EFFECT OF CITRIC ACID ON BIOFILM FORMED BY *P. FLUORESCENS* STRAINS ISOLATED FROM RAW MILK SAMPLES OFFERED FOR CONSUMPTION

ABSTRACT

Aims: In this study, the antibiofilm activity of citric acid on *P. fluorescens* isolated from raw milk samples was studied.

Background: Due to the resistance it gives to *Pseudomonas* bacteria, the presence of biofilm has been mentioned in recent studies. Biofilm is defined as the irreversible mucoid layer that microorganisms form on any surface and milk biofilms, which are the cause of contamination in milk, are a major concern in the dairy industry.

Methods: In this study, antibiofilm activity of citric acid and chlorine was investigated in 16 *Pseudomonas fluorescens* strains isolated from raw milk samples. For this purpose, the prevention and removal of biofilm formation of *P. fluorescens* strains was determined comparatively after treatment with microtitration plates with chlorine or citric acid.

Results: It was found that after treatment of microplates with citric acid, biofilm formation in *P. fluorescens* isolates was prevented by 52% and eliminated by 71-78%. It was also found that after the microplates were treated with chlorine, biofilm formation was prevented by 48% and eliminated by 61%.

Conclusion: This study showed that it was observed that citric acid can be used as an antibiofilm against biofilms produced by *P. fluorescens* bacteria.

Keywords: *P. fluorescens*, Biofilm, Citric acid, Antibacterial activity

INTRODUCTION

Pseudomonas are bacteria that are classified in the *Pseudomonadaceae* family. *Pseudomonas* species commonly found in nature have gram negative, nonfermentative and aerobic

properties. Some of its types are pathogens for humans, animals and plants. *P.aeruginosa, P.fluorescens, P.putida and P.stutzeri* are important species that are infectious in humans [1]. *Pseudomonas* are opportunistic microorganisms that cause many important diseases such as endocarditis, pneumonia and bacteremia [2,3]. *Pseudomonas* bacteria are also very important for foods as they are a factor of degradation due to their rapid reproduction in foods due to their aerobics. They can also synthesize the factors and vitamins necessary for them to reproduce. They cannot reproduce in an oxygen-free environment and above 42ºC. *Pseudomonas* bacteria have psychrophil, mesophile or psychotrophic species [4]. Bacteria of *Pseudomonas* genus can decompose in many food compositions, especially meat, milk and fish, causing them to degrade. In addition, they have the ability to degrade vegetable carbohydrates, fatty and fatty acids [1]. Many types of microorganisms can be found in foods. While some of the bacterial species persist in food, some are used in the production of various foods. Many of them can cause deterioration in food structure or foodborne diseases [5]. Food-borne diseases caused by consuming foods contaminated with pathogenic microorganisms or toxins, often show gastrointestinal symptoms [6]. Milk and dairy products play an important role in strengthening the immune system. The composition of milk includes calcium, phosphorus and riboflavin. Besides vital amino acids and fatty acids, there are important factors such as lactose, milk fat, casein, lactoalbumin and lactoglobulin [7]. Since raw milk is high in nutrients, it creates an excellent environment for many microorganisms to reproduce and more than 160 species of bacteria have been detected in it [8]. The microbiological structure of milk and dairy products provides an environment for many diseases, but also constitutes an important problem in terms of public health. The most common *Pseudomonas* species identified in these foods are *P. fluorescens, P. gessardii, P. fragi and P. lundensis* [9,10]. *Pseudomonas* bacteria, especially *P. fluorescens* in raw milk, have difficulties in controlling their reproduction during cold storage, and consequently the

