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Antibiotic resistance and biofilm forming abilities of *Listeria monocytogenes* and effect of subMIC concentration of white vinegar on these virulence factors

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ABSTRACT

In the present study, antibiotic resistance profiles and biofilm forming abilities of 9 *Listeria monocytogenes* isolates obtained from out of 30 retail meat samples were determined, and the effect of commercial white vinegar on these virulence factors in isolates exposed to subMIC concentrations were investigated. All isolates were found to be resistant to cefotixin and oxacillin, 8 isolates (26.6%) to clindamycin, 1 isolate (3.3%) to rifampicin, and 1 (3.3%) isolate was found to show intermediate resistance against clindamycin. Biofilm formation was determined for all the isolates at 22 °C and 37 °C (24 h, 48 h and 72 h). MIC values of white vinegar samples were determined at 3.12% for all isolates. MIC/2 and MIC/4 concentrations of white vinegar increased the biofilm forming capacity of the isolates by 21.2% and 17.1%, respectively. After exposure to MIC/2 concentration of white vinegar for seven days, the antibiotic resistance status of the isolates to tetracycline, rifampicin, and clindamycin changed, and the biofilm forming abilities significantly decreased at 4 °C and 37 °C for 48 h and at 37 °C for 72 h ($P < 0.05$). The results showed that the use of subMIC concentrations of white vinegar should be avoided in routine sanitation applications.

KEYWORDS

Listeria monocytogenes, ground meat, antibiotic resistance, biofilm, white vinegar, subMIC

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1. INTRODUCTION

Listeria monocytogenes is a Gram-positive foodborne pathogen that causes human listeriosis particularly in susceptible individuals such as pregnant women, cancer and AIDS patients, and people over 65 years of age. *L. monocytogenes* can survive under stress conditions such as low temperature, high salt concentration, and low pH, which causes widespread survival of the microorganism in nature (Radoshevich and Cossart, 2018).

Studies showed that *L. monocytogenes* is widely distributed in food processing environments and in farm, retail, and home environments. Biofilms are important components in food industry, because a wide variety of substances such as plastic, glass, and polypropylene are solid surfaces, on which bacteria can attach and form mature biofilms. In this context, equipment that is difficult to clean and disinfect, like cutting boards and knives, creates environments, where pathogens can easily grow and cause cross-contamination (Møretrø and Langsrud, 2004; Buchanan et al., 2017).

Antibiotic resistance is considered one of the greatest threats to global public health, and food security. The high mortality rate in listeriosis cases and the increase in multidrug resistance in foodborne pathogens necessitate monitoring of the change in the antibiotic resistance of *L. monocytogenes* isolates (WHO, 2018).

Vinegar is a household natural disinfectant widely used as an antifungal and antimicrobial agent due to its very low pH value and the presence of acetic acid as the main ingredient (Ramos et al., 2014). It is not only used in different application areas such as nutrition, medicine, and pharmaceutical fields but also commonly used in food sanitation. Vinegar and vinegar-based solutions are also used as dressing for salads and appetizers. Several studies reported that vinegar samples showed antibacterial activity at varying levels (Baldas and Altuner, 2018; Kara et al., 2022). However, there is no report on the effectiveness of vinegar on *L. monocytogenes* when used at low concentrations. A better understanding of the epidemiology of *L. monocytogenes* and virulence factors such as antibiotic resistance and biofilm-forming abilities of isolates, which contribute to survival, spread, and persistence of strains is needed to develop effective food processing applications. The aim of this study is to investigate the presence of *L. monocytogenes* in ground meat and to determine virulence factors such as biofilm-forming ability and antibiotic resistance profiles of the isolates. We also investigated the effect of commercial white vinegar on these virulence factors in isolates exposed to sub-MIC concentrations of vinegar.

