Acta Alimentaria 52 (2023) 2, 270–280
DOI: 10.1556/066.2023.00017

AKADÉMIAI KIADÓ

Effects of pH, sodium chloride, and temperature on the growth of *Listeria monocytogenes* biofilms

S. Labidi[1], A. Jánosity[1], A. Yakdhane[2], E. Yakdhane[1], B. Surányi[1], Cs. Mohácsi-Farkas[1] and G. Kiskó[1]*

[1] Department of Food Microbiology, Hygiene and Safety, Institute of Food Science and Technology, Hungarian University of Agriculture and Life Sciences, Somlói Street 14–16, HU-1118, Budapest, Hungary

[2] Department of Food Engineering, Institute of Food Science and Technology, Hungarian University of Agriculture and Life Sciences, Ménesi Street 44, HU-1118, Budapest, Hungary

ORIGINAL RESEARCH PAPER

Received: January 23, 2023 • Accepted: April 9, 2023
Published online: May 26, 2023

ABSTRACT

Listeria monocytogenes is able to form biofilms on food contact surfaces. Effectiveness of salt concentration, pH, and temperature on the formation of *L. monocytogenes* biofilms was evaluated individually and in combinations using microtiter plate assay by measuring the optical density. The tested strains differed in their biofilm formation (low, moderate, and strong) ability. At 37 °C, decreasing amounts of biofilms was observed in almost all *L. monocytogenes* strains when the NaCl concentration increased from 0.05 to 15%, but all strains were able to form biofilm even at 1 °C. There was no significant difference in biofilm formation between pH 4, 5, and 6, except for some strains. When stress conditions were tested in combination, the addition of 15% NaCl significantly inhibited the growth of *L. monocytogenes* at 1 °C and 4 °C, and the weak biofilm-forming strains were less sensitive to the temperature and to NaCl treatments than the strong biofilm-forming strains. These results enhance our knowledge of the application of NaCl, temperature, and pH stresses in the food industry and provide basis to develop new strategies for control of biofilm formation of this pathogen.

KEYWORDS

Listeria monocytogenes, biofilm formation, NaCl, pH, temperature

* Corresponding author. Tel.: +36 1 305 7610, E-mail: kisko.gabriella@uni-mate.hu

1. INTRODUCTION

Listeria monocytogenes represents a major public health concern for consumers, the food industry, and regulatory agencies (Farber and Peterkin, 1991).

It is estimated that up to 80% of bacteria on Earth live in biofilms, which provide protection against environmental stresses (e.g., heat, desiccation) and antimicrobial treatments (e.g., antibiotics, disinfectants) (Sturtevant et al., 2015). Biofilms are particularly important in food industry, because they provide persistent cross- and post-processing contamination in the food processing environment. Appearance of biofilms can cause reduced shelf-life of foods and even significant food safety hazards.

L. monocytogenes is frequently isolated from biofilms in food processing plants (Borucki et al., 2003), moreover, it is able to form biofilm on different industrial work surfaces such as stainless steel, rubber, glass, and polystyrene (Mráz et al., 2011). The biofilm formation of *L. monocytogenes* and its tolerance to low water activity and temperature explain its widespread distribution and persistence, thereby increasing the likelihood of its survival and subsequent cross-contamination potential in food processing environments. Understanding the impact of environmental factors on *L. monocytogenes* biofilm formation is crucial in the food industry.

The impact of environmental factors on bacterial growth is widely studied, however only a little is known about how these factors influence the biofilm formation of bacteria. Biofilm formation is one of bacterial stress responses under unstable environmental conditions (Toyofuku et al., 2016).

The ability of pathogenic microorganisms to form biofilms is usually tested at optimal growth conditions i.e., at 37 °C. However, in a food environment, it is important to know whether bacteria can form biofilms at not optimal temperatures, such as in freezing temperature (−18 °C) or chilled processing environments (10–15 °C). The optimum temperature for the bacterial biofilm formation was shown to be strain dependent (Sizova et al., 2012). Studies indicated that osmolarity increased biofilm production (Lee et al., 2019). Pilchova et al. (2014) observed that under acidic conditions, the first step of biofilm formation in *L. monocytogenes* was impaired.

