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Effectiveness of acetic and citric acid against *Staphylococcus aureus* contamination in parsley and dill

Maydanoz ve dereotunda Staphylococcus aureus kontaminasyonuna karşı asetik ve sitrik asitin etkinliği

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Abstract

Staphylococcal food intoxication from *Staphylococcus aureus* (*S. aureus*) species is an important food-borne disease that threatens public health in many countries. Products prepared with fresh vegetables that have not been heat treated and/or not sufficiently disinfected are particularly risky. In this study, the effects of various organic acid (acetic and citric acid) concentrations on *S. aureus* previously inoculated into parsley and dill vegetables were investigated at storage time of 0, 1., 3., 5, and 7 days. For this purpose, a total of 7 groups were formed: individually with 0.5% and 1.5% acetic and citric acid, as well as their combinations and control. The number of *S. aureus* was adjusted to 10^2 and 10^6 (log CFU/mL) numbers that known to produce toxins by spectrophotometric method. As a result of the study, acetic and citric acid were found to be more effective when combined in *S. aureus* inhibition. It was observed that this effect varies depending on the microbiological load, acid concentration and storage days of the vegetables. It was also determined that the organic acids used had a better effect on the parsley than the dill vegetable.

Keywords: Acetic acid, Citric acid, Dill, Parsley, *Staphylococcus aureus*

Öz

Staphylococcus aureus (S. aureus) türlerinden kaynaklı stafilokokal gıda intoksikasyonu birçok ülkede halk sağlığını tehdit eden gıda kaynaklı önemli bir hastalıktır. Isıl işlem görmemiş ve/veya yeterince dezenfekte edilmemiş taze sebzelerle hazırlanan ürünler özellikle risklidir. Bu çalışmada, daha önce maydanoz ve dereotu sebzelerine inokule edilen S. aureus'un 0., 1., 3., 5. ve 7. depolama sürelerinde çeşitli organik asit (asetik ve sitrik asit) konsantrasyonlarının etkileri araştırılmıştır. Bu amaçla toplam 7 grup oluşturulmuştur: ayrı ayrı %0,5 ve %1,5 asetik ve sitrik asit ile bunların kombinasyonları ve kontrol. S. aureus sayısı spektrofotometrik yöntemle 10^2 ve toksin ürettiği bilinen 10^6 (log KOB/mL) sayısına ayarlanmıştır. Çalışma sonucunda asetik ve sitrik asitin S. aureus inhibisyonunda kombinasyon halinde kullanıldığında daha etkili olduğu bulunmuştur. Bu etkinin sebzelerin mikrobiyolojik yüküne, asit konsantrasyonuna ve saklama günlerine bağlı olarak değiştiği görülmüştür. Ayrıca kullanılan organik asitlerin maydanoz sebzesine dereotu sebzesinden daha iyi etki ettiği belirlenmiştir.

***Anahtar kelimeler**: Asetik asit, Sitrik asit, Dereotu, Maydanoz, Staphylococcus aureus*

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1. Introduction

Staphylococcal food poisoning is a significant public health concern caused by the ingestion of food contaminated with *Staphylococcus aureus* bacteria. *S. aureus* can contaminate foods in a variety of ways. *S. aureus* is a bacterium that can colonize the skin and mucous membranes of normal people and animals, and usually does not cause infection. However, when the foods are contaminated with the species that can produce enterotoxins of this microorganism, poisoning occurs depending on the consumption of food, if the conditions are suitable. This is known to occur due to the presence of the pathogen in the raw material and / or foodstuff, contamination of food from the equipment and improper storage conditions (Lehel et al., 2021; Onyeaka et al., 2024). One third of foodborne gastrointestinal diseases are thought to be caused by *S. aureus*. 23 different heat-resistant staphylococcal enterotoxins (SE), which have been produced by certain *S. aureus* strains and have been identified so far, are held responsible for staphylococcal food poisoning (Abolghait et al., 2020).

