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Comparison of biofilm formation between non-pathogenic *Listeria* strains under different stress conditions

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ABSTRACT

Micro-organisms can attach to food surfaces and develop biofilms which present a concern in food and environmental safety. The main goal of the current study was to investigate the biofilm formation of six non-pathogenic *Listeria* strains under different stress conditions using a microplate assay. The effect of the weak biofilm-forming non-pathogenic *Listeria* strains on the biofilm formation of a strong biofilm-forming pathogenic *Listeria* strain (*Listeria monocytogenes* #8) was also examined. *Listeria innocua* CCM4030, *Listeria innocua* 2885 and *Listeria seeligeri/welshimeri* 292 showed the same patterns of biofilm formation with increasing NaCl concentrations from 0.05 to 15%, but all the other strains showed a continuously decreasing trend of OD_{595} in the same conditions. This study showed that in the case of non-pathogenic *Listeria* strains, higher concentrations of NaCl do not present a stress condition that enhances biofilm formation. Decrease in pH inhibited biofilm formation for all the non-pathogenic *Listeria* strains. The weak biofilm forming non-pathogenic *Listeria* strains (*Listeria innocua* 2885 and *Listeria innocua* CCM4030) overgrew the strong biofilm-forming *Listeria* strain (*Listeria monocytogenes* #8) during biofilm formation. This phenomenon could be beneficial and potentially be used as a novel control strategy to prevent the colonization of the pathogenic *Listeria* at food processing facilities such as in meat industry.

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INTRODUCTION

Food-borne diseases present a major problem throughout the world causing thousands of deaths each year from the consumption of food and water contaminated with toxins and pathogens. Contamination of food can occur at any point in the food chain, from the raw material to the consumers' table. Pathogens can enter and contaminate the food system through humans, animals, air, water, soil, and contaminated equipment. The increased level of knowledge on how, where, and when the contamination of food happens and how preventive measures should be applied to present a necessity for the food safety of the products we consume in our everyday life (Zaukuu et al., 2016).

Listeria species are widespread in the environment including in soil, raw foods, stream water, silage, sewage, plants, and animals. However, *Listeria monocytogenes* is a ubiquitous pathogen that can cause infections in humans, thus representing a major concern for public health and economic aspect. *Listeria* species commonly colonize the food processing environment and ready-to-eat products. Ready-to-eat foods (RTE) are products consumed without any heat-treatment and are usually associated with listeriosis outbreaks (Martín et al., 2014; Rückerl et al., 2014). Novel nonthermal decontamination technologies, like high hydrostatic pressure treatment, have been recognized to have a positive effect on *Listeria monocytogenes* inactivation and preserve the quality attributes of RTE foods (Bajovic et al., 2012; Dalmadi et al., 2007).

However, elimination of this bacterium from ready-to-eat foods and food processing equipment is difficult. The main reason is because of the ability of this bacterium to form biofilms that protect it from stresses in food processing environments. The difficulty of biofilm removal is also due to the increased resistance against disinfectants caused by factors such as the age of biofilm and different stress responses (Di Ciccio et al., 2012; Van Houdt and Michiels, 2010). Environmental factors, including temperature, sugar, salt, pH, and nutrients, have been demonstrated to have impacts on the adhesion and biofilm formation of Listeria species in food (Renner and Weibel, 2011). To understand the susceptibility of *Listeria* species biofilms to different stress conditions, six biofilm-forming non-pathogenic *Listeria* strains were analyzed. The effect of the weak biofilm forming non-pathogenic *Listeria* strains on the biofilm formation of the strong biofilm-forming *Listeria monocytogenes #8* was also examined. There are knowledge-gaps in literature for this particular topic, therefore the importance of this study lies in the possibility to develop new methods to control biofilm formation in the food industry and increase the knowledge on the role of the harmless bacteria in the food safety.

MATERIALS AND METHODS

Culture preparation

A total of 7 previously identified *Listeria* strains were isolated from various sources and included in this study (Table 1). All the isolates were obtained in a previous study at the Department of Microbiology and Biotechnology, Faculty of Food Science, Szent István University, Budapest,

Table 1. Listeria species used in this study

Species	Strains
Listeria innocua	CCM4030
Listeria innocua	2885
Listeria seeligeri/welshimeri	292
Listeria welshimeri	CCM3971
Listeria ivanovii	204
Listeria denitrificans	1157
Listeria monocytogenes	#8

Hungary. The different bacteria strains were stored at $-80\ ^\circ C$ in a mixture of tryptic soy broth (TSB) and glycerol. They were later recovered on Brain Heart Infusion (BHI) Agar, cultivated at 37 °C for 24–48 h and then streaked onto Trypto-Casein Soy Agar (TSA) before cultivating again at 37 °C for 24 h.

