Effectiveness of Organic Acid Treatments for Inhibition and Removal of *E. coli* Biofilms

E. coli Biyofilmlerinin Önlenmesi ve Ortadan Kaldırılmasında Organik Asit Uygulamalarının Etkileri

Research Article

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ABSTRACT

This study was carried out to evaluate the efficacies of organic acid (citric, malic and gallic acids) treatments at 1% and 2% concentrations for prevention and removal of *E. coli* biofilms. Antibiofilm effects of organic acids were compared with chlorine (100 ppm and 200 ppm) on both microtitration plate and stainless steel coupons for 5, 10 and 20 min. Results indicated that citric acid treatments when compared to the chlorine treatments were effective for inhibition and removal of *E. coli* biofilms. On the other hand, gallic acid treatments were found to be more effective than malic acid treatments. On stainless steel surfaces, the inhibition and removal of biofilms were observed to be higher than those found on microtitration plates. Moreover, the inhibition and removal ratios were calculated to be higher with increasing concentrations of sanitizers, on 24-h biofilm, on stainless steel coupons and with 20 min treatments. The results of this study indicates chlorine treatments could be replaced by organic acid treatments for inhibition and removal of biofilm formations of *E. coli* strains on different food contact surfaces. In addition, organic acid treatments are safe-to-use potential alternatives in industrial applications to chlorine treatments which is toxic to health and environment.

Key Words
Biofilm inhibition, biofilm removal, *E. coli*, food contact surfaces, organic acids.

ÖZET

Bu çalışmada, *E. coli* biyofilmlerinin önlenmesi ve ortadan kaldırılmasında %1 ve %2 derişimlerdeki organik asit (sitrik, malik ve gallik) uygulamalarının etkileri araştırılmıştır. Organik asitlerin antibiyofilm etkileri klor uygulamaları (100 ppm ve 200 ppm) ile 5, 10 ve 20 dakika süresince mikrotitrasyon plaklarında ve paslanmaz çelik kuponlarda karşılaştırılmıştır. Sonuçlar biyofilmlerin önlenme ve ortadan kaldırılmasında, klor uygulamaları ile karşılaştırıldığında, sitrik asit uygulamalarının etkili olduğunu göstermiştir. Gallik asit uygulamaları ise malik asit uygulamalarından daha etkili bulunmuştur. Çelik yüzeylerde, biyofilm önlenmesi ve ortadan kaldırılması oranları mikrotitrasyon plaklarından daha yüksek bulunmuştur. Ayrıca, biyofilm önlenme ve ortadan kaldırılma oranları, sanitizerların artan derişimlerinde, 24 saatlik biyofilm tabakası üzerinde, çelik yüzeylerde ve 20 dakikalık uygulamalarla daha yüksek değerlerde bulunmuştur. Çalışmanın sonuçları, farklı gıda ile temas eden yüzeylerde, *E. coli* biyofilmlerinin önlenmesi ve ortadan kaldırılmasında, organik asit uygulamalarının klor uygulamalarının yerini alabileceğini göstermektedir. Ayrıca organik asitler, endüstriyel uygulamalarda kullanılan sağlığa ve çevreye toksik etkili olan klorun kullanımının güvenli potansiyel alternatifleri olabilir.

Anahtar Kelimeler
Biyofilm önlenmesi, biyofilm ortadan kaldırılması, *E. coli*, gıda ile temas eden yüzeyler, organik asitler.

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INTRODUCTION

The control of food borne pathogens has received a great deal of attention in recent years. The reason for this is due to the fact that these organisms can form biofilms on different surfaces [1]. It is highly likely that they can cause significant health problems when they contaminate lifeline systems such as drinking water transmission lines and food processing environments [2].

In food industry, microbial cell transfer from biofilms to foods is a potential source of hazard for food safety and quality. In addition, food spoilage due to biofilms results in huge economic losses and has potential health risks [3]. Biofilm formation facilitates survival under stressed conditions such as ultraviolet radiation, physicochemical stresses and insufficient supply of nutritive resources. Previous studies indicate that bacteria in biofilms can be up to 10-1000 times more resistant to the effects of antimicrobial agents than planktonic bacterial cells [4,5]. The chemical control of biofilms is important due to the increased resistance of biofilm embedded bacteria to disinfectants. Resistance against various disinfectants gets higher as the biofilm matures, especially, after about 24 hours [6]. Therefore, one of the best ways of controlling biofilms is to prevent their development.

Food contact surfaces has great potential for allowing biofilm development. The adhesion surface properties and the bacterial cells are key factors that affect the biofilm formation. Since the food contact surfaces such as stainless steel, glass, rubber, and polypropylene surfaces can be contaminated easily by pathogenic microorganisms, the selection of surface material has substantial impact in designing food contact surfaces. Among these, stainless steel surfaces are preferred for food equipment and are specified in many industries [7].