Therefore, we aimed to study the effect of different factors (temperature, pH, and osmolarity) applied both individually and in combination on the biofilm formation of *L. monocytogenes* strains.

2. MATERIALS AND METHODS

2.1. Culture preparation

Nine *L. monocytogenes* isolates were tested (Table 1) that were maintained at the Department of Food Microbiology, Hygiene and Safety of the Hungarian University of Agriculture and Life Sciences. Fresh cultures were prepared on Tryptic Soy Agar plates (Basingstoke, Hampshire, UK) and incubated at 37 °C for 24 h.

2.2. Microtiter plate biofilm production assay

The biofilm forming ability of the strains was investigated using the crystal violet 0.4% (w/v) staining method by Mouwakeh (2018) with minor modifications (incubation time and

Table 1. 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*	$11/4.12t_03$	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

wavelength of optical density (OD) measurement was different). Briefly, biofilms were grown in ELISA titer plates with a final volume of 200 µL of M9 minimal media (1 g L^{-1} NH_4Cl, 6 g L^{-1} Na_2HPO_4, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl, 2 mL L^{-1} $MgSO_4$ 1M, 0.1 mL L^{-1} $CaCl_2$ 1M, and 10 mL L^{-1} glucose 20%). Initial cell counts were adjusted to an OD of 0.3 (~10^7 cells mL^{-1}) using a DEN-1B McFarland densitometer (Biosan).

To study the effect of different stress factors on the biofilm formation of *L. monocytogenes* strains, the composition of M9 minimal media was altered. Three factors were tested at multiple levels: (i) NaCl addition (0%, 5%, 10%, or 15%), (ii) the effect of pH (pH 4, 5, and 6 adjusted by HCl 1N solution), 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.

Supernatants from the wells were discarded. Each well was washed three times with phosphate-buffered-saline (PBS) solution (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1,000 mL distilled water; pH adjusted to 7.4 with HCl) followed by 15 min air drying in a laminar flow hood.

Finally, each well was stained with 200 µL Crystal Violet 0.4% (w/v) solution in ethanol. The plates were incubated for 15 min 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). Chemicals were derived from Oxoid (Basingstoke, Hampshire, 123 UK).

2.3. Data analyses

IBM SPSS Statistics (version 27) was used for experimental data analysis. One-factor ANOVA determined statistically significant differences between the strains' biofilm formation ability after incubation in an optimal environment (37 °C, pH = 6, and 0% NaCl). As a follow-up analysis, discriminant analysis (DA) was applied. For the multivariate analyses, the assumptions were tested as (i) homogeneity of variances was not violated based on Levene Statistic = 1.58; $P = 0.20$, (ii) based on the Shapiro-Wilk's tests (test values > 0.874; $P > 0.30$), the normality assumption was satisfied across the replicates and (iii) in the dataset no outlier was detected using Z score values after standardisation (values < 2). The recorded OD values indirectly

indicated the level of produced biofilms; thus, DA examined our hypothesis whether the tested 9 strains could be classified into three groups (weak-, moderate-, or strong biofilm producers) based on their biofilm-forming abilities.

For further analysis, OD values under optimal environmental conditions were used as positive controls, while OD values recorded after the incubation in the most adverse tested environment (1 °C, pH = 4, and 15% NaCl) inferred as negative controls.

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 normalised. Thus, the biofilm formation capacity (BFC, %) could be expressed (Eq. (1)) as:

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

Using the BFC values, strains classified into different groups could be compared.

3. RESULTS AND DISCUSSION

3.1. Biofilm formation ability of strains

After incubation at 37 °C, pH 6, and 0% NaCl, OD_{595} values were recorded (Fig. 1). Among the examined strains, 6 and 8 formed the highest amounts of biofilms ($OD_{595} > 0.25$), while the weakest biofilm-forming strain was found to be strain 5 ($OD_{595} < 0.15$) (Fig. 1).