Culture media

Components of M9 Minimal Media (NH_4Cl [1.9 mM], Na_2HPO_4 [42.3 mM], KH_2PO_4 [22 mM], NaCl [8.56 mM], $MgSO_4$ [2 mM], $CaCl_2$ [0.1 mM], and glucose [10 mM]), Brain Heart Infusion Agar and Trypto-Casein Soy Agar were obtained from Sigma Aldrich (Schnelldorf, Germany); tryptic soy broth and Muller-Hinton Agar was obtained from Lab M (England).

Microtiter plate biofilm formation assay

Biofilm formation was evaluated based on the measurement of the optical density (OD) of biofilms which is in correlation with the biofilm mass developed in microtiter plate wells, after crystal violet staining (Bakke et al., 2001; Djordjevic et al., 2002). The main aim of this investigation was to test the capability of *Listeria* strains to form biofilms in different stress conditions using the microtiter plate assay (Mouwakeh et al., 2019). For this purpose, Minimal Media M9 was used to assess the biofilm formation of *Listeria* strains under different sodium chloride (NaCl) concentrations (0.05, 5, 10, and 15%) and pH values of 4, 5, and 6 respectively. M9 Minimal media contained water, inorganic salts, and glucose as the sole carbon source. Constant pH of 4, 5 and 6 were obtained by adding hydrochloric acid (HCl 37%, Sigma Aldrich, USA) or sodium hydroxide (NaOH, Sigma Aldrich, USA) to the M9 Minimal Media. The first step of the microtiter plate assay was the preparation of an overnight culture of *Listeria* strains on Muller-Hinton Agar media. The next step was the adjustment of the OD_{600} of *Listeria* strains to 0.3 in tubes with Minimal Media M9 using a McFarland densitometer. After that, 200 μL of the adjusted strain in M9 was added to each well in the same row of the 96 wells microtiter plates. Similarly, in case of mixed-biofilm experiment, 100 μL of the adjusted *Listeria* pathogenic strain and 100 μL of the adjusted *Listeria* non-pathogenic strain were added to the wells of the microtiter plates. They were then incubated at 37 °C for 72 h.

The Crystal Violet method was applied for the detection of the total biofilms formed (Mouwakeh et al., 2019). For this purpose, the supernatant of the 96-wells plate was firstly removed and then each well was washed three times with 200 μL of phosphate-buffered saline (PBS) before discarding. The formed biomass was stained by adding the Crystal Violet 0.4% [v/v] to each well and kept at

room temperature for 15 min. After that, the microtiter plate washing step with PBS was applied again as before and then the plates were dried for 15 min in a sterile air flow hood. Once dried, 200 μL of acetic acid 33% [v/v] were added to each well followed by the incubation at room temperature for 15 min. The samples from each well (100 μL) were then transferred to a sterile microtiter plate and the OD level of the crystal violet present in the solution was measured in a Multiskan Ascent (ThermoLab System) Plate Reader at 595 nm. All experiments were carried out in triplicate.

Statistical analysis

The significances of the differences between the groups were verified by one-way ANOVA, and the mean comparison were performed by Tukey's test using SPSS-20 software (SPSS Inc., IBM Company, USA). The results were considered significant when $P \leq 0.05$.

RESULTS AND DISCUSSION

From literature, Listeria strains prefer different temperatures and media when forming biofilms (Pan et al., 2010). However, there is a deficit of studies in the literature showing the biofilm formation of non-pathogenic *Listeria* strains. According to our results, *Listeria ivanovii* 204 formed larger quantities of biofilms than the other non-pathogenic *Listeria* strains when incubated in M9 Minimal Media at 0.05% NaCl concentration (without adjusting NaCl concentration) at temperature 37 °C (Fig. 1). Nevertheless, it can be concluded that most of the studied non-pathogenic *Listeria* strains formed lower amounts of biofilms at the mentioned conditions compared to the pathogenic *Listeria monocytogenes* #8 (Fig. 3).

From Fig. 1, the OD_{595} of the *Listeria ivanovii* 204 incubated in M9 Minimal Media at 37 °C with increasing NaCl concentration from 0.05 to 10% dropped faster from 0.145 to 0.090 respectively, probably because the bacteria growth was inhibited. When NaCl concentration is adjusted to 15%, biofilm formation of the *Listeria ivanovii* 204 did not decrease any further. With the exception of *Listeria seeligeri/welshimeri* 292 ($P \leq 0.05$), all the other strains in the study showed no significant differences of OD_{595} values at 10 and 15% NaCl concentrations.