negative effects on milk or dairy products [11]. While biofilm is found to be attached to the surface or to each other in terms of reproduction, genetic structure, protein synthesis, planktonic cells; microbial substances that have been embedded within the matrix or extracellular polysaccharide substance (EPS) [12]. Biofilm layer can protect microorganisms from living tissue surfaces as well as protecting against nutritional deficiencies, pH changes, toxins and antibiotics [13]. Biofilms can occur quickly in food processing environments. First of all, it is the arrangement of the material surface and the cells are reversibly connected to that surface. Then, attachment becomes irreversible and the development of microcolonies begins. Later, the three-dimensional structure of the biofilm occurred and a complex ecosystem ready to disintegrate was formed [14]. Biofilms in food environments become the place where pigment-producing *Pseudomonas* bacteria accumulate. In addition, biofilms can make bacteria more resistant to various environmental stresses such as cooling, acidity, salinity and disinfection in food processing [15]. Microorganisms in Biofilm can be protected from disinfectants due to their environmental relations, the presence of extracellular polymeric substances and the grounding of contamination of processed dairy products. Bacteria in milk can cause the formation of biofilm in milk storage tanks and milk processing departments, as it has the ability to adhere and accumulate on stainless steel surfaces. And pathogenic microorganisms can grow in this structure and cause milk deterioration. Reducing the biofilms formed by *Pseudomonas* species on the materials and equipment used in the milk processing stages are very important processes in reducing milk pollution [16,17,18]. In addition to causing serious economic and health problems, biofilms formed in food production may cause the metal surfaces used to be corroded by some bacteria. In addition, *Pseudomonas*, *Bacillus* and some other types of bacteria can secrete many proteolytic and lipolytic enzymes that can create unpleasant odor and taste [19]. In microorganisms, the basis of the bacteriostatic and bactericidal properties of organic acids is that they have the ability to

dissolve in the pH-neutral cytoplasm by passing through the semipermeable membrane and to decrease the pH of the cytoplasm [20, 21]. The antibacterial effect of citric acid on *E. coli, S. typhimurium, S. aureus, L. monocytogenes, Y. enterocolitica* was investigated. In addition, citric acid was reported to be more effective than lactic and hydrochloric acid in a study on the inhibition of S. typhimurium species in milk that was acidified with citric acid [22]. In their study, they investigated the effect of ultrasound application with weak organic acids (citric, lactic and malic acid) on *E. coli* O157: H7, *S. typhimurium* and *L.monocytogenes*, and as the concentration of citric acid is increased (0.3-2.0%), more microorganisms are inhibited and stated that 2.0% citric acid concentration is the most effective [23]**.** The effect of citric acid and lactic acid has been investigated to inactivate *V. parahaemolyticus* isolated from oyster. Accordingly, the natural and strong effects of lactic acid and citric acid solutions on bacterial species have been demonstrated [24]. The most preferred disinfectant in eliminating biofilm formation is chlorine. However, it has been observed that chlorine affects the biofilm structure to a limited extent. Chlorine can form harmful residues in the cell by forming compounds with organic carbon [25]. In addition, some types of reactive chlorine can be deactivated on the surface without affecting the interior of the biofilm [26]. In addition to chlorine, various organic acids can be used to prevent and remove the biofilms of *Pseudomonas* species, which is one of the important problems related to food production, especially in raw milk. For this purpose, the antibiofilm effect of citric acid and chlorine was investigated against *P. fluorescens* biofilms.

MATERIALS AND METHODS

In this study, 16 *P. fluorescens* isolates isolated from the raw milk samples offered for consumption were used. Bacterial isolates were stored in glycerol liquid medium (glycerol, 20% v / v; Sigma Aldrich, St. Louis, MO, USA) to maintain its viability from freezing. The

ability to form biofilms in *P. fluorescens* isolates was determined by quantitative method. For this purpose, after incubating the isolates in nutrient agar, 2 cc suspension of 0.5 Mc Farland (~ 1.5 × 108 CFU / ml) was prepared in the tubes containing 1% glucose luria bertoni (LB) medium from their fresh cultures. Then, 200 microliters of 96-well polystyrene microtitration plates were distributed from the prepared suspension and kept in an aerobic environment for 24 hours at 37 ° C. After incubation, the microplate was washed three times with 0.2 ml of phosphate buffered water (PBS; pH 7.4) and dried at room temperature. Then 200 microliters of 0.1% crystal viole (Sigma-Aldrich, St. Louis, MO) solution was distributed to all wells and kept at room temperature for 15 minutes. Biofilm formation was observed macroscopically on the walls of the wells. These wells were dissolved by adding 200 microliters of 95% ethanol. Then, it was read in a spectrophotometer device (Versamax Tunable, Microplate Reader; Molecular Devices®) with a wavelength of 570 nm [27,28]. In the study, microplate wells were treated with chlorine (200 mg / kg) or citric acid (2%, w / v) for 20 minutes to prevent biofilm and dried at room temperature. Then, after incubating the isolates in nutrient agar according to the recipe mentioned above, the presence of biofilm was investigated. In order to remove biofilm, the microplate wells prepared and incubated according to the above recipe were washed three times with distilled water in the study. The wells were then treated with chlorine (200 mg / kg) or citric acid (2%, w / v) for 20 minutes and dried at room temperature. Then, 200 microliters of 0.1% crystal viole (Sigma-Aldrich, St. Louis, MO) solution was distributed to all wells and kept at room temperature for 15 minutes. These wells were dissolved by adding 200 microliters of 95% ethanol. Then, it was read in a spectrophotometer device (Versamax Tunable, Microplate Reader; Molecular Devices®) with a wavelength of 570 nm. Biofilm experiments were performed three times for each strain and the average absorbance value was determined. The wells with liquid medium without bacterial isolate were used as negative control, the wells not treated with *Pseudomonas aeruginosa* PAO1

