OF *Listeria monocytogenes* ISOLATES COLLECTED IN DELICATESSEN MEAT PRODUCTS

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Abstract – *Listeria monocytogenes* isolates (n=113) were collected from food samples (n=120) and from food contact equipments (n=60) in the industrial and retail segments of the meat delicatessen food-chain.

An experimental protocol was used to evaluate the development of a 5-day mono-cultural biofilm of *L. monocytogenes* (n=19) by the crystal violet stain and by the enumeration of total viable cell counts. The majority of the strains revealed to be moderate biofilm-formers (47%), although some were strong biofilm formers. When relating the biofilm-forming capacity with the serogroups presented by each isolate, significant differences were found for IIc and IVb serogroups that exhibited a significantly higher ability to form biofilm.

A better knowledge on *L. monocytogenes* biofilm-forming ability is needed in the meat delicatessen food-chain, in order to reduce the consumer's potential risk to acquire foodborne listeriosis.

Key Words – *L. monocytogenes*, ready-to-eat meat-based foods, PFGE.

I. INTRODUCTION

Listeria monocytogenes is an opportunistic pathogen that causes severe foodborne disease in humans, with low incidence but high mortality rates among those cases [1, 2]. This pathogen is associated with ready-to-eat foods consumption, and is a real concern in the delicatessen meat food-chain, in which the final products are handled in operations such as cutting, slicing and packaging after the listericidal treatment [3]. In addition to the public health threat, the presence of *L. monocytogenes* in foods has important economic consequences for the stakeholders involved in the ready-to-eat meat-based food products (RTEMP) food chain.

L. monocytogenes ability to persist in food processing environments is associated with the capacity to adhere to abiotic surfaces and form biofilms, favored by the environment temperature and presence of food residues [4]. Biofilms consist of microbial communities that grow attached to biotic or abiotic surfaces and are embedded in a matrix of highly hydrated extracellular polymeric substance [5]. Biofilms are frequently formed by different species that inhabit the same niche in food processing environments [5]. Biofilms usually establish and grow in a solid substrate, enduring air and liquid interfaces, and exhibit a greater resistance to environment stresses and antimicrobial substances than in the planktonic form, which seems to be due to an altered phenotype with respect to growth rate and gene transcription [6]. A better knowledge of *L. monocytogenes* behavior from farm to fork is needed to establish effective preventive control measures. The aim of the present study was to characterize the biofilm-forming ability of different *L. monocytogenes* strains collected in the delicatessen meat food-chain (producing industry and retail establishments) and to relate it with virulence-associated genetic characteristics.

II. MATERIALS AND METHODS

A collection of 113 *L. monocytogenes* strains isolated in the industrial and retail segments of the delicatessen meat food-chain was obtained, from 120 final food products and 60 food related surfaces (in-use and clean surfaces). *L. monocytogenes* detection was performed according to ISO11290-1 and up to 10

presumptive colonies per sample were collected for PCR identification as described by Simon *et al*. (1996). *L. monocytogenes* isolates (n=113) were serogrouped using a multiplex PCR [8].

Nineteen strains were selected to represent a range of serogroups and sources, in order to have representatives with different virulence-related characteristics and of distinct sampling points along the RTEMP food-chain.

The protocol of Romanova *et al.* (2007) was used with modifications to form a 5-day mono-cultural biofilm of *L. monocytogenes*. *L. monocytogenes* CECT911 was used as a control due to its known adherence characteristics previously described. A single colony of each selected strain was inoculated in buffered peptone water (BPW) and incubated at 30°C overnight. Bacterial suspension optical density at 600nm (OD) was assessed to obtain 8 log cfu/ml concentration. Triplicate wells of a microtiter plate were inoculated with the initial suspension, to obtain a final concentration of 5 log cfu/ml. Microtiter plates OD was read in a SpectraMax 340PC. Plates were incubated at 30 °C for 5 days and spent nutrients were daily removed and replaced with fresh BPW. By the end of the incubation period, the OD was measured. Afterwards, the wells were washed with sterile distilled water (SDW) and left air drying. Each well was stained with 0.1% crystal violet solution for 15 minutes, and the wells were washed with SDW and left to air dry. Then, 220µl of ethanol:acetone 80:20 v/v was added to each well for 15 minutes. The microtiter plate was shaken for 5 minutes and the absorbance was measured in SpectraMax 340PC. Each absorbance value was corrected by subtracting the means of absorbance readings from the negative control wells.

For the enumeration of viable cells in biofilms, the wells were rinsed with SDW; BPW was added to each well and the biofilms were detached from the well surface with a mini cell scrapper. The microtiter plate was sonicated to detach and collect the sessile cells. 10-fold dilutions were prepared in BPW that were dropped on the surface of a tryptone soy agar agar plate. After overnight incubation at 30°C, colonies were counted in a stereoscopic magnifier.