Pathogenic bacteria can initiate cellular growth to make biofilms when adhered to the surface under certain conditions [8]. *Bacillus, Salmonella*, *Listeria*, *Staphylococcus* and *Escherichia* bacteria when adhered in food processing environment may cause food spoilage or transmission of food-borne diseases [3,9].

The presence of *E. coli* in foods such as milk, meat and vegetables is an indicator of fecal contamination causing outbreaks of many diseases [10]. A recent study has also shown that *E. coli* had the ability to attach strongly to leafy structures [11].

Food contact surfaces are commonly disinfected with different compounds such as peroxides, chloramines or hypochlorites [12]. These compounds should be effective enough as to inactivate the pathogens while maintaining organoleptic properties of the product [11]. Moreover, corrosion and toxicity limit the use of these commercial compounds [13].

Among the commercial sanitizers, chlorine in its various forms, is the most commonly used sanitizer in food industry. Liquid chlorine, hypochlorites, inorganic or organic chloramines are widely used chlorine compounds. Chlorine is a relatively cheap disinfectant and known to be active at low temperature. One of the major drawbacks of chlorine is that it causes corrosion on metal surfaces as well as skin irritation and mucous membrane damage. Moreover, the use of chlorine as a drinking water disinfectant causes the formation of potentially carcinogenic trihalomethanes (THMs) under certain conditions. This fact indicated the need of developing new and safe biofilm control strategies. The interest in antimicrobials derived from natural sources has increased in recent years [14,15]. One approach may be the use of organic acids (citric, malic, gallic acids etc.) as antimicrobial agents. Organic acids are widely distributed in plants and animals. They are considered to be safe in terms of human and animal health with no toxic residues. Organic acids can be as effective as chemical disinfectants [16-18]. Eswaranandam *et al.* [16] found that organic acids such as malic, citric, lactic, and tartaric acid had antibacterial activity at specific pH conditions. Over *et al.* [18] treated *Salmonella Typhimurium* inoculated boneless chicken breast meat with 150 mM citric acid, malic acid and tartaric acid. The researchers found that *S. Typhimurium* numbers stored at 4°C were near undetectable levels by the end of day 9.

Citric acid is a hydroxy tricarboxylic acid produced naturally by various plants. It is approved and classified as GRAS (21CFR184.1033) for use in the manufacture of fresh and processed poultry and meats [19]. Citric acid inhibits bacterial cells via metal chelation. In a previous study, dipping of fresh-cut iceberg lettuce in citric acid solution (0.5%, w/v) was found to be effective as chlorine solution (100 ppm) for inactivating pathogenic microorganisms [17].

Malic acid is a dicarboxylic acid found in many foods. The studies determined that it can be used to treat food borne pathogens [20,21]. It was reported that malic acid could inhibit the growth of *Listeria monocytogenes*, *Salmonella gaminara*, and *Escherichia coli* O157: H7 [22]. Malic acid affects food borne microbial pathogens by lowering the pH value [23] by causing severe damage to the cytoplasm of bacteria [16]. Singla *et al*. [24] found that malic acid was effective in food industry for complete inhibiton of *S. Typhimurium* biofilm in carrot and other food contact surfaces.

Gallic acid is one of the abundant polyphenol and widely distributed among plants [25]. It has been shown to possess strong antimicrobial activity [26]. Borges *et al*. [27] investigated the activity of gallic acid at 1000 μg/ml on the prevention and control of biofilms formed by *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *L. monocytogenes*. The researchers found that gallic acid has potential to prevent and control these pathogens by promoting reductions in biofilm activity higher than 70% of all tested microorganisms.

This study investigates the activity of organic acids (citric, malic and gallic acids) at 1% and 2% concentrations in order to prevent and remove (from 5 to 20 min treatment) 24-hour, 48-hour and 72-hour *E. coli* biofilms on both microtitration plate and stainless steel coupons. The surface materials (polystyrene microtitration plate and stainless steel), concentrations, treatment times and the biofilm age on the antibiofilm effects of these organic acid treatments are compared with a commercial, chlorine (100 ppm and 200 ppm) treatments on both surfaces.

MATERIALS AND METHODS

Microorganism and Culture Conditions

In this work, *E. coli* ATCC 25922 was used as a model microorganism. Stock culture of this strain was stored at -20ºC in TSB supplemented with 20% glycerol. Working culture was maintained on nutrient agar containing plates at 4ºC.

