THESIS OF THE PHD DISSERTATION

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Budapest,

2025

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AGRICULTURE AND LIFE SCIENCES

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BIOFILM FORMATION OF *LISTERIA* SPECIES UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

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Thesis of Ph.D. dissertation

Budapest, 2025

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1. INTRODUCTION AND OBJECTIVES

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 pathogens. The most known organisms linked with these diseases include *Listeria monocytogenes*, *Salmonella* spp., and *Escherichia coli*.

Listeria species are widespread in the environment including in soil, raw foods, stream water, silage, sewage, plants, and animals. The European Food Safety Authority reported 2536 confirmed human cases and 247 deaths in 2016, and 1887 confirmed cases with 166 deaths were reported in 2020. *Listeria* species also commonly colonize the food processing environment and ready-to-eat products. Ready-to-eat foods are products consumed without any heat-treatment and often are associated with listeriosis outbreaks. *Listeria* species can colonize and persist in specific ecological niches within the food processing environment. A variety of hurdles such as heat treatment, disinfection, reduction of water activity, reduction of pH, or removal of oxygen, can be used to prevent or eliminate pathogens from foods. The response of foodborne pathogens to these stresses is a major concern because

it can result in an enhanced survival and enhanced virulence of the pathogen.

Elimination of this bacterium from ready-to-eat foods and food-processing equipment is difficult because they adhere and grow on food surfaces such as stainless steel, rubber, glass, and polystyrene. The main reason is because of the ability of this bacterium to form biofilms that protects it from stresses in food-processing environments, which can lead to reduced shelf-life of food as well as significant food safety hazards.

To understand the susceptibility of *Listeria* species biofilms to different stress conditions, fifteen biofilm forming *Listeria* strains were analyzed. Five of the chosen strains were non-pathogenic strains and ten of them were pathogenic (*L. monocytogenes* strains and *L. ivanovii*) which were taken to compare the difference of the susceptibility between the pathogenic and non-pathogenic strains to different stress conditions (applied separately and in combination) when grown under biofilm conditions.

Different treatments of chlorine, balsamic vinegar, acetic acid, white vinegar, and lactic acid were evaluated to determine the role of those solutions at the stage of washing lettuce to remove *L. monocytogenes.*

OBJECTIVES:

The overall objective of my research was to better understand the influence of different environmental conditions on the biofilm formation of different *Listeria* species and to investigate the efficiency of some antimicrobial compounds against *L. monocytogenes* present in fresh produce (lettuce).

The specific objectives of my research are:

- to analyze the biofilm formation ability of different *Listeria* species,
- to investigate the differences on biofilm formation of different *L. monocytogenes* strains under different stress conditions,
- to compare the effect of stress conditions on biofilm formation of *L. monocytogenes* strains (NCAIM B1454, 3b T1, CCM5576, CCM7202, NCAIM B01966T, 11/4.12t03, 10887 ½ a, CCM 4699, and 5105 3a),
- to examine the efficacy of chlorine, acetic acid, balsamic vinegar, white vinegar, and lactic acid compounds on the decline of biofilms of *L. monocytogenes* strains on the surface of lettuce.

2. MATERIALS AND METHODS

Fifteen *Listeria strains* were selected for this study (table 1,2), obtained from the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary and further maintained at the Department of Food Microbiology, Hygiene and Safety of the Hungarian University of Agriculture and Life Sciences. The cultures, which were derived from frozen stocks (−80 °C), were cultivated on Tryptic Soy Agar plates and incubated at 37 °C for 24 hours.

Table 1. Non-*monocytogenes Listeria* species used in this study

Species	Isolate ID	Origin
L. innocua	CCM4030^{T}	cow brain
L. innocua	2885	unknown
L. seeligeri/welshimeri	292	unknown
L. welshimeri	CCM3971^{T}	decaying
L. ivanovii	204	vegetation
L. denitrificans	1157	unknown

Table 2. *Listeria monocytogenes* strains used in this study

Notation	Species	Isolate ID	Origin
1	*L. monocytogenes*	NCAIM B1454	unknown
2	*L. monocytogenes*	3b T1	unknown
3	*L. monocytogenes*	11/4.12$t_0$3	isolated from cheese
4	*L. monocytogenes*	CCM 5576	guinea pig
5	*L. monocytogenes*	CCM 7202	spinal fluid of a child
6	*L. monocytogenes*	NCAIM B1966	guinea pig
7	*L. monocytogenes*	NCTC 10887	chinchilla
8	*L. monocytogenes*	NCTC 5105	human
9	*L. monocytogenes*	CCM 4699	sheep