Vegetables are an essential component of a healthy diet, providing a plethora of nutrients and benefits to our overall well-being. However, they can also be a breeding ground for contamination if proper precautions aren't taken. On the other hand, the trend towards healthy foods with a low-calorie value and low weight gain caused the demand for salads to increase (Otto et al., 2020). The fact that these foods are consumed fresh and rich in vitamin content are among the reasons why they are preferred for healthy nutrition. The microbiological loads of fresh vegetables are high and the main reason for this is the use of contaminated irrigation water in agriculture. Contamination also occurs during transportation, storage and processing (Rahman et al., 2022). In this context, sandwiches and salads prepared with fresh vegetables contaminated with pathogenic microorganisms can cause food poisoning.

The disinfection of fresh green leafy vegetables is important in the light of all that is told. For this purpose, many disinfection methods are used. However, problems such as the disinfectants used to leave residue or inadequate disinfection depending on the surface properties were encountered (Mendoza et al., 2022). The washing process is a critical step in the processing of fresh vegetables to block microorganism's growth and maintain the food quality. However, frequent and repeated use of chemicals used in this method to remove microorganisms may cause pathogens to develop resistance (Park et al., 2013). The growing concern for the disinfectants used in this (Dandie et al., 2020) context has revealed the need to use more natural methods. To overcome this problem, the use of natural disinfectants has been tried. For this purpose, the use of organic acids has been in question. The antimicrobial effect of organic acids results from lowering the ambient pH and impairing the permeability of the cell membrane. In addition, since organic acids are weak acids, they can migrate from the microorganism membrane to the cytoplasm where they dissolve, lowering the pH of the cytoplasm and causing the bacteria to die (Ji et al., 2023). Organic acids such as acetic and citric acid used for these purposes are also used as food additives. These acids are often suitable for use in ready-to-eat foods in the form of vinegar and lemon juice (Karam et al., 2023; Lopes et al., 2022). Indeed, these organic acids have Generally Recognized As Safe (GRAS) status and are used in plant disinfection due to their antimicrobial effects (Bermúdez-Aguirre & Barbosa-Cánovas, 2013; Islam et al., 2022; Park et al., 2013).

S. aureus is the most common pathogen associated with food poisoning resulting from improper handling of ready-to-eat products such as vegetables (Tang et al., 2015). Although its antimicrobial effect is known, parsley, one of these vegetables, showed some bactericidal properties against all bacteria tested except *S. aureus*. A study reported that parsley extract has antibacterial activity against Gram-negative bacteria, including *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*), but no inhibitory effect was observed against *S. aureus* (El Astal et., 2004). In this context, it is understood that parsley does not have the ability to eliminate *S. aureus* by internal effect as a result of possible contamination. Dill, another important vegetable, has a significant potential for contamination by *S. aureus*. While the presence of more than log CFU/g of *E. coli* was reported in dill-flavored cheeses, the presence of *S. aureus* was reported only in dill (3.66±1.86 log CFU/g), one of the ingredients used in its production (Petróczki et., 2018). It is clear that there is a need to eliminate *S. aureus*, which may be a potential risk in the vegetables in question. As a matter of fact, the combination of natural technologies with antimicrobial/preservative effects is used in the food industry to protect food quality, especially in vegetables, and to destroy or control pathogens (Ijabadeniyi et al., 2020). Thus, the effects of various concentrations of acetic and citric acid on *S. aureus* were investigated in this study. For this purpose, parsley and dill plants were used. The effects of these acids on *S. aureus* have been observed for storage days.

2. Material and Method

2.1. Study design

The following experimental design has been created for the experiments to be carried out primarily. All experiments were done in three replicates for parsley and dill (Table 1).

Table 1. Experimental groups used in this study

Groups	Description
1	0.5% Acetic acid
2	1.5% Acetic acid
3	0.5% Citric acid
4	1.5% Citric acid
5	0.5% Acetic acid + 0.5% Citric acid
6	1.5% Acetic acid + 1.5% Citric acid
7	Control

Organic acids are prepared by taking into consideration the above concentrations. Citric acid was obtained from the local market (Bağdat Baharat, Turkey) and acetic acid was obtained from Merck (Merck, 100058, Darmstadt, Germany).