Preparation of Solutions

All sanitizing solutions were prepared daily using sterilized distilled water. For the purpose of obtaining a solution with concentrations of 100 mg/l (ppm) and 200 mg/l (ppm) free chlorine, chlorinated water was prepared by adding sodium hypochlorite (NaOCl) solution to pre-sterilized distilled water containing 13% active chlorine (Merck, Darmstadt, Germany). Chlorine concentrations within the solutions were measured by using a commercial chlorine test kit (Merck, Darmstadt, Germany). Citric, malic and gallic acids were provided from Merck (Darmstadt, Germany). They were used to prepare solutions containing citric acid (1% and 2%, w/v), gallic acid (1% and 2%, w/v), or malic acid (1% and 2%, w/v). The prepared fresh solutions were used within 30 min.

Biofilm Forming Ability on 96-well Polystrene Microtitration Plate

The modified microtitration plate test proposed by Stepanovic *et al*. [28] was performed to assess the biofilm formation ability of *E. coli* on microtitration plate. *E. coli* strain was grown overnight at 37ºC in tryptic soy broth (TSB, Becton, Dickinson, and Company, France) supplemented with 2% (w/v) D-glucose (TSBGlc). Following incubation at 37ºC for 24 hour, culture supernatant was diluted at a rate of 1:200 in fresh TSBGlc. An aliquot bacterial suspension (200 μl; about 10^5 cfu/ml, final cell density) was transferred into 96 well polystyrene microtitration plate (Nunc, Roskilde, Denmark). The medium without the bacterial suspension was used as the negative control during the test. The plates were incubated at 37ºC for three different time periods (24-, 48- or 72-hours). Then, culture supernatants from each well were first gently removed by pipetting and washed three times with distilled water. The attached cells were stained with 0.5% (w/v) crystal violet (150 μl) solution and the bound crystal violet was resolubilized with 33%

(v/v) glacial acetic acid (150 μL) for 10 min. The OD value of each well at 570 nm was measured using microplate reader (BMG Labtech Fluostar Omega ELISA, Germany). The microtitration plate biofilm assay was performed in triplicate. Biofilm formation was interpreted as positive for cases where OD570 > 0.1 or negative for cases where OD570 < 0.1 These OD values were considered for an index of bacteria adhering to surface and forming biofilms.

Biofilm Forming Ability on Stainless Steel

Stainless steel coupons (20x40x1 mm) were rinsed with distilled water and autoclaved at 121°C for 30 min. After autoclaving, the sterile steel coupons were placed in a 100 ml sterile glass container with 50 ml fresh TSBG. Then, the medium was inoculated with overnight culture of *E. coli* strain. The final cell density in TSBG was about 10^5 cfu/ml. The containers were incubated in an incubator at 37°C for 24, 48 or 72 hours. The steel coupons were transferred to a new 100 ml sterile container with 50 ml of 0.5% (w/v) crystal violet solution. The crystal violet bound to the biofilm was solubilized with 50 ml of 33% (v/v) glacial acetic acid for 10 min and the absorbance was determined at 570 nm by using the microplate reader (BMG Labtech Fluostar Omega ELISA, Germany). 150 μl of TSBG was dispensed into each well for negative controls. The biofilm assay was again performed in triplicate as in the case of microtitration plate assay.

All experiments on microtitration plates and stainless steel coupons were repeated three times on different days and with all solutions freshly prepared.

Inhibition of Biofilm Formation

Organic acids (citric, gallic and malic acids) at concentrations of 1% and 2% (w/v) were tested for comparison with chlorine solution (100 ppm and 200 ppm) ffor their potential to prevent biofilm formation of *E. coli* strain after 24-hour, 48-hour or 72-hour.

Inhibition of Biofilm Formation on 96-Well Polystrene Microtitration Plate

For this purpose, the wells of microtitration plates were loaded with citric, malic or gallic acids (1% or 2%, w/v) or chlorine (100 ppm or 200 ppm) solutions. After each treatment, each well was first decanted by pipetting and then, the wells were air dried at room temperature for 30 min. *E. coli* strain was grown overnight at 37°C on tryptic soy broth (TSB, Becton, Dickinson, and Company, France) supplemented with 2% (w/w) D-glucose (TSBGlc). Following the incubation at 37°C for 24-hour, the culture supernatant of *E. coli* strain was diluted at a rate of 1:200 in fresh TSBGlc. Aliquots of bacterial suspension (200 μL; $5x10^5$ cfu/ml, final concentration) were transferred into citric acid, malic or gallic acid (1% or 2% w/v) or chlorine solution (100 ppm or 200 ppm) treated polystyrene microtitration plate (Nunc, Roskilde, Denmark) for incubation at 37°C for 24-hour, 48-hour or 72-hour. For negative controls, 150 μl of TSBG were dispensed into each well. Biofilm formation experiments on microtitration plates were carried out similar to the ones in previous sections.