1. The effect of pH, sodium chloride, and temperature on the biofilm formation of *Listeria* strains

Microtiter plate biofilm production assay

a. The biofilm forming ability of the strains was investigated using the crystal violet 0.4% (w/v) staining method by Mouwakeh (2018). Biofilms were grown in ELISA titer plates with a final volume of 200 µL of M9 minimal media. Initial cell counts were adjusted to an OD of 0.3 ($\sim 10^7$ cells mL^{-1}) using a DEN-1B McFarland densitometer (Biosan).

b. To study the effect of different stress factors on the biofilm formation of *Listeria* strains, the composition of M9 minimal media was altered. Three factors were tested at multiple levels (separately and in combination): (i) NaCl addition (0%, 5%, 10%, or 15%), (ii) the effect of pH (pH 4, 5, and 6), and (iii) the influence of temperature (1 °C, 4 °C, 20 °C, and 37 °C). Plates were incubated for 7 days at 1 °C or 4 °C and for 48 h at 20 °C and 37 °C.

c. Supernatants from the wells were discarded. Each well was washed three times with phosphate-buffered-saline (PBS) solution followed by 15 min air drying in a laminar flow hood.

d. Finally, each well was stained with 200 µL Crystal Violet 0.4% (w/v) solution in ethanol. The plates were incubated for 15min at room temperature, washed three times with PBS solution, then air dried for 15 min under a laminar flow hood. Then 200 µL of acetic acid of 33% (v/v) was added to each well, and OD readings were carried out at 595 nm using a microtiter plate reader (Multiscan Ascent, ThermoLab System) controlled via Ascent Software Version 2.6 (ThermoLabsystems).

2. Effect of sub-lethal temperature on the cells of *L. monocytogenes* biofilms

Nine *Listeria monocytogenes* isolates (NCAIMB1454, 3b T1, 11/4.12$t_0$3, CCM5576, CCM7202, NCAIM B1966, NCTC 10887, NCTC 5105, CCM 4699) were selected for this experiment.

We used the same protocol for the determination of the effect of pH and sodium chloride. Shortly, fresh cultures were prepared in Trypto-Casein Soy Broth (TSB), cultivated at 37 °C for 24 hours. Biofilm formation was investigated using the crystal violet 0.4% (w/v) staining method. Laboratory-generated biofilms were grown in an ELISA titer plate with a final volume of 200 µL of M9 minimal media. Initial cell-counts were adjusted to an optical density of 0.3 using a McFarland densitometer (Biosan).

To study the behavior of *Listeria monocytogenes* biofilm cells when submitted to the sub-lethal temperature, after creating the biofilms and before reading the optical density, the cultures were placed in a thermal bath at 50 °C for 60 min.

Finally, in the last step, the optical density at 595 nm from the three biofilm assays was measured using a microtiter plate reader (Multiscan Ascent, ThermoLab System). Multiskan Ascent instrument was controlled through a computer using the Ascent Software Version 2.6 (ThermoLabsystems) and the heat-treated and non-heat-treated strains were compared regarding their biofilm formation ability.

3. The effectiveness of different antimicrobials on *L. monocytogenes* biofilms formed on the surface of lettuce leaves

For this experiment, two strains (selected based on previous experiments) of *L. monocytogenes* were used:

- NCAIM B1966: strain with strong biofilm forming capacity.
- CCM 5576: strain with weak biofilm forming capacity.

Experiments were performed to analyse the effect of some antimicrobials (industrial/natural)(chlorine, acetic acid, balsamic

vinegar, white vinegar, and lactic acid) against the growth of these two strains on the surface of lettuce leaves.

a. The experimental media (TSB) was inoculated with 1% (v/v) of an overnight *Listeria* culture. The lettuce leaves (1x1cm squares) were disinfected with alcohol (70%), and then they were dipped into the inoculated media and incubated for 1 hour at room temperature.

b. After 1 hour of incubation, both sides of the lettuce leaves were treated with one of the following antimicrobial compounds:

- chlorine (for 5 minutes),
- acetic acid or lactic acid (for 10min),
- undiluted balsamic vinegar or white vinegar (for 15min).

c. The lettuce leaves were rinsed with 10 ml of sterile distilled water. This was followed by mechanical removal of the attached cells using a vortex.

d. The biofilm formation was monitored by determining the attached cell number after 24h, and 48h of incubation. Then, serial ten-fold dilutions were done, and the cell number was determined by spread plate technique.