Since the OD values are proportional to the biofilm-forming ability of the strains, the nine strains were classified visually as weak ($OD_{595} < 0.19$), moderate ($0.19 \leq OD_{595} \leq 0.25$), or strong ($OD_{595} > 0.25$) biofilm formers (Fig. 1).

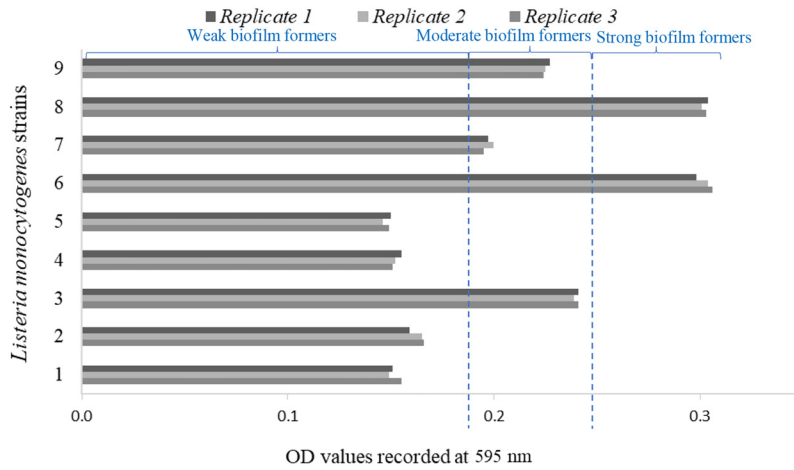

Fig. 1. Biofilm formation of coded *L. monocytogenes* strains incubated at 37 °C for 48 h in M9 minimal medium (pH 6, NaCl 0%) based on the measured OD_{595} values

One-factor ANOVA showed a significant difference between the groups ($F = 1696.03$, $P = 2.76 \times 10^{-24}$ value, $F_{crit} = 2.51$). DA was used as a follow-up analysis and tested our hypotheses regarding the strain's classification. The Chi-square test was significant (Wilks $\lambda = 0.015$, Chi-square = 21.1, Canonical correlation = 0.998, $P = 0.002$), confirming the different classification of strains. The classification was based on Mahalanobis distances and it was highly successful: 100% of the cases were correctly reclassified into the 3 groups indicating that the strains can be divided into 3 groups regarding their biofilm-forming ability (Fig. 2).

3.2. Individual effect of temperature, NaCl concentration, and pH on biofilm formation

BFC of strains was investigated using the normalised OD values. BFC was assumed 100% under optimal conditions (37 °C, pH 6, 0% NaCl) and 0% under the most adverse conditions (1 °C, pH 4, 15% NaCl) as negative control.

3.2.1. The effect of temperature on the BFC (%). The effect of temperature was tested at 37, 20, 4, and 1 °C at 0% added NaCl and pH 6 (Fig. 3).

Most of the analysed strains showed good biofilm formation at 37 °C. At 20 °C, the BFC of the strains decreased by an average of 16%. However, at 4 °C, the BFC of weak biofilm formers decreased by an average of 48%, that of moderate biofilm formers by 76%, and that of strong biofilm formers by 80%. In most cases similar reduction was observed for biofilm formation at 1 °C.

The BFC of the strains at lower temperatures decreased gradually, which is consistent with other findings (Djordjevic et al., 2002; Fan et al., 2020). Although biofilm formation was weaker at refrigerated temperatures, the investigated strains were able to form biofilm. The ability of *L. monocytogenes* to form biofilms at low temperatures can increase the potential for cross-contamination during food production. Higher temperature may have positive effects, i.e., improves the growth of *L. monocytogenes* through increasing reaction rate and bacterial growth resulting more biofilm formation (Garrett et al., 2008); increases the necessary number of cells adhered in the initial attachment (Mai and Conner, 2007); and also by its effect on