2. MATERIALS AND METHODS

2.1. Detection of *L. monocytogenes*

Thirty unpacked ground meat samples were randomly purchased from different butchers and markets in Edirne, Türkiye, in November and December 2019. Detection of L. *monocytogenes* in the samples (25 g of each) was performed in agreement with the standard method ISO 11290. Gram and catalase positive and oxidase negative presumptive colonies with typical aspects of *L. monocytogenes* on Palcam agar (Sigma Aldrich, USA) were biochemically identified using the API Listeria system (BioMérieux, Marcy l'Etoile, France).

2.2. Minimum inhibitory concentrations (MIC) of white vinegar samples

The MIC values of the white vinegar samples were determined by using the microdilution method according to Clinical Laboratory Standards Institute guidelines (CLSI, 2018). Three commercial white vinegar samples of different manufacturers were used. Two hundred microlitres of Mueller–Hinton broth (Merck, Germany) was added in each well of a microplate, then an equal volume of white vinegar sample was added to the first well to obtain 50% concentration of white vinegar. The serial two-fold dilutions of white vinegar samples (25%, 12.5%, 6.75%, 3.12%, and 1.56%) were prepared in the same manner.

2.3. Exposure to *L. monocytogenes* isolates to white vinegar

Overnight bacterial cultures were transferred to 10 ml TSB (Tryptic soy broth, Merck, Germany) containing MIC/2 (1.56%) concentration of white vinegar with 1% inoculation ratio. After 24 h of incubation, the incubated cultures were transferred into fresh TSB containing MIC/2 white vinegar concentration. This process was repeated for 7 days.

2.4. Antibiotic susceptibility

Disk diffusion method according to Clinical Laboratory Standards Institute guidelines (CLSI, 2018) on Mueller-Hinton agar (Merck, Germany) plates was used for determination of antibiotic resistance of *L. monocytogenes* isolates before and after exposure to white vinegar. A total of 13 antibiotics, erythromycin (15 μg), penicillin G (10 U), gentamicin (10 μg), trimethoprim-sulfamethaxazole (1.25/23.75 μg), ciprofloxacin (2 μg), clindamycin (2 μg), vancomycin (30 μg), ampicillin (30 μg), chloramphenicol (30 μg), tetracycline (30 μg), cefotixin (30 μg), rifampicin (5 μg), and oxacillin (1 μg) were used. The breakpoints of *Staphylococcus* and *Enterococcus* species resistance were used for interpretation of results.

2.5. Biofilm formation on polystyrene and effect of sub MIC of white vinegar on biofilm formation

96-well polystyrene microplates (Grenier bio-one, Austria) were used for quantification of biofilm production based on the previously described method with some modifications (Stepanović et al., 2004). Overnight bacterial cultures (18 h old) diluted in TSB broth (Merck, Germany) to OD570 = 0.2 were used (three parallels of each strain). The negative control wells contained broth only. Plates were incubated for 24 h, 48 h, and 72 h at 4 °C, 22 °C, and 37 °C. After incubation, the absorbances were measured at 590 nm by using a Multiskan EX reader (Bio-Rad, ABD). Strains were classified as no, weak, moderate, and strong biofilm producers according to Stepanović et al. (2000). To determine the effect of sub MIC of white vinegar on biofilm formation, subMIC concentrations (MIC/2 and MIC/4) of white vinegar were added to microplate wells. Plates were incubated statically at 37 °C for 24 h. Biofilm formation was determined as above. The following formula was used to calculate the percentages of change (Sandasi et al., 2010):

$$\% \text{ change} = \frac{OD \text{ growth control} - OD \text{ sample}}{OD \text{ growth control}} \times 100$$

2.6. Statistical analyses

The Student-*t* test was used to calculate the significance of the differences between the biofilm formation abilities of isolates and isolates exposed to white vinegar at different temperatures and times ($P < 0.05$). Analyses were performed with the GraphPad Prism software (Intuitive Software for Science, San Diego, CA).