4. Data analyses

For data analyses, statistical software IBM SPSS Statistics (version 27) was used. One-factor analysis of variance (ANOVA) was used to determine statistically significant differences between the *L. monocytogenes* strains' biofilm formation ability after incubation at optimum environment (37 °C, pH=6, and 0 % NaCl).

Discriminant analysis was applied to conduct a multivariate analysis of variance test of the hypothesis that the 9 *L. monocytogenes* strains could be classified into 3 groups (weak, moderate, or strong biofilm former). The assumptions were tested as homogeneity of variances was not violated based on Levene Statistic=1.58; p=0.20, based on the Shapiro-Wilk's tests (test values>0.874; p>0.30) the normality assumption is satisfied across the replicates and in the dataset no outlier was detected using Z score values after standardization (values<2).

For further analysis, OD values at optimum environment were used as a positive controls and OD values recorded after the incubation at the most adverse tested environment (1 °C, pH=4, and 15 % NaCl) inferred as negative controls.

In further analyses, the average of the three measurements was taken and OD values based on the positive ($ODmax$ – average of positive control values) and negative ($ODmin$ – average of negative control values) controls were normalized. In that way the biofilm formation capacity (%) could be expressed (Eq. 1) as:

$$Biofilm\ formation\ capacity\ (\%) = \frac{OD_x - ODmin}{ODmax - ODmin} \quad (1)$$

Using the biofilm formation capacity (%) values, the responses of *L. monocytogenes* strains classified into different groups could be compared.

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 Past program. The results were considered significant when $P \leq 0.05$.

3. RESULTS AND DISCUSSION

Examining our strains in optimal conditions, we found that the 9 strains of *L. monocytogenes* could be divided into 3 groups: weak, moderate, and strong biofilm formers.

In general *L. monocytogenes* strains showed higher biofilm forming ability than other examined *Listeria* strains. All *L. monocytogenes* showed decreasing patterns of biofilm formation when the sodium chloride concentration increased from 0 to 15%, especially for the strong biofilm formers they were the most sensitive to the sodium chloride treatment. Same results obtained for the non-pathogenic *Listeria* strains, which higher concentrations of sodium chloride did not present a stress condition that enhance the biofilm forming ability.

Regarding the temperature effect, as it was expected only 1 and 4 °C had the most relevant effect in terms of inactivating the biofilm formation of *Listeria monocytogenes* strains. Also, we found that the strains which had originally lower biofilm-forming capacity were less sensitive to the temperature effect.

The decrease of pH showed an inhibition effect for biofilm formation of all the non-pathogenic *Listeria* strains which formed lower amounts of biofilms than most of pathogenic *L. monocytogenes* strains. However, we found that *L. monocytogenes* prefers to grow and form biofilms in an acidic environment especially for strains 6 and 8.

The combination of these treatments (15% NaCl concentration and low temperatures) resulted better inhibition of biofilm formation of *L. monocytogenes* compared to the results of individual stresses. We also found that the strong biofilm formers were more sensitive to all the treatments compared to the moderate or weak biofilm forming groups.

L. monocytogenes is tolerant to environmental stresses (applied individually or in combination). But we can conclude that the temperature treatment (1°C and 4 °C) was the most active inhibitor followed by the NaCl and the pH.

Studying the effect of sub-lethal temperature (50 °C) on the biofilm formation of *L. monocytogenes* strains we found that

the heat treatment accentuates the inhibition of *L. monocytogenes*, and it decreased their associated biofilms. However, some strong and even weak biofilm-forming strains showed considerable resistance to intermediate heat.

Examining the effect of some disinfectants on the growth of the selected two strains of *L. monocytogenes*, we found that the lactic acid is not efficient in removing *L. monocytogenes* from lettuce, the white vinegar, and the acetic acid solutions showed similar efficiency and they were better than the lactic acid.

