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

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Abstract

Micro-organisms attach to surfaces and develop biofilms which are a concern in food and environmental safety. The main goal of the current study was to investigate the biofilm formation by 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 (*L. monocytogenes #8*) was also examined. While *L. innocua CCM4030* and *L. seeligeri/welshimeri 292* showed same patterns of biofilm formation with just slightly increase of OD 595 when grown on 0.05 to 15 % NaCl concentrations, all the other strains have showed a continuously decreasing trend of OD 595. This study showed that in case of non-pathogenic *Listeria* strains, higher concentrations of NaCl do not present a stress condition that enhance the biofilm forming ability. Nonetheless the decrease of pH showed to be an inhibition effect for biofilm formation of all the non-pathogenic *Listeria* strains. Our results showed also that the weak biofilm forming non-pathogenic *Listeria* strains (*L. innocua 2885 and L. innocua CCM4030)* may overgrow the strong biofilm forming *Listeria* strain (*L. monocytogenes #8*) during biofilm formation. This phenomenon could be beneficial for the food industry, since *L. innocua* is not a human pathogenic bacterium.

Keywords: *Listeria* species, biofilms, stress condition

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 toxin and pathogens. Contamination of food can occur at any point of the food chain, from the raw material to the consumer 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 present a necessity for the food safety of the products we consume in our everyday life.

Listeria species are widespread in the environment including in soil, raw foods, stream water, silage, sewage, plants, and animals. However, *L. monocytogenes* is a ubiquitous pathogen that can cause infections in humans, thus representing a major concern for the public health and economical aspect. *Listeria* species commonly colonize the food processing environment and ready-to-eat products. Ready-to-eat foods are products consumed without any heat-treatment and usually are associated with listeriosis outbreaks (Martin et.al., 2014, Rückerl et.al., 2014).
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 protects 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 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 that are common in foods and food-processing environments, have been demonstrated to have impacts on the adhesion and biofilm formation of *Listeria* species (Renner and Weibel, 2011). To understand the susceptibility of *Listeria* species biofilms to different stress conditions, six biofilm forming non-pathogenic *Listeria* strains were analysed. The effect of the weak biofilm forming non-pathogenic *Listeria* strains on the biofilm formation of the strong biofilm forming *L. 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 the biofilm formation in the food industry and increasing the knowledge on the role of the harmless bacteria in the food safety.

Materials and Methods

A total of 7 previously characterized and serotyped *Listeria* strains isolated from various sources were included in this study (Table 1). All the isolates were obtained in a previous study in the Department of Microbiology and Biotechnology of the Szent István University. The different bacterial strains were stored at - 4 °C in a mixture of TSB broth and glycerol, which were then recovered on Brain Heart Infusion (BHI) Agar, cultivated at 37 °C for 24-48 hours and then streaked onto Trypto-Casein Soy Agar (TSA), cultivated at 37 °C for 24 hours.

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*

The main aim of this investigation was to test the capability of *Listeria* strains to form biofilms using the microtiter plate essay. 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. M9 Minimal media contained inorganic salts, a carbon source and water. After that, 200 μL of the adjusted strain was added to each well of the same row of the 96 wells microtiter plate, which was incubated at 37 °C for 48/72 hours.

Then the absorbance was measured in the Multiskan Ascent (ThermoLab System) Plate Reader at 595 nm using the Crystal Violet method. The OD 595 results were imported to Microsoft Office Excel software and the average values and standard deviations from the three biofilm assays were calculated for each *Listeria* strain.

Results and discussion

According to similar studies different 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, L. ivanovii 204 and *L. innocua CCM4030* formed bigger amounts of biofilms than the other *Listeria* strains when incubated in M9 Minimal Media at temperature 37 °C. Nevertheless, we can still conclude that most non-pathogenic *Listeria* strains analysed formed relatively good amounts of biofilms.

According to Figure 1, the OD_{595} reading of the *L. ivanovii 204* incubated in M9 Minimal Media at 37 °C with NaCl concentration adjusted from 0.05 to 10%, OD_{595} drops faster from 0.145 to 0.090 respectively, probably because the growth of bacteria was inhibited. Whereas when NaCl concentration is adjusted to 15%, biofilm formation didn't decrease more. Same happened with all the other analysed strains except *L. seeligeri/welshimeri 292*, that showed similar OD_{595} at 10 and 15% NaCl concentration**.**