Similar patterns of biofilm formation (OD_{595}) were observed in *Listeria innocua* CCM4030, *Listeria innocua* 2885, *and Listeria seeligeri/welshimeri* 292 when grown on 0.05–15% NaCl
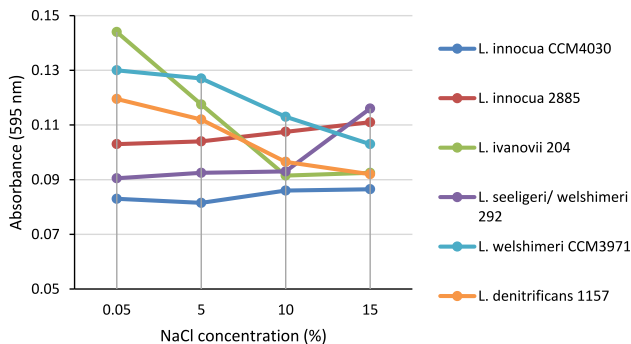

Fig. 1. Biofilm formation (OD_{595}) comparison between the studied *Listeria strains* following 72 h incubation at 37 °C under different NaCl concentrations (0.05, 5, 10, and 15%) using M9 Minimal Media

concentrations. A continuously decreasing trend of OD_{595} was detected for all the other strains, indicating that more biofilm was formed at 0.05 and 5% than at 10 and 15% NaCl concentrations. From these results, it can be concluded that the higher concentrations of NaCl did not present stress conditions that enhanced biofilm formation from non-pathogenic *Listeria* strains. Similar results were obtained when biofilm formation of *Listeria monocytogenes* strains in different NaCl concentrations were studied (Pan et al., 2010; Xu et al., 2010).

From Fig. 2, all the *Listeria* strains incubated in M9 Minimal Media at 37 °C at different pH values (4, 5, and 6) showed a continuously decreasing trend of OD_{595}, except *Listeria ivanovii* 204. Therefore, more biofilm amounts were detected at pH of 6 than at pH of 4 and 5, respectively, which proves the inhibition effect of acidic conditions for the biofilm formation of *Listeria* strains.

The lowest amounts of biofilms were observed at pH 5 compared to pH 6 in M9 Minimal Media for all the strains ($P \leq 0.05$). At pH 4, *Listeria ivanovii* 204 and *Listeria denitrificans* 1157 were found to produce the most biofilm thus showing resistance to low pH. The most sensitive strain at pH 4 was *Listeria welshimeri* CCM3971 that showed weak biofilm formation. At pH = 5 the lowest amounts of biofilm formed were observed from *Listeria ivanovi* 204 and *Listeria innocua* CCM4030, these were the weakest biofilms forming strains.

The biofilm formation (OD 595) from strong biofilm-forming strain *Listeria monocytogenes* #8 and its interaction with the weak biofilm forming non-pathogenic *Listeria* strains, were also studied. Significantly, ($P \leq 0.05$) more biomass was produced by *Listeria monocytogenes* #8 ($OD_{595} = 0.251 \pm 0.015$) compared to *Listeria innocua* 2885 ($OD_{595} = 0.185 \pm 0.02$) and *Listeria innocua* CCM4030 strains ($OD_{595} = 0.182 \pm 0.01$). The OD_{595} values of the biofilms formed when *Listeria monocytogenes* #8 was mixed with the non-pathogenic *Listeria* strains were significantly ($P \leq 0.05$) less than that of the biofilms formed by *Listeria monocytogenes* #8 only (Fig. 3). Biofilm formation of the strong biofilm-forming *Listeria monocytogenes* #8 was overgrown when mixed with the weak biofilm-forming *Listeria innocua* 2885 strain among the conditions described above. The same results were also obtained when *Listeria monocytogenes* #8 was grown in presence of *Listeria innocua* CCM4030, thus reducing its biofilm formation. From Fig. 3, the OD 595 values for mixed-biofilm *Listeria monocytogenes* #8 with *Listeria innocua* 2885 strain were significantly ($P \leq 0.05$) lower compared to mixed-biofilm *Listeria monocytogenes* #8 with *Listeria innocua* CCM4030 strain.