Inhibition of Biofilm Formation on Stainless Steel Coupons

Sterile stainless steel coupons were treated with 50 mL organic acids (1% or 2%,w/v, citric, malic or gallic acid) or chlorine (100 ppm or 200 ppm) solution in 100 ml sterile glass containers. After each treatment, the coupons were air dried at room temperature for 30 min. Sterile steel coupons were placed in a new 100 ml sterile glass container with fresh 50 ml TSBG. The medium was inoculated with overnight culture of *E. coli* strain. The final cell density was about 10^5 cfu/ml. The medium without the bacterial suspension was used as the negative control. The containers were incubated in an incubator at 37°C for 24-hour, 48-hour or 72-hour. Biofilm formation experiments on stainless steel coupons were again carried out similar to the ones in in previous sections.

Removal of Biofilm Formation

For this purpose, biofilm formation assays were performed as described in the previous sections on both microtitration plates and stainless steel coupons. Organic acid (citric, malic and gallic acids) at 1% or 2% (w/v) concentrations were tested and compared with chlorine solution (100 ppm or 200 ppm) for 5, 10 and 20 min to explore their potential biofilm removal activities on 24-hour, 48-hour and 72-hour aged biofilm formations of *E. coli*. The experiments were performed on both microtitration

plates and stainless steel coupons.

The percentages of biofilm prevention and removal were calculated as [29]:

Prevention or removal of biofilm (%) =

$$[(C-B)-(T-B)]/(C-B) \times 100 \quad [1]$$

where B was the average absorbance per well with no biofilm (negative control), C was the average absorbance per well for non-treated wells (positive control) and T was the average absorbance per well for the sanitizer treated wells.

Statistical Analysis

Three independent trials were conducted. Analysis of variance (anova) was performed with SPSS (SPSS Inc.,version 11.5, Chicago, Illinois, USA) followed by post hoc Tukey's test with a level of significance at $P < 0.05$.

RESULTS

The experiments yielded in enhanced biofilm formation on microtitration plates as compared to stainless steel coupons. The biofilm formation was determined at 24-hour (early, O.D. values 0.3) of incubation and enhanced at 48-hour (mature, OD values 0.5) and 72-hour (more mature, OD values 0.9) in microtitration plates. On the stainless steel coupons, lower OD values were determined for early (OD values 0.2), mature (OD values 0.3) and more mature (OD 0.6) biofilm formations.

Chlorine treatments on microtitration plates

Inhibition of biofilm formations was in the range of 74-80% ($p < 0.05$) after 24-, 48- and 72-hour when 100 ppm chlorine was used before the biofilm formation. On the other hand, the inhibition ratios increased significantly up to 83-87% ($p < 0.05$) by using 200 ppm chlorine before the biofilm formation (Figure 1A).

Removal of biofilm formation was about between 42-50 % ($p < 0.05$) by using 100 ppm for 5 to 20 min while up to 57-69 % ($p < 0.05$) removal ratios were determined when 200 ppm chlorine was used for the same treatment times on 24-, 48- and 72-hour pre-formed biofilm formations (Figure 2A).

Organic Acid Treatments on Microtitration Plates Citric Acid Treatments

Biofilm formation was prevented about 80-85% ($p < 0.05$) when 1% citric acid was used before the biofilm formation. When the citric acid concentration was increased from 1% to 2%, substantially increased inhibition rates were detected up to 84-86% ($p > 0.05$) (Figure 1B).

Removal of biofilms were up to 47-55% ($p < 0.05$) by using 1% citric acid in 24-, 48- and 72-hour old *E. coli* biofilms. Increasing the concentration of citric acid from 1% to 2% and the treatment time from 5 min to 20 min resulted in significant decrease of biofilm formation up to 55-64% ($p < 0.05$) (Figure 2B).

Malic Acid Treatments

Biofilm formation was inhibited about 52-65% ($p< 0.05$) with 1% malic acid treatment. However, the inhibition ratio of biofilm formation was increased significantly 55-68% ($p > 0.05$) after 24-, 48- and 72-hour when the concentration of malic acid was increased from 1% to 2% (Figure 1C).

Biofilm formation was removed up to 39-52% ($p < 0.05$) when 1% malic acid was used for 5 to 20 min for 24-, 48- and 72-hour aged biofilms. When the concentration of malic acid and the exposure time were increased, the removal ratios of biofilm formations substantially increased up to 42 to 59% ($p < 0.05$) after 5 to 20 min treatments on 24-, 48- and 72-hour pre-formed biofilms (Figure 2C).

Gallic Acid Treatments

The inhibition rates of biofilm formations were between 61-70% ($p < 0.05$) after 24-, 48- and 72-hour when 1% gallic acid treatments were used before the biofilm formation while the ratios significantly increased up to 67-76% ($p < 0.05$) for 5 to 20 min (Figure 1D).

Biofilm formation was reduced only about 42-62% ($p< 0.05$) after using 1% gallic acid for 5 to 20 min for 24-, 48- and 72-hour old biofilm formations. When the gallic acid concentration was increased from 1% to 2%, the reduction ratios of biofilm formation increased significantly by 52-70% ($p < 0.05$) by using the same treatment times on 24-, 48- and 72-hour aged biofilms (Figure 2D).