strain added, chlorine (200 mg / kg) or citric acid (2%, w / v) were used as positive control [29,30,31]. In the investigation of the presence of biofilm using microplate, there is no internationally approved reference value [32]. In the prevention and elimination of biofilm formation, the percentage of its effectiveness is explained according to the formula below.

Biofilm reduction value: ((C-B) - (T-B) / C-B)) X 100

A: Average absorbance value in wells with Biofilm

B: Average absorbance value in wells without bacterial isolate

T: Average absorbance value in wells treated with citric acid or chlorine [33]

In the study, the results obtained from microplate wells that are not treated with citric acid or chlorine and the reduction rates in the prevention and elimination of biofilm formation were determined.

Statistical analysis

In the strains included in the study, the statistical significance of comparing the effect of chlorine or citric acid on biofilm formation was evaluated by chi-square test using SPSS 21.0 (Chicago, Illinois) program. In statistical evaluation, (P) value was considered significant if $P < 0.05$.

FINDINGS AND DISCUSSION

In our study, the rates of prevention and elimination of biofilm formation in wells in polystyrene microtiter plates, which were applied chlorine (200 mg / kg) or citric acid (2%, w / v), were determined in percent (%). For this purpose, the effect of chlorine or citric acid on the biofilm formation of 16 *P. fluorescens* isolates in microplates isolated from raw milk was determined using dyes called crystal violet. All biofilm formation experiments were repeated three times and the mean value was determined. Standard deviations are shown in brackets.

In the study, as a result of the treatment of microplates with citric acid (2%, w / v), 16 *P. fluorescens* isolates, 52% in one strain (P <0.05), 45% in one strain (P <0.05) and 4% in four strains. 21- 37% (P <0.05) rate of biofilm formation was prevented (Table 1). In another study, after treatment with polystyrene microplates with citric acid (2%, w / v), 71-78% (P <0.05) in three strains, 63-69% (P <0.05) in four strains, 51-58% in three strains It was observed that the formation of biofilm was eliminated in the ratio (P <0.05) (Table 1).

Table 1. P. fluorescens biofilm formation rates (%) in polystyrene microplates treated with citric acid (2%, w / v)

Strain No.	A	B
1	7 (0.00)	64 (0.00)
2	15 (0.00)	58 (0.01)
3	34 (0.02)	71 (0.06)
4	2 (0.00)	38 (0.00)
5	0 (0.02)	63 (0.02)
6	9 (0.00)	48 (0.05)
7	1 (0.00)	42 (0.01)
8	3 (0.01)	51 (0.00)
9	52 (0.01)	78 (0.00)
10	27 (0.00)	53 (0.03)
11	12 (0.01)	65 (0.01)
12	45 (0.02)	72 (0.00)
13	4 (0.05)	46 (0.01)
14	21 (0.08)	37 (0.03)
15	37 (0.00)	69 (0.00)
16	3 (0.01)	41 (0.01)

A: Prevention of Biofilm B: Biofilm Elimination
(Results are averaged over three replicates. Standard deviations are shown in parentheses).

In the study, as a result of the treatment of microplates with chlorine (200 mg / kg), 16 *P. fluorescens* isolates were 48% (P <0.05) in one strain, 35% (P <0.05) in one strain and 20-29% in five strains. It was observed that the formation of biofilm (P <0.05) was prevented at the rates (P <0.05) (Table 2).

In addition, as a result of application of chlorine (200 mg / kg) to microplates, 16 *P. fluorescens* isolates, 61% (P <0.05) in one strain, 58% (P <0.05) in one strain, 51% (P <0.05)

in one strain, and 41-32% (P <0.05) biofilm formation was eliminated in four strains(Table 2).