3. RESULTS AND DISCUSSION

3.1. Detection of *L. monocytogenes*

Raw meat products are considered an important vehicle for *L. monocytogenes* transmission in the meat industry (Mazaheri et al., 2021). In our study, *L. monocytogenes* was detected in 9 (30%) out of 30 retail ground meat samples. *L. monocytogenes* was detected within the range of 12.4–41.9% in raw meat and meat products in recent studies in different regions of Türkiye and in various countries in the world (Wang et al., 2013; Arslan and Baytur, 2019; Matle et al., 2019; Uludağ et al., 2023). The results of these studies showed that the contamination rates of *L. monocytogenes* in raw meat samples were quite high. Although it is difficult to associate any retail environment with an outbreak of infection, these environments play an important role in the contamination of food and the growth of *L. monocytogenes*. Interventions directed at these environments might reduce sporadic disease, which are the main type of *L. monocytogenes* infection (Varma et al., 2007).

3.2. Antibacterial effect of white vinegar

In our study, MIC values of three commercial white vinegar samples were determined at 3.12% for all *L. monocytogenes* isolates. There are studies showing the antimicrobial activity of vinegar against many pathogens, including *L. monocytogenes* (Baldas and Altuner, 2018; Pedroso et al., 2018). Acetic acid, the main active ingredient of vinegar, has been used to clean and disinfect the surfaces in home environments for many decades. The antimicrobial efficacy of cleaning procedures is particularly critical for more fragile populations such as youngs, the elderly, and those who are immunocompromised, but may also concern other groups, particularly with regard to the COVID-19 pandemic (Zinn and Bockmühl, 2020). Considering the similarity of their MIC values, one of the white vinegar samples was chosen and used for ongoing experiments.

3.3. Antibiotic resistance profiles and biofilm forming capacities of *L. monocytogenes* isolates before and after white vinegar exposure

All 9 *L. monocytogenes* isolates showed resistance against at least one of the 13 antibiotics tested, but none of the isolates were resistant to ciprofloxacin, vancomycin, ampicillin, penicillin G, chloramphenicol, erythromycin, gentamicin, trimethoprim-sulfamethaxazole, and tetracycline. All isolates were found to be resistant to cefotixin and oxacillin, 8 isolates (26.6%) to clindamycin, 1 isolate (3.3%) to rifampicin, and 1 (3.3%) isolate was found to show intermediate resistance against clindamycin (Table 1).

Table 1. Antibiotic resistance patterns of *L. monocytogenes* isolates before and after exposure to white vinegar

Strain code	Antibiotic resistance pattern	
	Antibiotics that the isolates are initially resistant to	Antibiotics that the isolates showed resistance to after exposure to white vinegar
DLM2	FOX, CLI, OX	TET(I), FOX, CLI, OX
DLM5	FOX, CLI(I), OX	TET(I), FOX,CLI, OX
DLM6	FOX, CLI, OX	TET(I), FOX, CLI, OX
DLM8	FOX, RD, CLI, OX	TET(I), FOX, CLI, OX
DLM9	FOX, CLI,OX	TET(I), FOX, CLI, OX
DLM13	FOX, CLI, OX	TET(I), FOX, CLI, OX
DLM14	FOX, CLI, OX	TET(I), FOX, CLI, OX
DLM16	FOX, CLI, OX	TET(I), FOX, CLI, OX
DLM28	FOX, CLI, OX	TET(I), FOX, CLI, OX

I: intermediate resistance; FOX: cefotixin; CLI: clindamycin; RD: rifampicin, OX: oxacillin; TET: tetracycline.

Our findings are in agreement with data reported on antibiotic resistance of *L. monocytogenes* isolated from meat and meat products in other studies (Wang et al., 2013; Arslan and Baytur, 2019; Matle et al., 2019; Uludağ et al., 2023). Ampicillin or penicillin G in combination with an aminoglycoside such as gentamicin is a first choice for treating human listeriosis. Trimethoprim in combination with a sulfonamide, i.e. sulfamethoxazole-co-trimoxazole, is considered the second choice of therapy (Matle et al., 2020). In our study, it was determined that none of the *L. monocytogenes* isolates were resistant to the antibiotics used in the treatment of listeriosis.