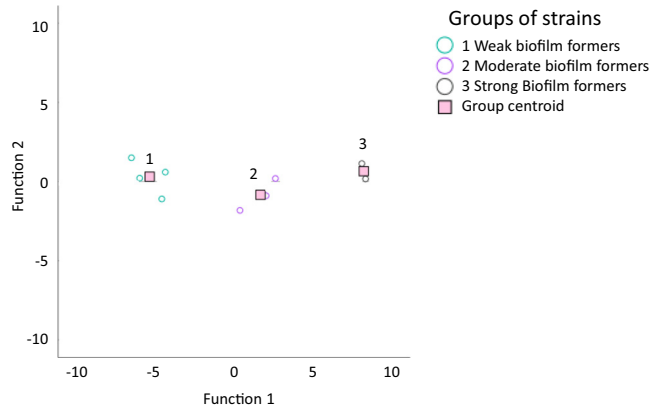

Fig. 2. Canonical discriminant functions are used for the classification of the strains, based on their biofilm formation ability following incubation under optimal conditions

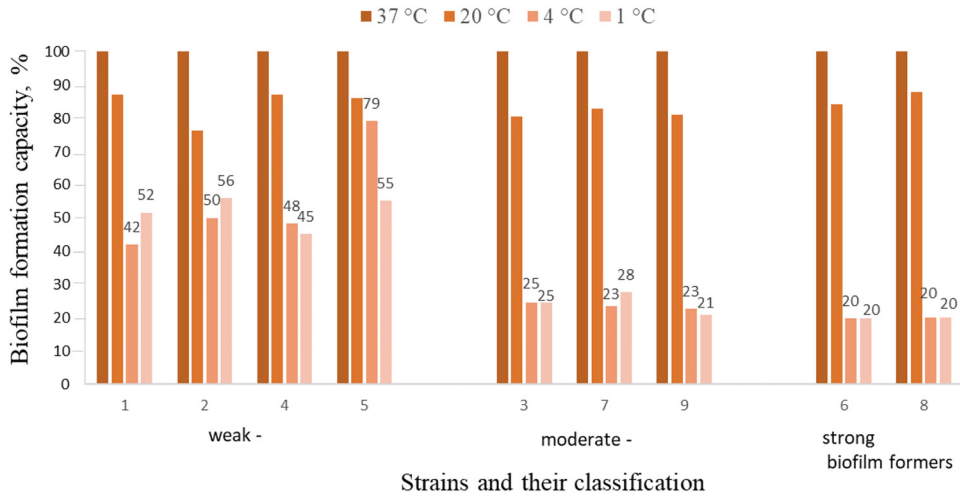

Fig. 3. The effect of different incubation temperatures on BFC of coded *L. monocytogenes* strains incubated for 7 days at 1 °C and 4 °C and 48 h at 20 °C and 37 °C in M9 minimal medium (pH 6, and NaCl 0%)

hydrophobicity, which increases as growth temperature does (Koga and Yamamoto, 2018) and positively correlates with biofilm formation (Fan et al., 2020). Temperature also affects the viscosity of the polysaccharides in bacterial extracellular polymeric substances (EPS), which also has an effect on biofilm formation (Villain-Simonnet et al., 2000) and on appendages (e.g., flagella) of bacteria that help them adhere to surfaces (Garrett et al., 2008).

Our results showed that strains with originally lower BFC were less sensitive to 4 °C temperature. Strain *5* seemed to be the most resistant to the effect of temperature, since its capacity was still 79% at 4 °C. Moreover, different strains prefer different temperatures when forming biofilms (Pan et al., 2010).