Table 2. P. fluorescens biofilm formation rates (%) in polystyrene microplates applied with chlorine (200 ppm)

Strain No.	A	B
1	1(0.00)	23(0.00)
2	11(0.02)	27(0.01)
3	3(0.01)	58(0.03)
4	20(0.00)	34(0.00)
5	0(0.03)	16(0.00)
6	22(0.00)	18(0.01)
7	0(0.00)	32(0.00)
8	3(0.02)	61(0.00)
9	6(0.01)	47(0.02)
10	29(0.00)	37(0.04)
11	48(0.01)	25(0.00)
12	23(0.02)	51(0.02)
13	9(0.00)	24(0.05)
14	35(0.03)	0(0.02)
15	27(0.00)	0(0.00)
16	2(0.01)	41(0.01)

A: Prevention of Biofilm B: Biofilm Elimination
(Results are averaged over three replicates. Standard deviations are shown in parentheses).

Foodborne pathogens and the biofilms they create are commonly found in the natural environment and in many habitats. In addition, food pathogens cause bacterial food poisoning, which seriously endangers human health and can cause major economic losses [34,35]. In recent years, diseases caused by food borne pathogens, morbidity and mortality factors in many parts of the world have become an important public health problem. In addition, many other identified pathogen outbreaks have been found to be associated with biofilms [36,37]. According to the World Health Organization (WHO) report, foodborne diseases are seen as an important public health problem that occurs in both developed and developing countries [38]. Biofilms are bacterial communities located in a matrix of nucleic acids, polysaccharides, lipids and proteins, which are formed by the ability of microorganisms to hold on to a suitable surface and then multiply [18]. Many *Pseudomonas* species use biofilm formation on different

surfaces during their colonization and lead to the production of various biofilm matrix molecules [39]. Biofilms are of great importance for the milk and milk processing industry. Because it allows bacteria to adhere to various surfaces such as stainless steel, plastic and polypropylene in a short time, and biofilms can mature within a few hours or even a few days [18]. Raw milk can be contaminated by milk collection materials and utensils during milking and storage due to inadequate hygiene. Milk is very sensitive to contamination. It can be an effective source of transmission of foodborne pathogens, especially gram negative bacteria, in humans [40]. *Pseudomonas, Enterococcus, Listeria, Bacillus* are among the most common bacterial species in the dairy industry [41,42]. **(**In a study in raw milk, *Pseudomonas, Lactococcus* and *Acinetobacter* are the 62% most common bacteria among all isolates [8]. The materials used from the collection of raw milk to the milk production stage, mostly *Pseudomonas* bacteria, provide a favorable physicochemical environment for the formation of a wide spectrum of microorganisms. Among the *Pseudomonas* species, *P. fluorescens* is considered as the most important deterioration factor in milk tanks [43]. In the research on microorganisms and biofilms formed in raw milk tanks, they found a large number of microorganisms that determine the presence of microbial biofilms. In addition, *Pseudomonas* were found to be the most common species and biofilm was detected in 50.2 of the isolates [44]. Many bacterial species need the optimum pH environment for their reproduction and cannot survive under extremely acidic conditions. Organic acids can prevent or prevent the growth and reproduction of bacteria sensitive to the acidic environment by lowering the pH value. Weak organic acids such as acetic, citric, benzoic, sorbic and lactic acids are often used to limit microbial growth [45, 46]. In the study of Bjarnsholt et al., they showed that using both acetic acid, which is one of the organic acids, biofilms formed by both gram positive and gram negative bacteria can be completely destroyed [47]. They found that organic acids such as malic, citric, lactic and tartaric acid have antibacterial properties under specific pH