Pathogens can become more resistant to antibiotics during their adaptation to environmental stress. Therefore, it is important to understand how specific protection and environmental stress factors affect the antibiotic susceptibility of *L. monocytogenes* (Olaimat et al., 2018).

When antibiotic resistance profiles of the isolates before and after exposure to white vinegar were compared, it appeared that the antibiotic resistance status of the isolates against tetracycline, rifampicin, and clindamycin changed. While all isolates were susceptible against tetracycline, after exposure to white vinegar, they became intermediately resistant. One isolate that was intermediately resistant against clindamycin became resistant and another isolate that was resistant against rifampicin became susceptible (Table 1). The increase in tetracycline resistance observed as a result of exposure to white vinegar in all *L. monocytogenes* isolates is quite remarkable. In previous studies, an increase in tetracycline resistance was observed in *L. monocytogenes* strains as a result of exposure to sub-lethal chlorine, hydrogen peroxide, and heat (45 °C) (Faezi-Ghasemi and Kazemi, 2015; Bansal et al., 2018). Rifampicin and tetracyline are used to treat listeriosis, and food chain is one of the most probable ways for the spread of emerging antibiotic resistant strains (Olaimat et al., 2018).

Polystyrene is a hydrophobic material and is widely used in food contact surfaces, food packaging, and household daily use materials (Genualdi et al., 2014). In our study, biofilm

formation was determined for all *L. monocytogenes* isolates at different temperatures (22 °C and 37 °C) and incubation times (24 h, 48 h and 72 h). At 4 °C, biofilm formation was determined in five isolates (55.5%) at 24 h; in eight isolates (88.8%) at 48 h, and in seven isolates (77.7%) at 72 h (Fig. 1).

When the average OD590 values of the isolates were compared, the results showed that there were significant differences ($P < 0.05$) between incubation times at all three temperatures except for 24 and 48 h at 22 °C and 37 °C. The comparison of the average OD590 values of the isolates at different incubation temperatures on time basis revealed significant differences for all temperatures for all times except 4 °C and 22 °C for 48 and 72 h ($P < 0.05$).

The results showed that the strongest biofilm forming abilities of the isolates were observed at 37 °C for 72 h, for which 8 isolates (88.8%) were determined as moderate biofilm producers and 1 isolate (11.1%) was determined as strong biofilm producer. At 22 °C for 72 h, 8 isolates (88.8%) were determined as weak biofilm producers and 1 isolate (11.1%) was determined as moderate biofilm producer. At 4 °C for 72 h, 7 isolates (77.7%) were determined as weak biofilm producers and 2 isolates (22.2%) produced no biofilm. The lowest OD590 value (0.180 ± 0.015) was in DLM9 strain obtained with the incubation at 4 °C for 24 h, and the highest OD590 value (1.031 ± 0.184) was in DLM13 strain obtained with the incubation at 37 °C for 72 h. Detection of *L. monocytogenes* in retail meat and meat products does not indicate that contamination occurs solely in the retail environment. It has also been identified that the main source of *L. monocytogenes* in retail products is the persistence of strains and cross-contamination from equipment, surfaces, and workers. This cross-contamination is associated with the ability of *L. monocytogenes* to attach to different food equipment and to form biofilm (Sauders et al., 2016; Matle et al., 2020). The results obtained in the present study revealed that *L. monocytogenes* isolates can form biofilms on plastic surfaces at temperatures representing routine food storage conditions, room temperature, and optimum growth condition of *L. monocytogenes*.