The two best treatments with the highest bacterial reductions were chlorine solution and balsamic vinegar. The efficacity of balsamic vinegar to decontaminate *L. monocytogenes* from lettuce surfaces was similar or higher to chlorine, and this seems to be a promising method to reduce *L. monocytogenes* present in product at home and retail environments since it is a natural product.

Further investigation on the biofilm formation of *Listeria* strains and their interaction under different environmental

conditions are still necessary to provide more information on conditions that may inhibit biofilm formation and could be used to control the production of biofilms in food industry. Further investigations are needed also to study the effect of vinegars, since they are an important resource for households and food establishments due to its availability and organic nature.

4. CONCLUSION AND SUGGESTIONS

The results reported here demonstrate that strong biofilm former strains produced more biofilms under the investigated environmental conditions, indicating strain-dependent biofilm formation. *L. monocytogenes* is tolerant to environmental stresses (applied individually or in combination) such as high NaCl concentrations, refrigeration temperatures, and low-pH conditions. The different treatments had a more expressed effect in the case of strong biofilm former strains, but we can conclude that the temperature treatment was the most active inhibitor followed by the NaCl and the pH.

The sub-lethal heat treatment accentuates the inhibition of *L. monocytogenes*, and it decreased their associated biofilms. However, some strong and even weak biofilm forming strains showed considerable resistance to intermediate heat. Although, a detailed study is needed to better identify other factors affecting the growth and death of *Listeria* in foods.

All tested solutions (chlorine, acetic acid, balsamic vinegar, white vinegar, and lactic acid) showed higher bactericidal effects against the *L. monocytogenes* strains than balsamic vinegar having a clearly higher activity. Balsamic vinegar may be a promising effective solution to inhibit other food pathogens present on produce surface or other foods. There is a lack of studies with these vinegars, and it is an important resource for households and food establishments due to is availability and organic nature.

In the future, it is proposed to use new analytical methods to study the structure and formation of *Listeria monocytogenes* and other food borne pathogenic bacterial biofilms in vivo and in vitro, e.g. laser scanning microscopy etc.

As the use of enzymes is becoming increasingly important in the fight against biofilms, it would be useful to investigate their effect (e.g. the application of pectinase, arabanase, cellulase, hemicellulase, beta-glucanase, and xylanase activities on *Listeria monocytogenes* biofilms). In addition to enzymes, photoinactivation is also a possible method for inactivating biofilms. This would represent an additional

potential for inactivation of *Listeria* species and other foodborne pathogens, either on food contact or on food surfaces such as leafy vegetables e.g. lettuce.

5. NEW SCIENTIFIC RESULTS

1) Based on the optical density readings in optimal conditions, in terms of the biofilm-forming ability, the examined 9 strains of *L. monocytogenes* could be divided into 3 groups: weak (NCAIM B1454, 3b T1, CCM5576, CCM7202), moderate (11/4.12t03, 10887 ½ a, CCM 4699), and strong (NCAIM B01966T, 5105 3a) biofilm formers. Based on their normalized OD values (Labidi et al. 2023):

- It was found that strains which had originally strong biofilm-forming capacity were more sensitive to the temperature effect (their reduction in biofilm forming capacity with decreasing temperature (1°C and 4 °C) was significantly higher compared to weak biofilm formers (<p 0.05)).
- It was shown that strains which had originally higher biofilm-forming capacity were more sensitive to the sodium chloride treatment (their reduction in biofilm forming capacity with increasing NaCl concentration from 5% to 15% was significantly higher compared to weak biofilm formers (<p 0.05)).

- It was proved that strains which had originally stronger biofilm-forming capacity were the most sensitive to low pH (pH 4) (their reduction in biofilm forming capacity with decreasing pH from 6 to 4 was significantly higher compared to weak biofilm formers (<p 0.05)).
- The results evidenced that originally weak or moderate biofilm formers were more resistant to the effects of temperature, NaCl and pH which can bring survival advantages to these strains.

2) Undiluted balsamic vinegar proved to be as effective as chlorine in inhibiting *L. monocytogenes* on lettuce surface which provide a promising method to reduce *L. monocytogenes* presence in fresh produce at home and retail environments.

3) It was presented for the first time that *Listeria monocytogenes* is able to form biofilms at the lower limit of its growth temperature range (1 °C). This property seems to be strain independent as all ten strains tested showed it.

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