3.2.2. Effect of NaCl on BFC (%). The effect of NaCl was tested by adding 0, 5, 7.5, and 15% NaCl to the media (37 °C and pH 6) (Fig. 4). There was no significant difference between the responses of the weak biofilm former strains ($F = 0.68$, $P = 0.53$, $F_{crit} = 4.26$) between 0 and 7.5% NaCl, however, 15% NaCl decreased the BFC of the strains to 31–59%. A similar decreasing pattern was observed by Pan et al. (2010) using *L. monocytogenes* after increasing the NaCl concentration from 0.5 to 7% (37 °C). In moderate biofilm formers, 15% NaCl had a strong influence, and decreased the BFC of the strains by 26–68%. Although, the disruption of biofilm formation of strain *9* was only 26%, indicating its resistance and adaptation to high salt concentration. The strong biofilm formers were the most sensitive to the effect of 15% NaCl as their capacity decreased by 73–80%. Only the highest applied NaCl concentration (15%) decreased significantly the BFC.

3.3. The effect of pH on the BFC (%)

The effect of pH was tested by adjusting its value to 4, 5, and 6 (37 °C and 0% NaCl) (Fig. 5). All tested strains formed biofilms at all three pH values. Slight decrease in biofilm formation was

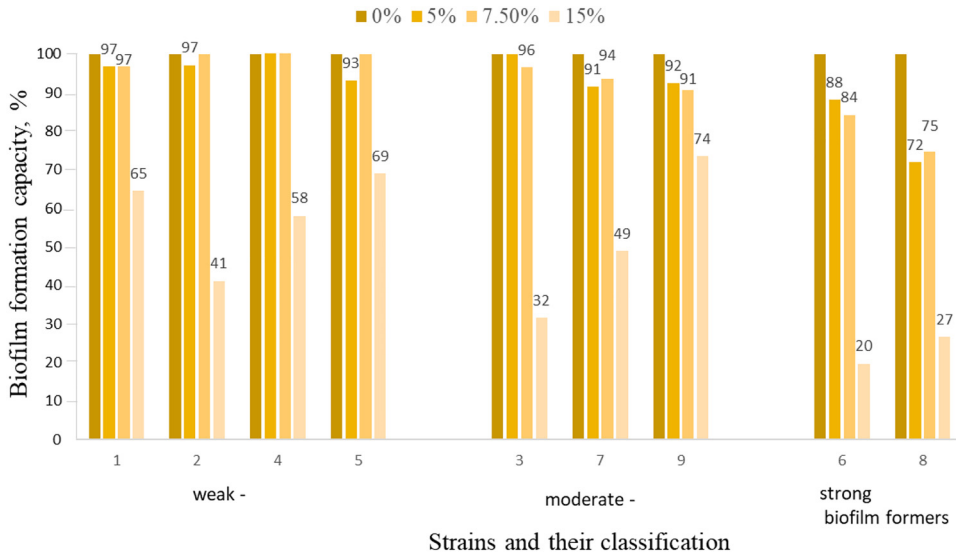

Fig. 4. The effect of different NaCl concentrations on BFC of coded *L. monocytogenes* strains incubated at 37 °C for 48 h in M9 minimal medium