conditions. They also found that organic acids can affect food-borne microbial pathogens or cause significant damage to the cytoplasm as microorganism cells can spread throughout the cell membrane [48]. In a study to investigate the ability of *Pseudomonas* bacteria to form biofilms, it was revealed that they tend to form biofilms on both polystyrene and stainless surfaces [49]. In another study investigating the antimicrobial effect of citric acid, the cattle carcass was washed with citric acid solution and it was found that the number of *P. fluorescens* decreased when the pH decreased to 4 [22]. The inhibitory effect of organic acids was investigated using citric acid on *Y.enterocolitica*, and in their study at different concentrations and at different temperatures, they stated that although there was an exponential inactivation at 4 and 20 ° C, no inactivation was observed at 40 ° C. The inactivation effect of citric acid was found to be dependent on time, the temperature it was exposed to, and the acid concentration [50]. They also investigated the effect of different concentrations of citric acid for the inhibition of pathogens in the egg and determined that citric acid reduced the population of *E. coli, S. typhimurium, S. aureus, L. monocytogenes, Y. enterocolitica* [51]. In this study, it was observed that by the treatment of polystyrene surfaces of microplates with citric acid (2%, w / v), in 16 *P. fluorescens* isolates, biofilm formation was highest, 52%, and 71-78% was eliminated. Faot et al. explained that citric acid can reduce the production and viability of biofilm created by *C. albicans* [52] In another study, they report that citric acid isolated from tomato inhibits microorganisms, shows very little bacteriostatic activity at pH 5.0, and inhibition increases with decreasing pH [22]. In their study using media containing glucose and milk, it was determined that biofilms formed by *B. cereus* vegetative cells were prevented by 59% and removed by 38-63% with the effect of citric acid and reported that citric acid affects the biofilms of *B. cereus* vegetative cells as much as chlorine [30]. In their study, Tsai et al. Investigated the effect of citric acid on the biofilm formed by bacteria colonized on the surface of the water pipes and showed that citric acid is highly effective [53].

In the study, it was reported that citric acid delayed the development of E. coli O157: H7 colonies, but did not completely stop it [54]. Sommer et al. investigated the effect of citric acid on *L. monocytogenes*' radiation resistance and the quality of sausages, and reported that citric acid reduced the radiation resistance of *L. monocytogenes* inoculated on the surface of sausages [55]. They demonstrated that the formation of *P. aeruginosa* biofilms occurring in 24 hours was highly reduced with a concentration of 4% chlorine solution [17]. In the study, it was found that by treating the polystyrene surfaces of microplates with chlorine (200 mg / kg), in *P. fluorescens* isolates, biofilm formation was prevented at the highest rate, 48% and eliminated by 61%. In the results obtained from the study, the role of citric acid in the prevention and elimination of biofilm formation was found to be more effective compared to chlorine.

CONCLUSION

Studies show that especially *P. fluorescens* strains have a high biofilm ability in milk and dairy products. In order to prevent contamination and biofilm control in various tools and devices used in the food industry, more detailed studies should be carried out at different times, temperatures and concentrations in order to use non-toxic, citric acid or other natural substances. In our study, it was observed that citric acid can be used as against biofilms produced by *P. fluorescens* bacteria. As a result, it has been determined that it is preferable to use environmentally friendly citric acid in the dairy industry to prevent and eliminate biofilm formation of *P. fluorescens* strains.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advance-

ment of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

REFERENCES

1. Palleroni NJ. Family 1. *Pseudomonadeceae;* Genus: *Pseudomonas,* Brenner DJ, Krieg NR, Staley JT: Bergey's Manual of Sistematic Bacteriology., Garrity GM, Sc. D. East Lansing-U.S.A. 2005; (2): 323-379.

2. Todar K. *Pseudomonas*. Todar's Online Textbook of Bacteriology. Science Magazine. 2004; 304:1421-1425.

3. Pollack M.*Pseudomonas aeruginosa*. In:Mandell GL, Dolan R, Benett J E. (eds): Principles and Practice of Infectious Diseases. Churchill Livingston Inc. New York. 4. th. ed. 1995; pp.1980-2003.

4. Şen A and Halkman A K. Çiğ Sütte *Pseudomonas aeruginosa* Sayılması için Yöntem Modifikasyonları Üzerine Çalışmalar. Orlab On-Line Mikrobiyoloji Derg. 2006; 4(2), 02, p. 2-13.

5. Erkmen O. Gıda Mikrobiyolojisi. 3. Baskı, Eflatun Yayınevi, Ankara. 2011;pp. 40-172.

6. Carrique-Mas JJ, and Bryant JE. A review of foodborne bacterial and parasitic zoonoses in Vietnam. Eco Health. 2013;10(4): 465-489.
doi: 10.1007/s10393-013-0884-9

7. FitzGerald R J and Meisel H. Milk protein-derived peptide inhibitors of angiotensin-I-

converting enzyme. Br. J. Nutr. 2000; 84:33–37.
doi: 10.1017/s0007114500002221.