2.2. Preparation of samples

Fresh parsley and dill samples were obtained from central vegetable market in Erzurum, Turkey. Fresh parsley and dill bundles brought to the laboratory under a cold chain were washed with distilled water and cleaned from coarse dirt, then shaken to remove excess water and kept at room temperature for 30 min. It was then put in sterile polyethylene packages to prevent possible contamination.

2.3. Preparation of *S. aureus* culture

NCTC 10654 *S. aureus* strain was incubated in 100 mL Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, Hampshire, England) for 24 hours at 37 ℃ and then divided into 50 mL falcon tubes. These tubes were then centrifuged at x5000 g for 15 minutes. At the end of the centrifuge supernatant was discarded and the pellet was washed with 0.90% sterile saline. This process was repeated 3 times. Then, serial dilutions up to 10^{-6} from the pellet in 0.90% sterile saline was taken and absorbance was measured with a spectrophotometer at a wavelength of 696 nm. Of the prepared dilutions up to 10^{-6}, 0.1 mL was spread onto Baird Parker (BP) agar with egg yolk tellurite emulsion. The counting of microorganisms was performed after 24 hours incubation at 37 ℃ and the calibration curve was drawn and the formula $(y = mx + c)$ based on the number of $\text{bacteria} \times \text{absorbance}$ value was obtained by the data obtained from the spectrophotometric measurement. According to this formula, 10^{-2} and 10^{-6} bacteria numbers have been adjusted by making use of the equality (Equation 1) of

$$y=1.220x-0.043 \tag{1}$$

Here, y indicates the dilution factor and x indicates the absorbance value. It was matched with the dilution factor corresponding to the determined OD value, and then multiplied by our standard's log CFU/mL to calculate the number of microorganisms' present.

2.4. Inoculation of *S. aureus* to vegetable samples

The previously prepared inoculum was filled in polyethylene packages containing 250 g parsley and dill and 250 mL distillated water, and the vegetables were kept in this solution for 1 min. Then the water was drained and kept at 20 ℃ for 24 h.

2.5. Application of acetic acid and citric acid to vegetable samples inoculated with *S. aureus*

One of the polyethylene packages containing *S. aureus* inoculated vegetables was determined as a control, and the remaining packages were left for 10 min after adding the acids at the concentrations that we determined previously, and excess liquid was removed. Then vegetables in polyethylene bags were kept at +4 ℃ for 7 days.

2.6. Determination of count of *S. aureus* and growth inhibition level

Immediately after disinfection, 10 grams of samples were taken on days 0, 1, 3, 5 and 7 from samples stored at +4 ℃ and transferred to stomacher bags with filter containing 90 ml of sterile peptone water and homogenized on Stomacher for 1 min. 0.1 ml of suitable dilutions (10^{-1} to10^{-5}) prepared from this homogenate were spread onto Baird Parker (BP) agar with egg yolk tellurite emulsion. Typical colonies (1-1.5 mm diameter, transparent, black glossy colonies) were counted following incubation condition at 37 ℃ for 24 hours. The results obtained have been converted to logarithmic values.

In addition to bacterial counts, growth inhibition levels (GILs) of *S. aureus* resulting from the action of organic acids were calculated using the following equation (Equation 2) applied by Sağdıç (2003):

$$GIL(\%) = \frac{(P_C - P_T)}{P_C} x100 \tag{2}$$

Here, PC and PT represent the number of *S. aureus* in samples exposed to control and organic acids, respectively.

2.7. Statistical analysis

The results obtained from the experiment were evaluated using the SPSS statistics program (Version 19, IBM). The Duncan's Multiple Range Test was used to determine the difference between the groups. Significance range was set at 0.05.

3. Results

The evaluation of the results obtained with the experiments was done separately for parsley and dill. As a result of the trials, *S. aureus* could not be detected in both parsley and dill samples that were inoculated with *S. aureus* at 10^2 inoculum level after acid applications. Therefore, the findings below are based on trials with 10^6 inoculum level.