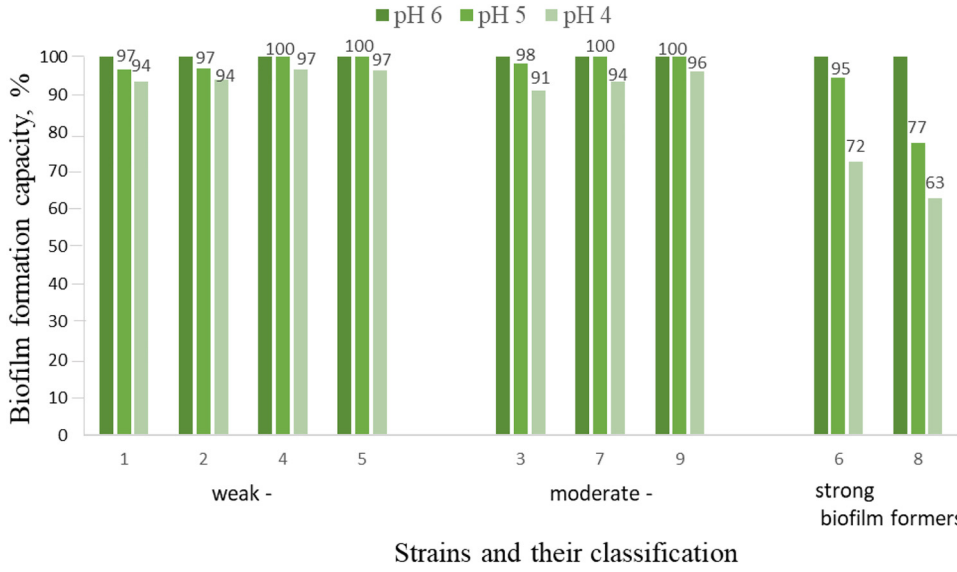

Fig. 5. The effect of different pH values on BFC of coded *L. monocytogenes* strains incubated at 37 °C for 48 h in M9 minimal medium

observed with decreasing pH, thus the strains preferred to grow and form biofilms in a less acidic environment. At the lowest tested pH, the capacity of the weak biofilm former strains decreased by an average of 5%.

Our results are in accordance with the study of Fan et al. (2020) that showed significantly inhibited biofilm formation of *L. monocytogenes* at pH 4 compared to neutral environment. The pH optimum of EPS secretion was found to be around neutral (Tilahun et al., 2016). The slight decrease in biofilm production at low pH can be explained partly by changes in the enzyme activity and EPS production (Chaieb et al., 2007). EPS production protects bacteria against environmental stressors such as pH, because the biofilm slime layer (EPS) acts as a diffusion barrier, resulting in a reduction of diffusion of hydrogen ions within the extracellular matrix (Nicolau Korres et al., 2013). In contrast, Nilsson et al. (2011) found the strongest biofilm formation in acidic environments. Again, the strong biofilm formers were the most sensitive ones against pH 4, their capacity decreased between 28 - 37%.

In summary, the tested strains differed in biofilm formation (low, moderate, and strong) ability. The smallest variability was found in the weakest biofilm-forming strains, consistently with the findings of Fan et al. (2020) that, in general, strong biofilm-forming strains are highly variable in response to environmental factors.

3.4. Biofilm formation under mixed stress conditions

Biofilm formation of the nine strains was analysed in M9 Minimal Media with different NaCl concentrations, pH values, and temperatures (Figs 6 and 7).

Temperature had the most dominant effect on the biofilm formation (Fig. 6) when 5% NaCl was added to the medium. The tested pH values had not influenced the biofilm formation, there were no significant differences in the measured OD values except at 4 °C. Same results were found with 7.5% NaCl (results are not shown).

When 15% NaCl was added, differences could be observed between the responses of the strains in terms of tested pH values at 37 °C. At pH 4 (37 °C) OD ≈ 0.1 value was measured for

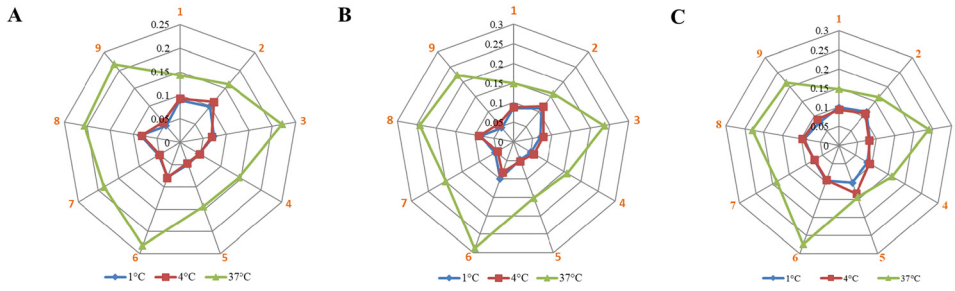

Fig. 6. Effect of combined treatments on the biofilm formation of *Listeria monocytogenes* using 5% NaCl in M9 minimal medium after 7 days incubation at 1 °C or 4 °C and 48 h at 37 °C using OD595 values. Capital letters refer to the different pH values i.e., A) pH = 4, B) pH = 5, C) pH = 6. Strains are notated with numbers