8. von Neubeck M, Baur C, Krewinkel M, Stoeckel M, Kranz B, Stressler T, Fischer L, Hinrichs J, Scherer S, Wenning M. Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. Int. J Food Microbiol. 2015; 211: 57-65.
doi.org/10.1016/j.ijfoodmicro.2015.07.001

9. de Buyser ML, Dufour B, Maire M, Lafarge V. Implication of milk and milk products food-borne diseases in France and in different industrialised countries. Int. J. Food. Microbiol. 2001; 20;67(1-2): p.1-17.
doi: 10.1016/s0168-1605(01)00443-3

10. Mallet A, Guéguen M, Kauffmann F, Chesneau C, Sesboué A, Desmasures N. Quantitative and Qualitative Microbial Analysis of Raw Milk Reveals Substantial Diversity Influenced by Herd Management Practices. International Dairy Journal. 2012; 27(1-2): pp.13-21.
doi.org/10.1016/j.idairyj.2012.07.009

11. de Oliveira GB, Favarin L, Luchese RH, McIntosh D. Psychrotrophic bacteria in milk: How much do we really know? Braz. J. Microbiol. 2015; 46(2): 313-321.
doi: 10.1590/S1517-838246220130963

12. Donlan RM. and Costerton JW. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clinical Microbiology Reviews. 2002;15 (2):167-193.
doi: 10.1128/CMR.15.2.167-193.2002

13. Lindsay D and vonHoly A. Bacterial biofilms within theclinical setting: what healthcare professionals should know. J. Hosp. Infect. 2006; 64 (4): 313-325. doi: 10.1016/j.jhin.2006.06.028

14. Coughlan LM, Cotter PD, Hill C, Alvarez-Ordóñez A. New Weapons to Fight Old Enemies: Novel Strategies for the (Bio) control of Bacterial Biofilms in the Food Industry. Front. Microbiol. 2016;7:1641. doi.org/10.3389/fmicb.2016.01641

15. Giaouris E, Heir E, Hébraud M, Chorianopoulos N, Langsrud S, Møretrø T, Habimana O, Desvaux M, Renier S, Nychas GJ. Attachment and biofilm formation by foodborne bacteria in meat processing environments: causes, implications, role of bacterial interactions and control by alternative novel methods. Meat Sci. 2014; 97(3):298-309. doi.org/10.1016/j.meatsci.2013.05.023

16. Simões M, Simões LC, Vieira MJ. A review of current and emergent biofilm control strategies. LWT- Food Sci. Technol. 2010; 43(4), 573-583. doi.org/10.1016/j.lwt.2009.12.008

17. Meesilp N and Mesil N. Effect of microbial sanitizers for reducing biofilm formation of Staphylococcus aureus and *Pseudomonas aeruginosa* on stainless steel by cultivation with UHT milk. Food Science and Biotechnology. 2019; 28(1): pp. 289–296. doi.org/10.1007/s10068-018-0448-4.

18. Marchand S, Block J De, Jonghe VDe, Coorevits A, Heyndrickx M, Herman L. Biofilm formation in milk production and processing environments; influence on milk quality and safety. Compr. Rev Food Sci. F. 2012; 11(2):133–147. **doi.org/10.1111/j.1541-4337.2011.00183.x**

19. Galié S, García-Gutiérrez C, Miguélez E M, Villar C J, Lombó F. Biofilms in the Food Industry: Health Aspects and Control Methods. Front Microbiol. 2018; 9: 898. doi.org/10.3389/fmicb.2018.00898

20. Cherrington CA, Hinton M, Mead GC, Chopra I. Organic acids: Chemistry, antibacterial activity and practical applications. Advances in Microbiology and Physiology.1991;32: 87-108. doi.org/10.1016/S0065-2911(08)60006-5

21. Booth IR. and Stratford M. Acidulants and low pH. In: (Russell NJ., Gould GW eds.) Food preservatives. Kluwer Academic/Plenum Publishers, New York. 2003; pp. 25–47.

22. Davidson MP and Branen AL. Antimicrobials in Foods. 2nd. Edition, New York. 1993; pp.647.

23. Sagong HG, Lee SY, Chang PS, Heu S, Ryu S, Choi YJ, Kang DH. Combined effect of ultrasound and organic acids to reduce *Escherichia coli* O157:H7, *Salmonella Typhimurium*, and *Listeria monocytogenes* on organic fresh lettuce. Int J Food Microbiol.2011;145(1):287-292. doi.org/10.1016/j.ijfoodmicro.2011.01.010

24. Barakat SMM. The efficacy of grape seed extract, citricacid and lactic acid on the inactivation of *Vibrio parahaemolyticus* inshucked oysters.Food Control.2014;41,13-16. doi:10.1016/j.foodcont.2013.12.027