The same inhibition phenomenon was obtained from the studies of Noorwood and Gilmour (2001) and Rodríguez-López et al. (2015) where a decrease in *Listeria monocytogenes*
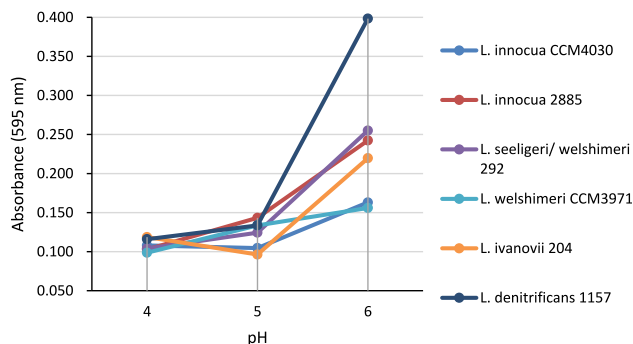

Fig. 2. Biofilm formation (OD_{595}) comparison between the studied *Listeria strains* following 72 h incubation at 37 °C under different pH values (4, 5 and 6) using M9 Minimal Media
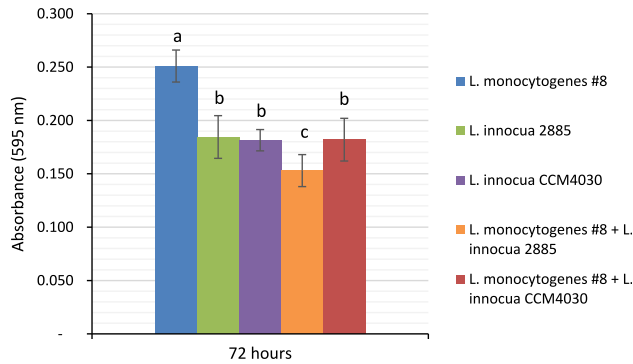
Fig. 3. Biofilm formation of *Listeria monocytogenes* #8 and its mixed biofilm formation with *Listeria innocua* 2885 and *Listeria innocua* CCM4030 following 72 h incubation at 37° C using M9 Minimal Media. Error bars represent the standard deviations of the means, from three individual measurements. Different letters above the bars indicate a significant difference ($P \leq 0.05$)

cell numbers was observed in multispecies biofilms formed with *Staphylococcus xylosus*, *Pseudomonas fragi*, and *Carnobacterium divergens* comparing with *Listeria monocytogenes* cell numbers grown in monocultures. Habimana et al. (2011) obtained similar results on prevention of *Listeria monocytogenes* subsequent biofilm formation by the presence of a technological flora resident namely *Lactoccocus lactis*, due to competition for adhesion sites. The studies of Zhao et al. (2006, 2013) have shown promising results on the use of *Lactoccocus lactis* against *Listeria monocytogenes* biofilm in food facility-testing. Contrarily, other studies showed a strong effect that the microorganisms present in food facilities can play on the number of adhered cells of *Listeria monocytogenes* in the biofilm. This can correspond to either mixed biofilm enhancement or inhibition (Carpentier and Chassaing, 2004; Fox et al., 2014). Nevertheless, a recent study by Tan et al. (2019) proved that the composition and diversity of microbiota from food processing surfaces indicates the persistence of the *Listeria monocytogenes*. This reiterates the need for further investigation on microbiota characterization particularly, on the role of the harmless bacteria in the food safety.

CONCLUSIONS

After studying growth conditions of non-pathogenic *Listeria* strains, higher concentrations of sodium chloride did not present a stress condition that enhanced biofilm formation. Decrease in pH however, exhibited some inhibition effect for biofilm formation in all of the non-pathogenic *Listeria* strains. The results also showed that the biofilm formation of the strong biofilm-forming *Listeria monocytogenes* #8 was overgrown when mixed with the weak biofilm-forming *Listeria* strains. Biofilm inhibition was 17.5 and 39.1% for *Listeria monocytogenes* #8, when it was grown in the presence of *Listeria innocua* CCM4030 or *Listeria innocua* 2885 non-pathogenic strains. The future perspective of these findings is the use of potential non-pathogenic strains of *Listeria* as biocontrol in the food industry. Therefore, biofilm formation of the pathogenic *Listeria* could be inhibited by the application of non-pathogenic strains on the food processing facilities as a

part of sanitation procedures. However, further investigation on the biofilm formation of *Listeria* strains and their interaction under different environmental conditions is still necessary. Their antagonistic effects could provide more information on conditions that may inhibit biofilm formation and could further be used to control the production of *Listeria monocytogenes* biofilms in the food industry.

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