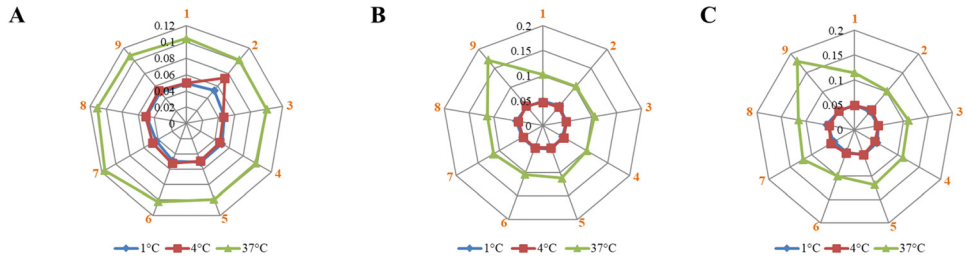

Fig. 7. Effect of combined treatments on the biofilm formation of *L. monocytogenes* using 15% NaCl in M9 minimal medium after 7 days incubation at 1 °C or 4 °C and 48 h at 37 °C using the OD_{595} values. Capital letters refer to the different pH values i.e., A) pH = 4, B) pH = 5, C) pH = 6. Strains are notated with numbers

all strains (Fig. 7). At pH 5 and pH 6 strain *9* could overcome the effect of NaCl, and OD values were >0.15. At 4 °C, pH 4 enhanced the biofilm formation of strain *2* showing adaptation to acidic environment.

The selected combined treatments had negative effect on the biofilm formation compared to the results of individual stresses. However, our results indicated that strains characterised as strong biofilm formers, seemed to be more sensitive to all of the treatments. As a response to the treatments, the BFC (%) of those strains decreased more, compared to the moderate or weak biofilm forming groups. This phenomenon may suggest that the originally weak or moderate biofilm forming ability can bring survival advantages to the strains.

Our results showed that the BFC of *L. monocytogenes* strains was influenced by a multitude of environmental factors and it was strain dependent. The differences in the NaCl, temperature, and pH tolerance highlighted the possibility that the adapted strains may alter the BFC as response to changing environmental factors. The result of this phenomenon can be the appearance of persistent strains that survive and even grow in varying environments over long periods of time in food processing environment.

4. CONCLUSIONS

The results reported here demonstrated that (i) the BFC of *L. monocytogenes* is strain dependent, (ii) the initially strong biofilm former strains can be more sensitive to the treatments, and (iii) among the investigated stresses, the temperature was the most active BFC inhibitor followed by NaCl and pH.

ACKNOWLEDGEMENT

The authors acknowledge the Hungarian University of Agriculture and Life Sciences' Doctoral School of Food Science for the support in this study.

REFERENCES

Borucki, M.K., Peppin, J.D., White, D., Loge, F., and Call, D.R. (2003). Variation in biofilm formation among strains of *Listeria monocytogenes*. *Applied and Environmental Microbiology*, 69: 7336–7342.

Chaieb, K., Chehab, O., Zmantar, T., Rouabhia, M., Mahdouani, K., and Bakhrouf, A. (2007). *In vitro* effect of pH and ethanol on biofilm formation by clinical *ica*-positive *Staphylococcus epidermidis* strains. *Annals of Microbiology*, 57: 431–437.

Djordjevic, D., Wiedmann, M., and McLandsborough L.A. (2002). Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Applied and Environmental Microbiology*, 68(6): 2950–2958.

Fan, Y., Qiao, J., Lu, Z., Fen, Z., Tao, Y., Lv, F., Zhao, H., Zhang, C., and Bie, X. (2020). Influence of different factors on biofilm formation of *Listeria monocytogenes* and the regulation of *cheY* gene. *Food Research International*, 137: 109405.

Farber, J.M. and Peterkin, P.I. (1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiological Reviews*, 55: 476–511.