When OD590 values obtained with the isolates before and after exposure to white vinegar were compared (Table 2), it was determined that biofilm forming abilities of the isolates exposed to white vinegar significantly decreased at 4 °C and 37 °C for 48 h and at 37 °C for 72 h ($P < 0.05$). The comparison of changes in biofilm formation categories after exposure to white

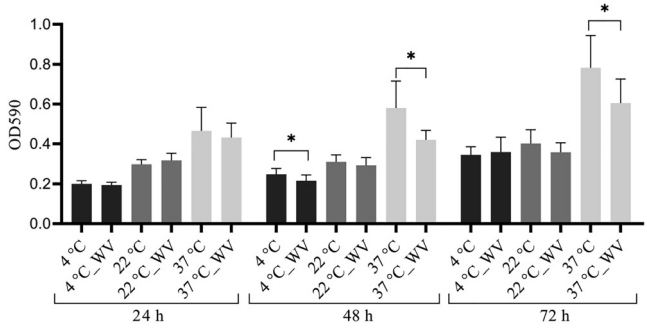

Fig. 1. The average of OD590 values of *L. monocytogenes* isolates and isolates exposed to white vinegar (MIC/2) on microtiter plates at different incubation temperatures and incubation times. * indicates significant differences between groups. 'WV' indicates *L. monocytogenes* isolates exposed to white vinegar

Table 2. Biofilm formation abilities of *L. monocytogenes* isolates before and after exposed to white vinegar