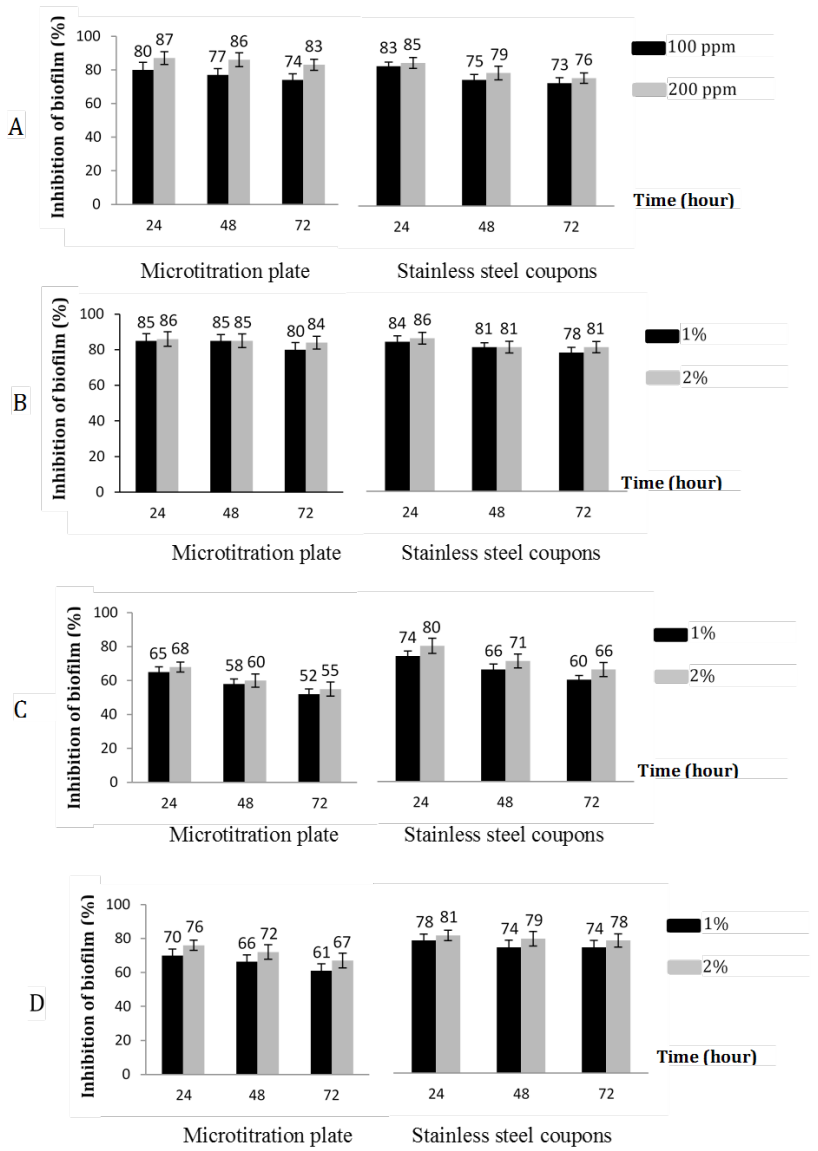

Figure 1. Effects of chlorine (A) and organic acid treatments (B: citric acid; C: malic acid; and D: gallic acid) for inhibition of 24- hour; 48- hour; and 72- hour *E. coli* biofilm formations on microtitration plate and stainless steel coupons. Mean values ± standard deviation for three independent experiments performed in duplicate.

Chlorine Treatments on Stainless Steel Coupons

On stainless steel coupons, biofilm formations were inhibited by up to 73-83% ($p < 0.05$) with 100 ppm chlorine treatment after 24-, 48- and 72-hour. When the concentration was increased from 100 ppm to 200 ppm, the inhibition rates remained at 76-85% ($p > 0.05$) (Figure 1A).

Removal of biofilm ratios was about 60-80% ($p < 0.05$) by using 100 ppm chlorine while the reduction ratios increased to 68-87% ($p < 0.05$) when the concentration and the treatment time were increased from 100 ppm to 200 ppm for 5 to 20 min on 24-, 48- and 72-hour old biofilm formations (Figure 3A).

Organic Acid Treatments on Stainless Steel Coupons

Citric Acid Treatments

Biofilm formations were inhibitied about 78-84% ($p < 0.05$) with 1% citric acid treatments while ratios remained about 81-86% ($p > 0.05$) by using 2% citric acid treatments after 24-, 48- and 72-hour (Figure 1B).