According to the experimental design, the results of the organic acid application applied to parsley are given in Table 2. Accordingly, it was observed that the amount of *S. aureus* generally decreases, including from the first day to the 7th day, after the application of all acids individually and in combination. Considering the storage days, the continuous decrease in the amount of *S. aureus* in parsley samples with low concentrations (0.5%) of organic acid was observed to be higher between 0 and 1 days compared to other consecutive days. On the other hand, when the single and combine high concentrations of both acids (1.5%) were used, it was observed that the continuous decrease in parsley samples was higher between the 5th and 7th days compared to the other consecutive days. Indeed, the lowest bacterial population was observed in the 1.5% CA, 1.5% AA and 1.5% AA + 1.5% CA groups at a reduction on 1.76, 2.53, and 2.41 log, respectively. This reduction seen between days was found statistically significant compared to the control group ($p<0.05$) (Table 2).

Organic acid results applied to dill in accordance with the experimental design are given in Table 3. Accordingly, the amount of *S. aureus* after application of all acids individually and in combination has shown a generally variable character from the day of application. As can be seen from the values given in Table 3, it was observed that the continuous decrease in dill samples, where single and combine high concentrations (1.5%) of both acids were used, was higher between the 5th and 7th days compared to the other consecutive days. As a matter of fact, a decrease of 3.21 log was found in the 1.5% AA + 1.5% CA group compared to the initial inoculation level. This decrease observed between days was statistically significant compared to the control group ($p<0.05$) (Table 3).

Table 2. Antibacterial activity of acetic and citric acid against 10^6 log CFU/mL *S. aureus* on parsley

Groups	Storage days (log CFU/g)				
	0	1	3	5	7
0.5% AA	5.46 ± 0.15^{Cb}	4.89 ± 0.15^{Ca}	4.58 ± 0.10^{Bc}	4.46 ± 0.18^{Ec}	4.34 ± 0.10^{Dc}
1.5% AA	5.03 ± 0.11^{Be}	4.32 ± 0.13^{Bd}	4.02 ± 0.16^{Ac}	3.66 ± 0.11^{Cb}	2.50 ± 0.10^{Aa}
0.5% CA	4.80 ± 0.15^{ABc}	4.39 ± 0.18^{Bb}	4.16 ± 0.10^{Aa}	4.37 ± 0.13^{Eb}	4.02 ± 0.15^{Ca}
1.5% CA	4.60 ± 0.15^{Ac}	4.04 ± 0.16^{Ab}	4.03 ± 0.21^{Ab}	4.04 ± 0.14^{Db}	2.84 ± 0.11^{Ba}
0.5%+0.5% (AA+CA)	4.84 ± 0.11^{Bd}	4.84 ± 0.11^{Cd}	$4.59\pm.014^{Bc}$	3.28 ± 0.10^{Ba}	4.12 ± 0.16^{CDb}
1.5%+1.5% (AA+CA)	4.94 ± 0.13^{Bd}	4.67 ± 0.10^{Cc}	4.35 ± 0.11^{Bb}	2.43 ± 0.11^{Aa}	2.53 ± 0.17^{Aa}
Control	5.74 ± 0.09^{Da}	6.41 ± 0.07^{Db}	6.8 ± 0.12^{Cc}	7.22 ± 0.10^{Fd}	8.55 ± 0.13^{Ee}

A–F, The same uppercase letters within the same column for each sample show that the results are not statistically significantly different ($p>0.05$).
a–e, The same lowercase letters within the same row for each sample show that the results are not significantly different ($p>0.05$).
AA, Acetic Acid; CA, Citric Acid.
The result was expressed as mean ± standard deviation

It was observed that there were irregular increases and decreases in both parsley and dill during the storage period. This may be due to competition with *S. aureus* due to activation and/or inactivation of natural microbial flora found in vegetables. Another scenario is that the irregular shape of the plants affected the ability of the bacteria to attach to the tested plants.