	Biofilm formation (mean of OD590 ± std deviation)								
	4 °C			22 °C			37 °C		
Strain code	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
DLM2	0.237 ± 0.13 weak	0.248 ± 0.016 weak	0.383 ± 0.074 weak	0.261 ± 0.046 weak	0.343 ± 0.048 weak	0.294 ± 0.036 weak	0.387 ± 0.105 moderate	0.432 ± 0.058 weak	0.843 ± 0.123 moderate
DLM2-WV	0.196 ± 0.019 no biofilm	0.254 ± 0.026 weak	0.330 ± 0.014 weak	0.283 ± 0.016 weak	0.323 ± 0.038 weak	0.285 ± 0.017 weak	0.353 ± 0.108 weak	0.439 ± 0.017 weak	0.517 ± 0.019 weak
DLM5	0.201 ± 0.025 weak	0.258 ± 0.016 weak	0.411 ± 0.025 weak	0.265 ± 0.028 weak	0.320 ± 0.022 weak	0.516 ± 0.016 moderate	0.469 ± 0.066 moderate	0.514 ± 0.047 moderate	1.010 ± 0.082 moderate
DLM5-WV	0.197 ± 0.193 no biofilm	0.239 ± 0.035 weak	0.357 ± 0.016 weak	0.278 ± 0.009 weak	0.272 ± 0.02 weak	0.354 ± 0.005 weak	0.548 ± 0.087 moderate	0.498 ± 0.057 moderate	0.542 ± 0.033 weak
DLM6	0.209 ± 0.009 weak	0.258 ± 0.044 weak	0.314 ± 0.009 weak	0.306 ± 0.010 weak	0.259 ± 0.008 weak	0.346 ± 0.017 weak	0.296 ± 0.072 weak	0.415 ± 0.021 weak	0.744 ± 0.095 moderate
DLM6-WV	0.207 ± 0.007 weak	0.235 ± 0.031 weak	0.278 ± 0.012 no biofilm	0.287 ± 0.028 weak	0.345 ± 0.026 weak	0.305 ± 0.019 weak	0.371 ± 0.012 moderate	0.485 ± 0.005 moderate	0.575 ± 0.084 moderate
DLM8	0.202 ± 0.017 weak	0.241 ± 0.058 weak	0.333 ± 0.024 weak	0.308 ± 0.015 weak	0.326 ± 0.022 weak	0.485 ± 0.035 weak	0.634 ± 0.068 moderate	0.791 ± 0.1 moderate	0.758 ± 0.033 moderate
DLM8-WV	0.200 ± 0.008 weak	0.222 ± 0.011 weak	0.320 ± 0.011 weak	0.317 ± 0.023 weak	0.309 ± 0.018 weak	0.448 ± 0.010 weak	0.563 ± 0.59 moderate	0.456 ± 0.051 weak	0.568 ± 0.014 moderate
DLM9	0.180 ± 0.015 no biofilm	0.205 ± 0.014 no biofilm	0.305 ± 0.005 no biofilm	0.303 ± 0.033 weak	0.277 ± 0.017 weak	0.347 ± 0.018 weak	0.288 ± 0.056 weak	0.581 ± 0.034 moderate	0.601 ± 0.060 moderate
DLM9-WV	0.191 ± 0.01 no biofilm	0.245 ± 0.024 weak	0.441 ± 0.061 weak	0.291 ± 0.020 weak	0.290 ± 0.010 weak	0.333 ± 0.023 weak	0.405 ± 0.056 moderate	0.362 ± 0.024 weak	0.636 ± 0.015 moderate
DLM13	0.0196 ± 0.019 no biofilm	0.260 ± 0.022 weak	0.377 ± 0.045 weak	0.297 ± 0.029 weak	0.293 ± 0.033 weak	0.447 ± 0.043 weak	0.518 ± 0.028 moderate	0.703 ± 0.174 moderate	1.031 ± 0.184 strong
DLM13-WV	0.180 ± 0.022 no biofilm	0.216 ± 0.005 no biofilm	0.504 ± 0.092 weak	0.342 ± 0.064 weak	0.284 ± 0.016 weak	0.387 ± 0.019 weak	0.424 ± 0.017 moderate	0.413 ± 0.56 weak	0.778 ± 0.129 moderate
DLM14	0.182 ± 0.002 No biofilm	0.244 ± 0.039 Weak	0.339 ± 0.018 Weak	0.340 ± 0.057 Weak	0.303 ± 0.036 Weak	0.343 ± 0.004 Weak	0.471 ± 0.041 Moderate	0.489 ± 0.077 Moderate	0.605 ± 0.034 Moderate
DLM14-WV	0.216 ± 0.006 weak	0.182 ± 0.008 no biofilm	0.385 ± 0.030 weak	0.297 ± 0.021 weak	0.250 ± 0.004 weak	0.344 ± 0.007 weak	0.362 ± 0.031 weak	0.390 ± 0.028 weak	0.578 ± 0.037 moderate
DLM16	0.205 ± 0.023 weak	0.219 ± 0.038 weak	0.345 ± 0.015 weak	0.303 ± 0.052 weak	0.293 ± 0.010 weak	0.429 ± 0.061 weak	0.540 ± 0.028 moderate	0.634 ± 0.017 moderate	0.616 ± 0.051 moderate
DLM16-WV	0.203 ± 0.006 weak	0.218 ± 0.012 weak	0.355 ± 0.094 weak	0.337 ± 0.052 weak	0.253 ± 0.031 weak	0.336 ± 0.005 weak	0.434 ± 0.043 moderate	0.410 ± 0.008 weak	0.685 ± 0.069 moderate
DLM28	0.193 ± 0.011 no biofilm	0.245 ± 0.014 weak	0.275 ± 0.021 no biofilm	0.298 ± 0.05 weak	0.381 ± 0.016 weak	0.425 ± 0.043 weak	0.442 ± 0.103 moderate	0.489 ± 0.045 moderate	0.706 ± 0.033 moderate
DLM28-WV	0.196 ± 0.011 no biofilm	0.173 ± 0.011 no biofilm	0.245 ± 0.034 no biofilm	0.349 ± 0.023 weak	0.352 ± 0.051 weak	0.391 ± 0.026 weak	0.447 ± 0.045 moderate	0.382 ± 0.016 weak	0.387 ± 0.091 weak

'WV' indicates *L. monocytogenes* isolates exposed to white vinegar.