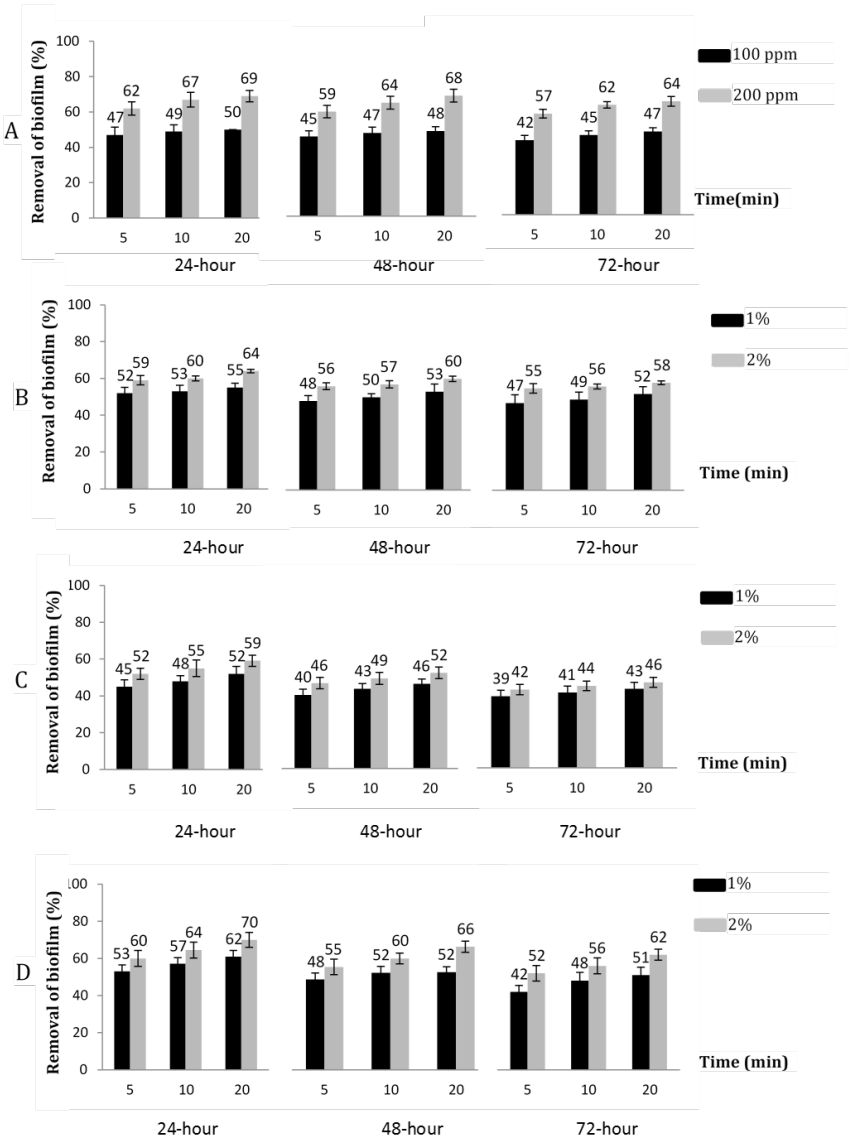

Figure 2. Effects of chlorine (A) and organic acid treatments (B: citric acid; C: malic acid; and D: gallic acid) for removal of 24- hour; 48- hour; and 72- hour *E. coli* biofilm formations on microtitration plate. Mean values ± standard deviation for three independent experiments performed in duplicate.

Biofilm formations were reduced about 66-85% p< 0.05) after using 1% citric acid for 5 to 20 min on 24-, 48- and 72-hour old biofilms. When the citric acid concentration was increased from 1% to 2%, the reductions increased quite signficantly up to 71-91% (p < 0.05) for the same treatment times on the same aged biofilm formations (Figure 3B).

Malic acid Treatments

The ratios of prevention of biofilm formations were about 60-74% (p < 0.05) by using 1% malic acid, whereas the rates substantially increased up to 66-80% (p < 0.05) when the concentration of malic acid was increased from 1% to 2% after 24-, 48- and 72-hour (Figure 1C).

Removal of biofilm formation ratios were between 47-68% (p < 0.05) with 1% malic acid treatments for 5 to 20 min in 24-, 48- and 72-hour *E. coli* biofilm formations. The rates were significantly enhanced up to 57-76 % (p < 0.05) when 2% malic acid was used for 5 to 20 min for 24-, 48- and 72-hour pre-formed biofilm formations (Figure 3C).