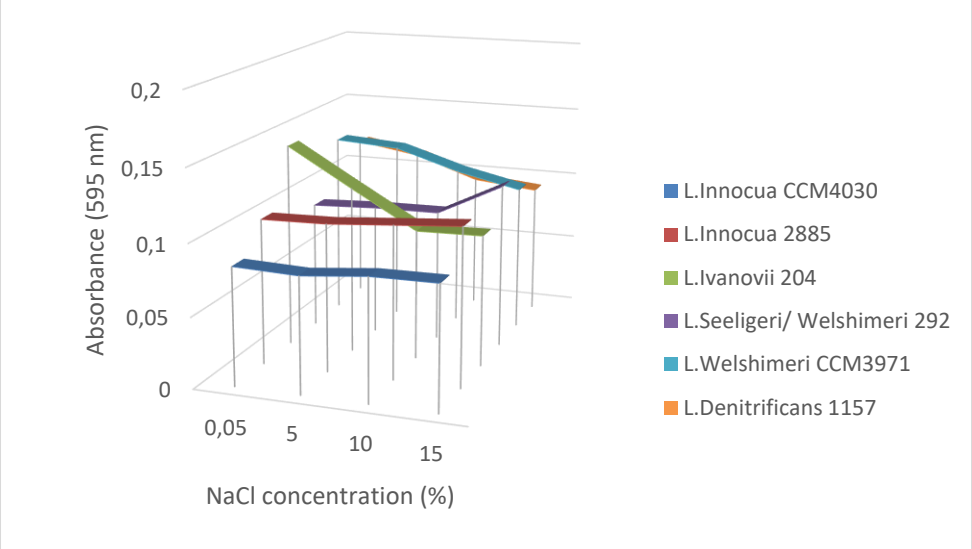

Figure 1. Comparison of biofilm formation (OD_{595}) between different *Listeria strains* (*L. innocua CCM4030, L. innocua 2885, L. seeligeri/welshimeri 292, L. welshimeri CCM3971, L. ivanovii 204 and L. denitrificans 1157)* under different NaCl concentrations (0.05, 5, 10 and 15%).

While *L. innocua CCM4030, L. innocua 2885 and L. seeligeri/welshimeri 292* showed same patterns of biofilm formation with just slightly increase of OD_{595} when grown on 0.05 to 15 % NaCl concentrations, all the other strains have showed a continuously decreasing trend of OD_{595} with more biofilm formed at 0.05 and 5% than at 10 and 15% NaCl concentrations. From these results we can conclude that the higher concentrations of sodium chloride didn't present a stress condition that enhance the biofilm formation of non-pathogenic *Listeria* strains. Similar outcomes were obtained also in other studies of biofilm formation of *L. monocytogenes* strains in different sodium chloride concentrations (Pan et.al., 2010, Xu et.al., 2010).

According to Figure 2, all the analysed *Listeria* strains except *L. ivanovii 204* incubated in M9 Minimal Media at 37 °C at different pH values (4, 5, and 6) showed a continuously decreasing

trend of OD_{595}. Therefore, more biofilm amounts were detected in pH of 6 than at pH of 4 and 5, that proves the inhibition effect of acidic conditions in biofilm formation of *Listeria* strains.

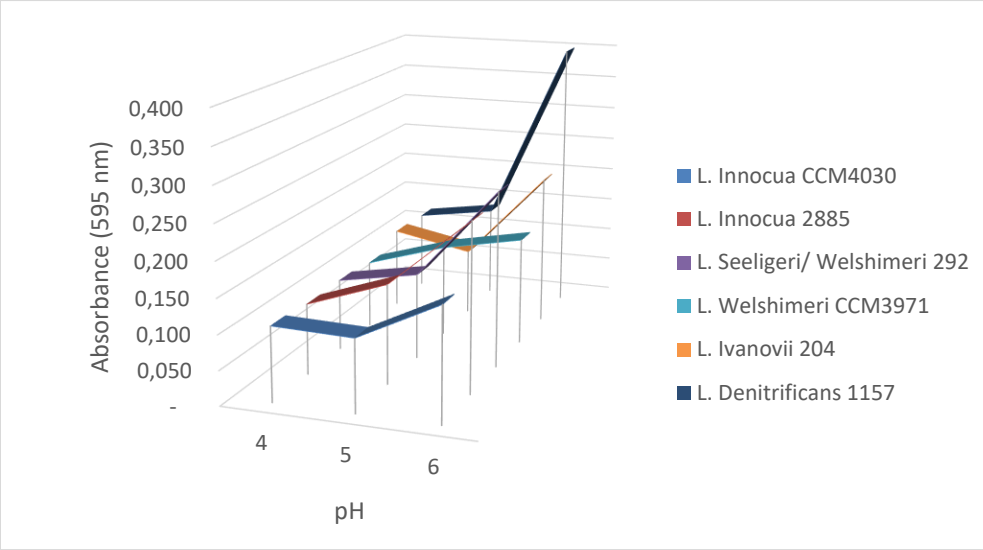

Figure 2. Comparison of biofilm formation (OD_{595}) between different Listeria strains (*L. innocua CCM4030, L. innocua 2885, L. seeligeri/welshimeri 292, L. welshimeri CCM3971, L. ivanovii 204 and L. denitrificans 1157)* under different pH values (4, 5 and 6).