vinegar showed that 2, 3, and 1 weak biofilm producer isolates became no biofilm producer at 4 °C for 24, 48, and 72 h, respectively, while the no biofilm producer isolates (1 isolate for each incubation time) became weak biofilm producers. At 22 °C, only 1 moderate biofilm producer isolate became weak biofilm producer (for 72 h). Moderate biofilm producer isolates at 37 °C for 24, 48, and 72 h (2, 3, and 4 isolates, respectively) became weak biofilm producers, 2 weak biofilm producer isolates became moderate biofilm producer for 72 h, and 1 strong biofilm producer isolate became moderate biofilm producer. In conclusion, when the changes in the biofilm formation categories of the isolates after exposure to white vinegar are considered, it is apparent that the results vary from isolate to isolate for each temperature and time.

3.4. Effect of the subMIC concentrations of white vinegar on biofilm formation

The results showed that MIC/2 and MIC/4 concentrations of white vinegar increased the biofilm forming capacity of *L. monocytogenes* isolates by 21.2% and 17.1%, respectively. For MIC/2 concentration, the biofilm formation of 2 isolates decreased by 5.4% and 12.8%. Biofilm formation of other 7 isolates increased by 6.4%–85.2%. For MIC/4 concentration, biofilm formation of 3 isolates decreased by 10.2%, 7.4%, and 3.3%. Biofilm formation of the other 6 isolates increased by 2.8%–90.9% (Fig. 2). Exposure of L. *monocytogenes* to disinfectants at subinhibitory concentrations may reveal the presence of certain strains after cleaning and disinfection. This situation can be explained not only by the emergence of resistant strains but also by the formation of the biofilm in niches where disinfectants cannot reach and consequently consisted of the protected microenvironment that leads to a reduction in the concentration of the disinfectants (Martínez-Suárez et al., 2016). The results obtained in our study also support this phenomenon. The presence of white vinegar at subinhibitory level caused increase in the biofilm forming capacity

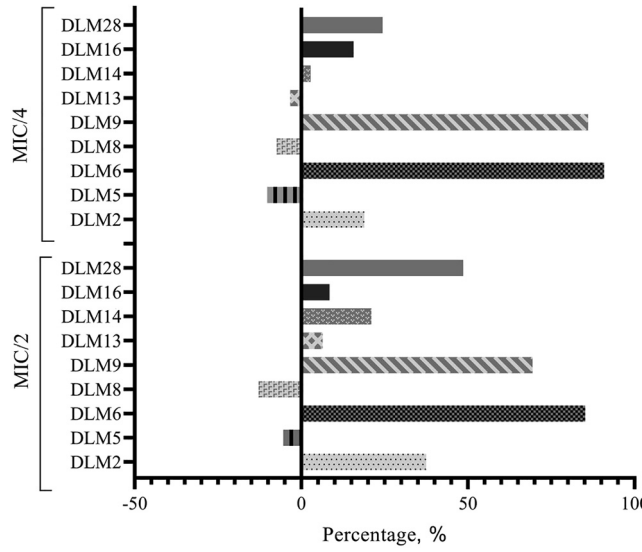

Fig. 2. Effect of the sub-MIC concentrations (MIC/2, MIC/4) of white vinegar on biofilm forming abilities of *L. monocytogenes* isolates

of *L. monocytogenes* isolates. It can also be seen that the change in the biofilm forming capacity of the isolates may show differences for each strain.

4. CONCLUSIONS

The increasing negative perception of consumers about chemical substances leads consumers to different environmentally friendly alternatives. The fact that vinegar is frequently used in households for cleaning purposes, increases the possibility of pathogenic microorganisms carried into homes with purchased foods of encountering with subMIC concentrations of vinegar. Investigating the changes in antibiotic resistance profiles and biofilm forming capacities of isolates exposed to subMIC concentrations of white vinegar will provide a source of information for the development of new cleaning and disinfection techniques. Our results showed that the use of subMIC concentrations of white vinegar should be avoided in order to prevent microbial persistance and changes in the antibiotic resistance profiles during the routine food processing applications.

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