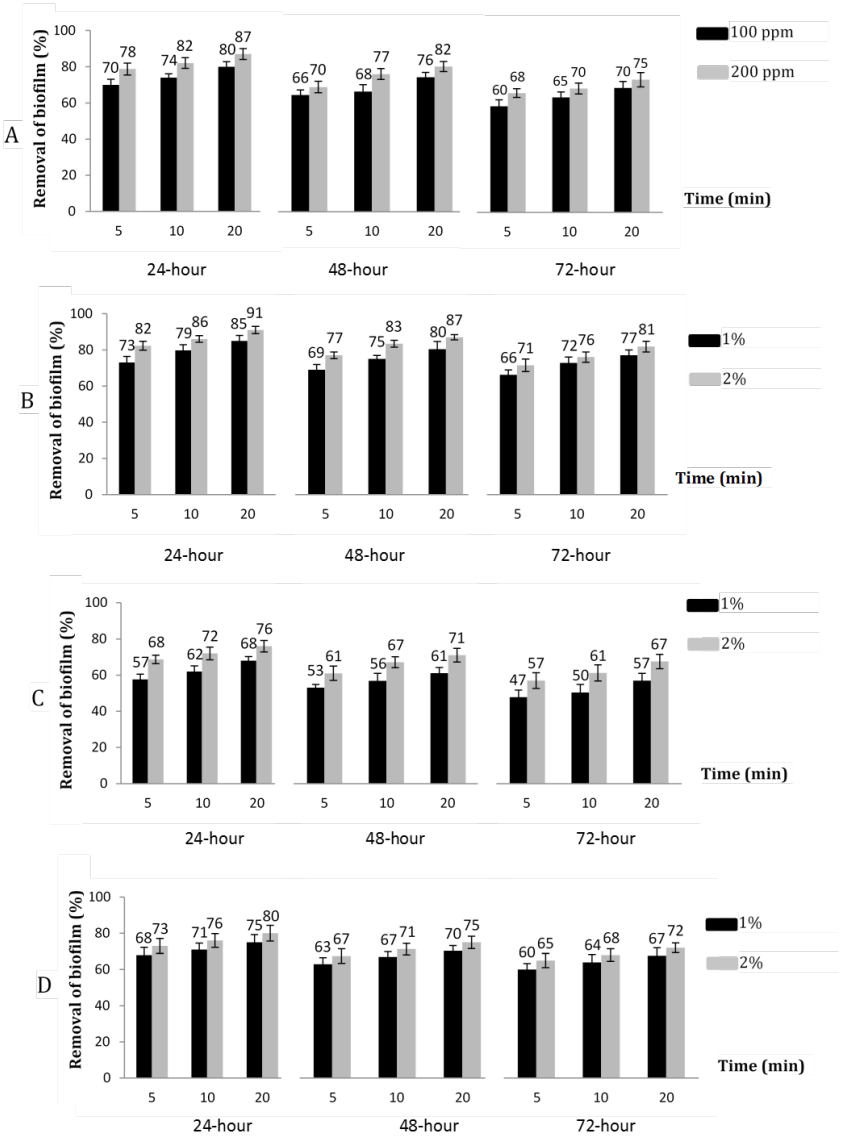

Figure 3. Effects of chlorine (A) and organic acid treatments (B: citric acid; C: malic acid; and D: gallic acid) for removal of 24 hour; 48 hour; and 72 hour *E. coli* biofilm formations on stainless steel coupons Mean values ± standard deviation for three independent experiments performed in duplicate.

Gallic Acid Treatments

Biofilm formations were inhibited by 74-78% ($p < 0.05$) with 1% gallic acid treatments. However, substantially increased inhibitions were obtained by up to 78-81% ($p < 0.05$) after 24-, 48- and 72-hour when the 2% of gallic acid was used before the biofilm formation (Figure 1D) .

Biofilm formations were decreased about 60-75% ($p < 0.05$) by using 1% gallic acid for 5 to 20 min on 24-, 48- and 72-hour old biofilms. When the concentration of gallic acid was increased from 1% to 2%, the removal ratios significantly increased to 65-80% ($p < 0.05$) after 5 to 20 min for 24-, 48- and 72-hour old biofilm formations (Figure 3D) .

DISCUSSION

The biofilm formation of microorganisms on food contact surfaces and processing facilities is a potential hazard for food processing industries [30]. Microorganisms can resist disinfection treatments when they form biofilms. Antimicrobial resistance becomes even worse when the microorganisms form biofilms [31]. Therefore, effective antimicrobial strategies are required.

In this work, inhibition and removal of 24-hour, 48-hour and 72-hour biofilms of *E. coli* strain by organic acids compared to chlorine treatments were investigated on both microtitration plates and stainless steel coupons.

The reduction in pre-formed biofilm and the inhibition of biofilm formation by chlorine and organic acids were observed to be dependent on the dose and time (for removal) on both surfaces. The inhibition and removal of biofilm formation ratios were increased by increasing the concentrations of organic acid treatments from 1% to 2% (w/v) or chlorine from 100 ppm to 200 ppm and the treatment times from 5 min to 20 min (for removal of biofilms). Even though the results indicated that 2% (w/v) organic acid treatments were more effective than 1% (w/v), 1% (w/v) concentration of organic acid treatments can be effectively used in practice to control biofilm formation.