Table 3. Antibacterial activity of acetic and citric acid against 10^6 log CFU/mL *S. aureus* on dill

Groups	Storage days (log CFU/g)				
	0	1	3	5	7
0.5% AA	5.13 ± 0.06^{Cc}	3.59 ± 0.02^{Ba}	5.15 ± 0.03^{Dc}	5.26 ± 0.06^{Dd}	4.58 ± 0.07^{Db}
1.5% AA	4.47 ± 0.08^{Be}	3.04 ± 0.06^{Ab}	2.43 ± 0.16^{Aa}	3.53 ± 0.07^{Bc}	3.90 ± 0.12^{Bd}
0.5% CA	5.02 ± 0.06^{Cc}	4.71 ± 0.22^{Eb}	4.57 ± 0.06^{Cab}	4.39 ± 0.08^{Ca}	4.35 ± 0.04^{Ca}
1.5% CA	5.30 ± 0.15^{Dd}	4.20 ± 0.15^{Da}	5.21 ± 0.13^{Dd}	5.02 ± 0.06^{Cc}	4.78 ± 0.17^{Db}
0.5%+0.5% (AA+CA)	5.30 ± 0.08^{Dd}	4.00 ± 0.13^{Ca}	5.24 ± 0.12^{Dd}	4.92 ± 0.12^{Cc}	4.68 ± 0.02^{Db}
1.5%+1.5% (AA+CA)	4.28 ± 0.12^{Ad}	3.72 ± 0.06^{Bc}	4.12 ± 0.16^{Bd}	3.48 ± 0.15^{Ab}	1.92 ± 0.14^{Aa}
Control	5.46 ± 0.08^{Da}	6.50 ± 0.06^{Fb}	6.66 ± 0.12^{Eb}	7.10 ± 0.09^{Ec}	7.70 ± 0.12^{Ed}

A–F, The same uppercase letters within the same column for each sample show that the results are not statistically significantly different ($p>0.05$).
a–f, The same lowercase letters within the same row for each sample show that the results are not significantly different ($p>0.05$).
AA, Acetic Acid; CA, Citric Acid.
The result was expressed as mean ± standard deviation

4. Discussion

Although different studies have been conducted in the food industry to control pathogens in foods, effective chemical methods to reduce the microbial load associated with fresh produce such as vegetables are not widespread. In different studies in the literature, most of the studies aimed at reducing the pathogen load have not been implemented on an industrial scale. In this context, it is necessary to ensure the applicability of scientific studies in the industrial field (Mostafidi et al., 2020). On the other hand, *S. aureus*, one of the gram-positive microorganisms, is relatively resistant to the methods used, and therefore this study investigated the potential of using organic acid-based agents for its mitigation in two selected vegetables (parsley and dill).

The effects of acetic and citric acid applications on the number of *S. aureus* in parsley and dill samples were found at different levels. Combined use of high doses of acetic and citric acid has significantly reduced the number of microorganisms in parsley and dill samples. It has been reported that the acetic and citric acid applications were more effective on the decreasing of number of *S. aureus* in Tabbouleh salad (Al-Rousan et al., 2018). The combination with 1.4% citric acid or 0.3% acetic acid has been reported to lead to a 3.2 log CFU/g reduction in the number of *S. aureus* in this study on the 7th day of storage at 4 °C.