The biggest reduced amounts of biofilm were detected after lowering the pH from 6 to 5 in all the analysed strains. Whereas at pH 4, *L. ivanovii 204* and *L. denitrificans 1157* found to be the biggest biofilm forming strains showing resistance to low pH, the most sensitive strain at pH 4 was *L. welshimeri CCM3971* showing low amounts of biofilm formation. Similar amounts of biofilm formation were obtained also from one of the *L. innocua* strains, namely *L. innocua 2885* as one of the weakest biofilms forming among the non-pathogenic *Listeria* strains.

The biofilm formation (OD 595) from strong biofilm forming strain *L. monocytogenes #8* and its interaction with the weak biofilm forming non-pathogenic Listeria strains (*L. innocua CCM4030 and L. innocua 2885*) was also measured and the standard deviations from the obtained results were calculated. According to our results, the weak biofilm forming *L. innocua 2885* strain overgrow the strong biofilm forming *L. monocytogenes #8* strain during biofilm formation at conditions. Same results were obtained also when *L. innocua CCM4030 was grown* in presence of *L. monocytogenes #8*, thus reducing the biofilm formation but in a lower rate.

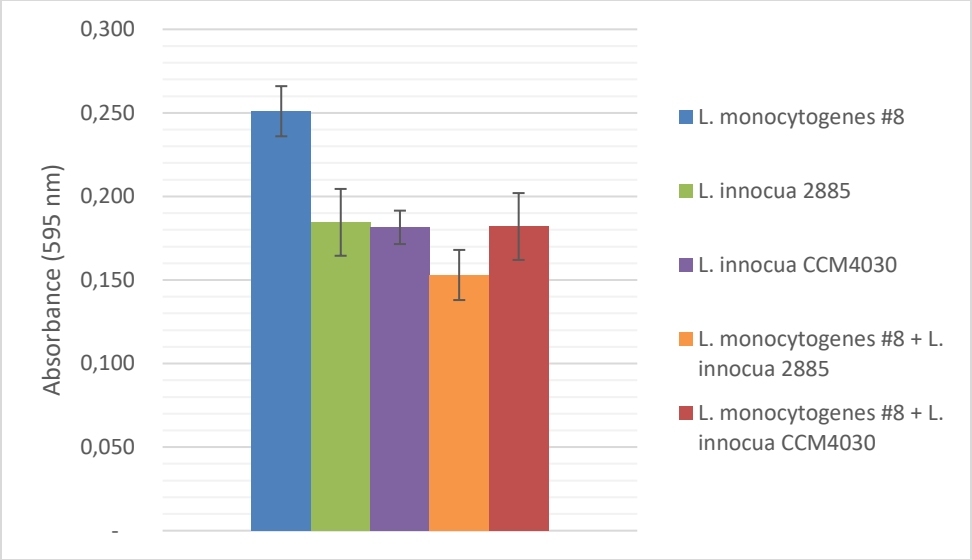

Figure 3. Biofilm formation of *L. monocytogenes #8* and its mixed biofilm formation with *L. innocua 2885* and *L. innocua CCM4030* under temperature 37°C using M9 Minimal Media. The results in the figure below are based on the means of OD595 from three individual biofilm assays. Errors bars represent the standard deviations of the means.

Same inhibition phenomenon was obtained from the study of Noorwood and Gilmour (2001), where a decrease in *L. monocytogenes* cell numbers was observed in multispecies biofilms formed with *Staphylococcus xylosus* and *Psudomonas fragi*, comparing with *L. monocytogenes* cell numbers grown in monocultures. However, different outcomes were observed in another study that showed the strong effect that can play the "house flora" on the number of adhered cells of *L. monocytogenes* in the corresponding mixed biofilm, either enhancing or inhibiting it (Carpenitier and Chassaing, 2004). Nevertheless, the most recent study conducted by Tan et.al., (2019) confirms the outcome of our study and shows that the composition and the diversity of microbiota of food processing surfaces indicates the persistence of the *L. monocytogenes* thus showing the role of the harmless bacteria in the food safety.

Conclusions

According to our study in case with the non-pathogenic *Listeria* strains, higher concentrations of sodium chloride did not present a stress condition that enhance the biofilm forming ability. Nonetheless the decrease of pH showed to be an inhibition effect for biofilm formation of all the non-pathogenic *Listeria* strains which formed lower amounts of biofilms. The results showed also that the weak biofilm forming *Listeria* strain may overgrow the strong biofilm forming *Listeria*

strain during biofilm formation. In our case of *L. monocytogenes #8, when it was grown* in presence of *L. innocua CCM4030* or *L. innocua 2885* non-pathogenic strains, the range of biofilm inhibition was 17.5 and 39.1 %, compared with the amount of biofilm formed from *L. monocytogenes #8* alone. The future perspective of these findings is the use of some of the non-pathogenic strains of *Listeria* as biocontrol in the food industry. Therefore, the pathogenic *Listeria* could be suppressed thus reducing food-safety risks with the application of non-pathogenic on the food surfaces of the equipment. However, further investigation on the biofilm formation of *Listeria* strains and their interaction under different environmental conditions and antagonistic effects between them is still necessary to provide more information on conditions that may inhibit biofilm formation and could be used to control the production of biofilms in the food industry.

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