A number of authors suggest that biofilm formed over an extensive period of time has increased resistance to antimicrobial substances [32,33]. Increased extra-polymeric substances and biofilm thickness over time are reported by some to enhance resistance to disinfectant agents [34]. It seems possible that hypochlorite may not have been able to penetrate effectively through the organic layers of the biofilm which may be the main reason for the loss of its effectiveness [35]. In the present work, biofilm formation of *E. coli* strain was developed in 24-hour and enhanced over 48-h with increased absorbance measurements. However, higher levels of biofilm formations were determined by using polystyrene microtitration plates than stainless steel coupons with lower absorbance values.

Bacterial adhesion to the surface depends on microbiological, physical, chemical, and material-related parameters. According to absorbance values, the attachment of *E. coli* strains was faster on microtitration plates than stainless steel coupons in the assays. The results agree with other studies [36,37]. It was determined that microorganisms attach more rapidly to hydrophobic surfaces such as polysytrene than to hydrophilic materials such as stainless surfaces. It was also indicated that the hydrophobicity of the cell surface was an important factor for the attachment of the bacteria to the surfaces [38-40]. This is due to the fact that hydrophobic interactions may lead to increase with an increasing nonpolar characteristics of surfaces involved [40]. Fimbriae or other proteins are needed for attachment on to hydrophobic surfaces [38]. Moreover, exopolysaccharides and lipopolysaccharides are important factors for attachment to hydrophilic surfaces [39]. Hyde *et al.* [41], found that biofilms consisting of *E. coli* grew better on polypropylene than stainless steel, while they did not grow well on glass. Therefore, it should be pointed out that hydrophilic materials should be used to render food contact surfaces like stainless steel, equipments and storage tanks.

Organic acids and chlorine were different in terms of their effectiveness for inhibition and removal of biofilm formations. This difference could be attributed to their mechanisms of antimicrobial action. Chlorine destroys microorganisms by chlorinating the lipid-protein substance in the bacterial cell wall to form toxic chloro compounds [42] and induces the leakage of macromolecules from the cells.

Organic acids were shown as inhibitors of many pathogenic bacteria [16,17]. The antimicrobial effect of organic acids could be related to several factors such as inhibiting metabolic acitivities or chain length [43]. It is also known that weak organic acids are lipophilic and penetrate plasma membrane acidifying the cell's interior. As bacteria maintain a neutral pH of the cytoplasm, the export of excess protons consumes cellular ATP and results in depletion of energy [44]. Various authors have hypothesized that organic acids may damage outer or cytoplasmic membrane, prevent synthesis of macromolecules and denature DNA reviewed by Ricke [44]. Ahn and Shin [45] reported that different types of organic acids exhibited different antimicrobial effects against different microorganisms.

Borges *et al.* [27] indicated that ferulic acid and gallic acids had effects in the prevention and control of biofilms of *E. coli*, *L. monocytogenes*, *P. aeruginosa* and *S. aureus*. Both phenolic acids had inhibition activity on biofilm formation and showed a higher potential to remove biofilm formations of the

Gram-negative bacteria. The authors emphasized that gallic acid and ferulic acid reduced biofilm activity about 70% for all the biofilms tested.

In conclusion, citric acid treatments for inhibition of biofilm on microtitration plates were as effective as chlorine treatments. Gallic acid was found to be more effective inhibitor than malic acid. Moreover, citric acid treatments were as effective as chlorine treatments for biofilm removal on microtitration plates. Inhibitory efffects of sanitizers were higher than removal rates on microtitration plates. The results obtained from microtitration plates are found to be somewhat related to those determined from stainless steel coupons. On stainless steel surfaces, in most cases the inhibition and removal of biofilm rates were higher than those found on microtitration plates. Citric acid treatments were even more effective than chlorine treatments for both inhibition and removal of biofilms on stainless steel coupons. Gallic acid treatments were found to be more effective than malic acid treatments. The results of the study show that the effectiveness of organic acids for inhibition and removal of *E. coli* biofilms can be ordered in descending order as citric acid, gallic acid, and malic acid treatments on both surfaces.

It is important to achieve maximum control of biofilm formation with lower concentrations of sanitizers. The use of citric or gallic acids at concentrations of 1% or 2% (w/v) can be considered as effective alternatives as commercial chemical sanitizers such as chlorine. Further studies are needed to determine the effects of these organic acids on different biofilm forming food related bacteria. Organic acid treatments to control *E. coli* biofilms are safe-to-use potential alternatives in industrial applications to chlorine treatments which are toxic to health and environment.

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CONFLICT OF INTEREST DISCLOSURE STATEMENT

No potential conflict of interest was reported by the author(s).

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