According to our best knowledge, studies examining the effects of these organic acids on the presence of *S. aureus* in vegetables are limited. In a study, it was stated that *Escherichia coli* O157: H7 growth had the highest inhibition effect on the first day of storage in those treated with basil leaf with 2% of different organic acids (acetic, lactic and citric) (Valiolahi et al., 2019). Osaili et al. (2015) reported that the number of *S. aureus* in eggplants stored at 4 °C decreased with increasing citric acid concentration and reached> 3.0 log CFU/g on the 15th day. The result in question matches our data. On the other hand, the most striking result is that *S. aureus*, which is found in plant samples at low inoculum level, is completely inhibited in the presence of organic acids (low/high concentration and/or combine). It has been reported that 1% and 2% acetic acids applied to *Salmonella enteritidis* inoculated into strawberries cause approximately 1 logarithmic decrease (Lepaus et al., 2020). Wu et al. (2000) reported that dipping and keeping all the parsley leaves in vinegar with an acidity of 5.2% for 5 minutes caused 6 log reduction in the number of *Shigella sonnei* (the number before the study was 7.07). In addition, it has been stated that parsley leaves have a 5-minute exposure with vinegar, whose acidity is 7.6%, and that the number of pathogens has decreased very much, even to a level that cannot be counted (<0.6 log). In another study on this subject, a significant decrease in the amount of *Escherichia coli* (1.3 log CFU/g) was observed in lettuce washed for two minutes with 0.5% acetic acid, and it was reported that this reduction did not change even when the acetic acid concentration was increased to 1% (Käferstein & Abdussalam, 1999). Mols and Abee (2011) reported that acetic acid promotes the formation of reactive oxygen species that can cause cell death of *Bacillus subtilis*. In our current study, this may be associated with the presence of this condition as a possible mechanism of the inhibitory effect of acetic acid on *S. aureus*.

One of the important results obtained in this study is that acetic acid is more effective than dill in parsley in decreasing the number of *S. aureus*. This situation is thought to be caused by antimicrobial substances in the structure of parsley. In previous studies with parsley (*Petroselinum crispum*), it has been reported that the leaf extracts of the plant have a strong antimicrobial effect (Ali-Shtayeh et al., 2000). It has been reported that tartaric acid, one of the organic acids, has an antimicrobial effect against *S. aureus* in freshly cut asparagus lettuce when combined with plasma-activated water. This effect is probably associated with the penetration of organic acid into the bacterial cell membrane and therefore increasing osmotic stress and/or inhibition of the synthesis of biomolecules in living organisms (Wu et al., 2024).

Organic acids destroy the cell membrane, causing the formation of a more acidic environment in the intercellular environment. Therefore, it inhibits the necessary metabolic reactions of microorganisms. In this case, accumulation of toxic anions and inhibition of cell growth can occur (Parish et al., 2003). This feature is influenced by some factors such as cell membrane permeability, undissociated acids, chain lengths and environmental conditions. In our current study, binary organic acid combinations showed significant effects on the reduction of *S. aureus*. This can be said to arise from external factors such as pH and heat shock stress, as explained in the previous sentence, impairing membrane functionality and inhibition of cell growth.

Organic acids have begun to be used as alternative and natural compounds for food antimicrobial activity due to their low cost and effectiveness. Although their use in high concentrations can have caustic and toxic effects, typical dilutions of use are considered not to cause irritation and toxicity (Deng et al., 2020). Acetic and citric acid have low toxicity and are widely used as pH regulators and pharmaceutical agents. Moreover, the antimicrobial activities of these acids against different types of microorganisms have been shown in studies (Červenka et al., 2004; Olaimat et al., 2017; *Turhan et al., 2022*). In this study, their bactericidal effects on *S. aureus* in real foods were observed. While a limited effect was observed individually for both organic acids they have been found to be more effective when used in combination with a synergistic effect.

5. Conclusion

It is known how important natural nutrition is today. Food additives and their suitability for health are discussed. All these discussions will be effective in continuing the searches on this subject by ensuring that the use of organic substances in the processing of foods remains current. Based on this study, different organic acids and their concentrations can also be tried to prevent the development of pathogenic microorganisms in vegetables. In addition, automation can be facilitated by establishing a system in food establishments, using organic acids in order to economically remove possible contaminations from *S. aureus* in vegetables

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Author contribution

A.U.: contributed to conceptualization of the study, data analysis and collection, and manuscript preparation; read and approved the final manuscript; and served as principal author. A.E.: contributed to conceptualization of the study, data analysis, and manuscript preparation and read and approved the final manuscript. A.B.: contributed to preparation and critical review of the manuscript and read and approved the final manuscript.

Declaration of ethical code

The authors of this article declare that the materials and methods used in this study do not require ethics committee approval and/or legal-special permission.

Conflicts of interest

The authors declare that there is no conflict of interest.

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