25. Chandy JP, and Angles ML. Determination of nutrients limiting biofilm formation and the subsequent impact on disinfectant decay. Water Res. 2001; 35(11): 2677-82. doi: 10.1016/s0043-1354(00)00572-8

26. de Beer D, Stoodley P, Roe F, Lewandowski Z. Effects of biofilm structures on oxygen distribution and mass transport. Biotechnol. Bioeng. 1994;43(11):1131-8. doi : 10.1002/bit.260431118

27. Musk DJ, Banko DA, Hergenrother PJ. Iron salts perturb biofilm formation and disrupt existing biofilms of *Pseudomonas aeruginosa*. Chem Biol. 2005;12 (7): 789-796. doi:10.1016/j.chembiol.2005.05.007

28. Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M. A modified microtiter-plate test for quantification of *Staphylococcal* biofilm formation. J Microbiol Methods.2000; 40(2):175–9. doi: 10.1016/s0167-7012(00)00122-6.

29. Rossi C, Chaves-López C, Serio A, Goffredo E, Goga B T C, Paparella A. Influence of Incubation Conditions on Biofilm Formation by *Pseudomonas fluorescens* Isolated from Dairy Products and Dairy Manufacturing Plants. Ital. J Food Saf. 2016; 3: 5(3): 5793.

doi: 10.4081/ijfs.2016.5793

30. Akbaş MY and Şar T. Control of *B. cereus* Biofilms By Citrıc Acid Treatments. GIDA. 2018; 43(4): p.605-616.
doi.org/10.15237/gida.GD18041

31. Meng L, Zhang Y, Liu H, Zhao S, Wang J, Zheng N. Characterization of *Pseudomonas* spp. and Associated Proteolytic Properties in Raw Milk Stored at Low Temperatures. Front Microbiol. 2017; 8: 2158.
doi: 10.3389/fmicb.2017.02158

32. Mendoza-Olazarán S, Camacho-Ortiz A, Martínez-Reséndez MF, Llaca-Díaz JM, Pérez-Rodríguez E, Garza-Gonzalez E. Influence of whole-body washing of critically ill patients with chlorhexidine on *Acinetobacter baumannii* isolates. Am. J. Infect. Control. 2014; 42: 874–878.
doi:10.1016/j.ajic.2014.04.009

33. Pitts B, Hamilton MA, Zelver N, Stewart P S. A microtiter-plate screening method for biofilm disinfection and removal. J Microbiol Methods. 2003; 54(2):269-76.
doi: 10.1016/s0167-7012(03)00034-4

34. Kusumaningrum HD, Riboldi G, Hazeleger WC, Beumer RR. Survival offoodborne pathogens on stainless steel surfaces and cross-contamination to foods. Int J Food Microbiol. 2003; 85(3):227-36.
doi: 10.1016/s0168-1605(02)00540-8

35. Camargo AC, Woodward JJ, Call DR, Nero LA. *Listeria monocytogenes* in food-processing facilities, food contamination, and human listeriosis: the Brazilian Scenario. Foodborne Pathog. Dis. 2017; 14(11): 623–636.
doi: 10.1089/fpd.2016.2274

36. Aarnisalo K, Lundén J, Korkeala H, Wirtanen G. Susceptibility of Listeria monocytogenes Strains to Disinfectants and Chlorinated Alkaline Cleaners at Cold Temperatures. LWT-Food Sci. Technol. 2007; 40(6), 1041–1048.
doi:10.1016/j.lwt.2006.07.009

37. Zhao X, Lin CW, Wang J, Oh D H. Advances in rapid detection methods for foodborne pathogens. *J. Microbiol. Biotechnol.* 2014; 24(3): 297–312.
doi: 10.4014/jmb.1310.10013

38. WHO. Fact Sheet 237: Food Safety and Foodborne Illness. World Health Organization, Geneva, Switzerland. www.who.int/mediacentre/factsheets/fs237/ en/, accessed 5/5/04. 2002.

39. Mann EE and Wozniak DJ. *Pseudomonas* biofilm matrix composition and niche biology. FEMS Microbiol Rev. 2012; 36(4):893–916.
doi: 10.1111/j.1574-6976.2011.00322.x.

40. Garedew L, Berhanu A, Mengesha D, Tsegay G. Identification ofgram-negative bacteria from critical control points of raw and pasteurized cow milk consumed at Gondar town and its suburbs, Ethiopia. BMC Public Health. 2012; 12: 950.

doi.org/10.1186/1471-2458-12-950

41. Sharma M, Anand SK, Prasad DN (2003). In vitro propagation of mixed species biofilms using online consortia for dairy processing lines. Milchwissenschaft. 58:270-273.