Garrett, T.R., Bhakoo, M., and Zhang, Z. (2008). Bacterial adhesion and biofilms on surfaces. *Progress in Natural Science*, 18: 1049–1056.

Koga, K. and Yamamoto, N. (2018). Hydrophobicity varying with temperature, pressure, and salt concentration. *The Journal of Physical Chemistry B*, 122(13): 3655–3665.

Lee, B-H., Cole, S., Badel-Berchoux, S., Guillier, L., Felix, B., Krezdorn, N., Hébraud, M., Bernardi, T., Sultan, I., and Piveteau, P. (2019). Biofilm formation of *Listeria monocytogenes* strains under food processing environments and pan-genome-wide association study. *Frontiers in Microbiology*, 10: 2698.

Mai, T.L. and Conner, D.E. (2007). Effect of temperature and growth media on the attachment of *Listeria monocytogenes* to stainless steel. *International Journal of Food Microbiology*, 120(3): 282–286.

Mouwakeh, A. (2018).The antimicrobial and resistance modifying activities of *Nigella sativa oil*. PhD thesis, Szent István Egyetem.

Mráz, B., Kiskó, G., Hidi, E.I., Ágoston, R., Mohácsi-Farkas, Cs., and Gillay, Z. (2011). Assessment of biofilm formation of *Listeria monocytogenes* strains. *Acta Alimentaria*, 40(Suppl.-1): 101–108.

Nicolau Korres, A.M., Aquije, G.M.D.F.V., Buss, D.S., Ventura, J.A., Fernandes, P.M.B., and Fernandes, A.A.R. (2013). Comparison of biofilm and attachment mechanisms of a phytopathological and clinical isolate of *Klebsiella pneumoniae* subsp. *pneumoniae*. *The Scientific World Journal*, 2013: 925375.

Nilsson, R.E., Ross, T., and Bowman, J.P. (2011). Variability in biofilm production by *Listeria monocytogenes* correlated to strain origin and growth conditions. *International Journal of Food Microbiology*, 150: 14–24.

Pan, Y., Breidt, F.Jr., and Gorski, L. (2010). Synergistic effects of sodium chloride, glucose, and temperature on biofilm formation by *Listeria monocytogenes* serotype 1/2a and 4b strains. *Applied and Environmental Microbiology*, 76: 1433–1441.

Pilchová, T., Hernould, M., Prévost, H., Demnerová, K., Pazlarová, J., and Tresse, O. (2014). Influence of food processing environments on structure initiation of static biofilm of *Listeria monocytogenes*. *Food Control*, 35: 366–372.

Sizova, Yu.V., Cherepakhina, I.Ya., Balakhnova, V.V., Burlakova, O.S., Sizova, E.V., Pomukhina, O.I., and Fetsailova, O.P. (2012). Variability of properties characterizing the ability of *V. cholerae* to survival in biofilm communities. *Problems of Particularly Dangerous Infections*, 3: 54–57.

Sturtevant, R.A., Sharma, P., Pavlovsky, L., Elizabeth, J., Stewart, E.J., Solomon, M.J., and Younger, J.G. (2015). Thermal augmentation of vancomycin against staphylococcal biofilms. *Shock*, 44: 121–127.

Tilahun, A., Haddis, S., Teshale, A., and Hadush, T. (2016). Review on biofilm and microbial adhesion. *International Journal of Microbiological Research*, 7(3): 63–73.

Toyofuku, M., Inaba, T., Kiyokawa, T., Obana, N., Yawata, Y., and Nomura, N. (2016). Environmental factors that shape biofilm formation. *Bioscience, Biotechnology, and Biochemistry*, 80(1): 7–12.

Villain-Simonnet, A., Milas, M., and Rinaudo, M. (2000). A new bacterial exopolysaccharide (YAS34). II. Influence of thermal treatments on the conformation and structure. Relation with gelation ability. *International Journal of Biological Macromolecules*, 27: 77–87.