Adherence capability of the tested strains was based upon the OD exhibited by bacterial biofilms, according to Stepanovic *et al*. (2004). The cut-off value (OD_{c-o}) was defined as 3 standard deviations above the negative control mean OD. The strains were classified as non-adherent ($OD \leq OD_{c-o}$), weakly adherent ($OD_{c-o} < OD \leq 2 \times OD_{c-o}$), moderately adherent ($2 \times OD_{c-o} < OD \leq 4 \times OD_{c-o}$) and strongly adherent ($4 \times OD_{c-o}) \leq OD$.

Analyses of variance to assess the biofilm-forming ability classification and the biofilm-forming ability of *L. monocytogenes* according to the corresponding serogroup, were performed with the SPSS statistics software v.21.0 and p values <0.05 were considered to be significant.

III. RESULTS AND DISCUSSION

The assessed strains revealed a crystal violet OD_{600nm} (cvOD) ranging from 0.067 (±0.023) to 0.265 (±0.004) units after 5 days of growth in the polystyrene microtiter wells. These values were all below the one observed for the positive control strain - CECT 911 - that exhibited an average cvOD of 0.285 (±0.003). When the biofilm-forming ability was assessed using total viable counts, these ranged from 6.81 (±0.080) to 8.68 (±0.042) log cfu/ml.

According to Stepanovic *et al.* (2004) classification, 32% (n=6) of the strains revealed a weak biofilm-forming ability, 47% (n=9) were moderate biofilm-formers and 21% (n=4) were strong biofilm producers, exhibiting significantly different degrees of biofilm-forming ability (p=0.000) based on their OD values.

Table 1. Biofilm-forming ability classification of the tested *L. monocytogenes* strains and corresponding log cfu/ml and cvOD of 5-day-old biofilms

Biofilm-forming ability	n	cvOD (mean ± SD)	Log cfu/ml (mean ± SD)
Weak	6	0.08 ±0.02 [a]	7.65 ±0.47 [a]
Moderate	9	0.13 ±0.03 [b]	8.03 ±0.51 [bc]
Strong	4	0.20 ±0.04 [c]	7.95 ±0.52 [ac]
Sig.		p=0.000	p=0.05

[a, b, c] - mean values with different letters are significantly different .

However, when assessing the biofilm-forming ability based on log cfu/ml, the weak biofilm-forming group is significantly different (p=0.05) from the moderate group, but cannot be differentiated from the strong biofilm-forming group (Table 1). Stepanovic *et al.* (2004) classification for biofilm-forming ability is based on crystal violet OD values, but in our results these are not coincident with log cfu/ml values.

This might be due to the nature of each determination, because while crystal violet stain measures total biomass (live and dead cells and extracellular matrix), in total viable cell count only live cells are considered (Kwasny & Opperman, 2010).

When relating the biofilm-forming capacity with the serogroups presented by each isolate, no significant differences were found in log cfu/ml values (p=0.414).

Table 2 – Biofilm-forming ability of the assessed *L. monocytogenes* strains and corresponding serogroup

Serogroup	n	cvOD (mean ± SD)	Log cfu/ml (mean ± SD)
IIa (n=5)	5	0.13 ± 0.03 [a]	7.83 ± 0.56
IIb (n=5)	5	0.11 ± 0.42 [a]	7.82 ± 0.45
IIc (n=2)	2	0.19 ± 0.08 [b]	8.20 ± 0.17
IVa (n=2)	2	0.09 ± 0.01 [a]	7.70 ± 0.46
IVb (n=5)	5	0.15 ± 0.06 [b]	7.99 ± 0.64
Sig.		p=0.001	p=0.414

[a, b, c] - mean values with different letters are significantly different .

But if the cvOD is used as an indicator of the biofilm-forming ability, significant differences were found between serogroups IIa, IIb and IVa strains and serogroups IIc and IVb that exhibited a significantly higher ability to form biofilm (p=0.001).

IV. CONCLUSION

All of the assessed *L. monocytogenes* strains formed biofilm in 5 days, even though none of those strains revealed a biofilm-forming ability comparable to the positive control used – *L. monocytogenes* CECT 911. The majority of the assessed strains were moderate biofilm-formers and only 21% revealed a strong biofilm-forming capacity.

Isolates from serogroups IIc and IVb revealed a significantly higher ability to form biofilm, compared with serogroups IIa, IIb and IVa strains.

Serogroup IVb strains presenting high biofilm-forming ability are worrisome, because this is the serogroup more associated with human listeriosis. A better knowledge about the way this pathogen attaches to food-related environments and persists in ready-to-eat foods, like meat delicatessen, is needed in order to reduce the consumer's potential risk to acquire foodborne listeriosis.

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