42. Salo S, Ehavald H, Raaska L, Vokk R, Wirtanen G. Microbial surveys in Estonian dairies. LWT Food Sci. Tech. 2006; 39(5):460-471.
doi.org/10.1016/j.lwt.2005.03.008

43. McPhee JD and Griffiths MW. *Psychrotrophic* bacteria *Pseudomonas* spp. In: Fuguay J W, editor. Encyclopedia of Dairy Sciences. Second Edition. Academic Press; San Diego. 2011; pp. 379–383.
doi:10.1016/B978-0-12-374407-4.00441-6

44. Flach J, Grzybowski V, Toniazzo G, Corcão G. Adhesion and production of degrading enzymes by bacteria isolated from biofilms in raw milk cooling tanks. Food Sci. Technol (Campinas). 2014; 34(3), pp.571-576.
doi.org/10.1590/1678-457x.6374

45. Stratford M and Eklund T. Organic acids and esters. In: (Russell NJ, Gould GW. eds) Food preservatives, Kluwer Academic/Plenum Publishers, New York. 2003; pp. 48-84.

46. Skrivanova E, Marounek M, Benda V, Brezina P. Susceptibility of *Escherichia coli*, *Salmonella sp.* and *Clostridium perfringens* to organic acids and monolaurin. Veterinarni Medicina. 2006; 51(3):81-88.
doi:10.17221/5524-VETMED

47. Bjarnsholt T, Alhede M, Jensen PQ, Nielsen AK, Johansen HK, Homøe P, Høiby N, Givskov M, Kirketerp-Møller K. Antibiofilm Properties of Acetic Acid. Adv Wound Care (New Rochelle). 2015; 1; 4(7): 363–372.
doi: 10.1089/wound.2014.0554

48. Eswaranandam S, Hettiarachchy NS, Johnson MG. Antimicrobial activity ofcitric, lactic, malic, or tartaric acids and nisin-incorporated soy protein film against *Listeria monocytogenes, Escherichia coli* O157:H7 and *Salmonella* gaminara. Journal of Food Science. 2004;69: 79-84.
doi.org/10.1111/j.1365-2621.2004.tb13375.x

49. Yuan L, Sadiq FA, Burmølle M, Wang N, He G. Insights into Psychrotrophic Bacteria in Raw Milk: A Review. Journal of Food Protection. 2019; 82(7): pp. 1148-1159.
https://doi.org/10.4315/0362-028X.JFP-19-032

50. Virto R, Sanz D, Alvarez I, Condón RJ. Inactivation kinetics of *Yersinia enterocolitica* by citric and lactic acid at different temperatures. Int. J. Food Microbiol. 2004;103(3): 251-257.
doi: 10.1016/j.ijfoodmicro.2004.11.036

51. Fisher JR, Fletcher DL, Cox NA, Bailey JS. Microbiological properties of hard cooked eggs in a citric acid-based preservation solution. J. Food Prot.1985;48(3):252.
doi: 10.4315/0362-028X-48.3.252

52. Faot F, Cavalcanti YW, Mendonça e Bertolini M, Pinto LR, Silva WJ, Cury AAD.

Efficacy of citric acid denture cleanser on the *Candida albicans* biofilm formed on poly (methyl methacrylate): effects on residual biofilm and recolonization process. BMC Oral Health. 2014;23;14:77.
doi: 10.1186/1472-6831-14-77

53. Tsai YP, Pai TY, Hsin JY, Wan TJ. Biofilm bacteria inactivation by citric acid and resuspension evaluations for drinking water production systems. Water Sci Technol. 2003; 48 (11-12): 463-72.
doi:10.2166/wst.2004.0895

54. Ryu JH, Deng Y, Beuchat LR. Behavior of acid adapted and unadapted E. coli O157:H7 when exposed to reduced pH activited with various organic acids. J Food Prot. 1999; 62(5):451-455.
doi: 10.4315/0362-028x-62.5.451

55. Sommer CH, Fan X, Handel AP, Sokarai KB. Effect of citric acid on the radiation resistance of *Listeria monocytogenes* and Frankfurter quality factors. Meat Science. 2003; 63 (3): 407-415.
doi: 10.1016/s0309-1740(02)00100-6