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Assessing the Effect of Ultrasonic Application Combined with Antimicrobial Solutions on *C. difficile* Spores and Microbial Contamination in Spinach

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ABSTRACT

The combined use of chemical and physical methods can increase the rate of microbial inactivation resulting in a low risk of foodborne diseases and good preservation of food quality. The aim of this study is to assess the inhibitory effects of various active-ingredient washing solutions on *Enterobacteriaceae*, total aerobic mesophilic bacteria (TAMB), and *Clostridioides difficile* spores both with and without the application of ultrasonic treatment. In this respect, spinach samples were washed with sodium hypochlorite (NaOCl), green tea extract–acetic acid (GTE-AA), and a natural disinfectant solution (NDS) for 3 and 6 minutes, either with or without ultrasonic treatment. Washing spinach with the mentioned antibacterial solutions resulted in reductions of 1.3–2.3, 2.23–4.05, and 2.38–3.52 log CFU/g for C. difficile, TAMB, and *Enterobacteriaceae*, respectively. NaOCl exhibited the highest antimicrobial effect against all microorganisms tested, while the lowest inhibition was observed in samples washed with GTE-AA. Ultrasound treatment alone did not result in a significant reduction in microbial levels on spinach; instead, high-frequency applications appeared to increase the detectable microbial load, likely due to the disruption of microbial clusters or biofilms that made cells more accessible for enumeration. No synergistic interaction was observed between antimicrobial washing treatments and ultrasonic application in terms of microbial reduction. These findings highlight the need for further studies to refine ultrasound parameters and explore optimized combinations with antimicrobial agents in order to enhance microbial inactivation on fresh produce.

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Introduction

Green leafy vegetables are popular in diets because they offer rich nutritional benefits. However, they can become contaminated with soil, parasites, and microorganisms during cultivation, harvesting, transportation, processing, and packaging (Abdelshafy et al., 2025; Yu et al., 2024). This contamination increases the risk of foodborne illnesses, making the implementation of effective decontamination methods essential (Yu et al., 2024).

C. difficile is an anaerobic spore-forming pathogen that plays a major role in diarrhea linked to hospital settings and antibiotic use. *C. difficile* infection has increasingly been reported as a community-acquired disease (Wang et at., 2025; Metcalf et al., 2010). Contaminated food is considered one of the potential sources of *C. difficile* transmission within the community (Alexiou et al., 2025; Lim et al., 2018). The presence of *C. difficile* in various meat products (Biasizzo et al., 2025), poultry (Biasizzo et al., 2025), seafood (Henigman et al., 2025), and dairy products (Taylan-Yalçın et al., 2025) has been reported. Moreover, leafy greens like spinach (Taylan-Yalçın et al., 2025) and lettuce (Han et al., 2018), root vegetables such as potatoes (Tkalec et al., 2022) and carrots (Lim et al., 2018), as well as in ready-to-eat salads (Scholtzek et al., 2022). have also been found to harbor *C. difficile*. Vegetables may become contaminated through organic fertilizers containing *C. difficile* spores or through the use of contaminated water during irrigation or washing (Rodriguez et al., 2016). Studies have explored the thermal inactivation of *C. difficile* in food products by applying heat treatments within the 80–85°C. (Marcos et al., 2023; Rodriguez-Palacios et al., 2010). However, the application of thermal treatments for the inactivation of *C. difficile* in vegetables is not feasible, making them potential vectors for human infections (Rodriguez et al., 2016).

Epigallocatechin gallate (EGCG), a predominant catechin in green tea extracts, is known for its antimicrobial properties (Yun et al., 2015). Studies suggest that EGCG may reduce the expression of *C. difficile* virulence factors by interfering with quorum sensing mechanisms, making it a potential natural agent against *C. difficile*-related infections (Yun et al., 2015). Additionally, green tea compounds have been shown to disrupt the integrity of *C. difficile* by interacting with its cell membrane and/or cell wall (McCully, 2013). Acetic acid exerts the most pronounced antimicrobial effect due to its high proportion of undissociated molecules and effective membrane penetration capability (Cho and Chung, 2017). Antimicrobial activity of acetic acid against spore-forming bacteria has been reported (Park et al., 2021).' Combining natural antimicrobials such as EGCG with organic acids like acetic acid may enhance their overall effectiveness, as multiple mechanisms can act synergistically to inhibit microbial growth more efficiently.

Alternative decontamination strategies in the food sector include high pressure, pulsed electric fields, electrolyzed water, ozone, irradiation, and ultrasound technologies (de São José et al., 2014). Sonication technology is gaining prominence as a non-thermal, eco-friendly technique for enhancing food safety (Alves de Aguiar Bernardo et al., 2023; Okafor et al., 2024). Most microbial inactivation studies have focused on low-frequency (20–44 kHz), high-power (>100 W) ultrasound, while high-frequency (>100 kHz) or low-power applications are relatively underexplored (Costello et al., 2021). This technology can be used alone or in combination with chemical disinfectants (de São José et al., 2014). There are studies that assess how combining antimicrobial washes with ultrasound can enhance the microbial safety of fresh produce (He et al., 2021; Hua et al., 2025; Irazoqui et al., 2024).

This study aimed to assess the impact of low- and high-frequency ultrasound treatments in combination with selected antimicrobial washing solutions on the reduction of *Clostridioides difficile*, total aerobic mesophilic bacteria, and *Enterobacteriaceae* on spinach leaves. Two different washing solutions, a natural disinfectant solution and a combination of green tea extract with acetic acid, were used based on their previously reported antimicrobial effectiveness. The primary focus of this study was to investigate whether ultrasound treatments enhance the antimicrobial performance of these solutions and to determine if synergistic effects occur. The outcomes are intended to inform the development of improved decontamination strategies for fresh produce, particularly in the fresh-cut vegetable industry.

Materials and Methods

Preparation of Spinach Samples

The fresh spinach (*Spinacia oleracea*) leaves were obtained from a market in Çanakkale, Turkey. The wilted and spoiled parts of them were excluded from the analyses. Before *C. difficile* spore inoculation, the samples were disinfected with NaOCl as described by Tosun et al. (2023) to remove anaerobic load in spinach. Spinach samples that were not inoculated were also not subjected to any disinfection procedures.

Decontaminating Agents

Natural disinfectant solution (NDS) was prepared using epigallocatechin gallate (EGCG, 1 mg/mL) (Biosynth, UK), borax (1 mg/mL) (Egaş, Turkey; PubChem CID: 10219853), and EDTA (0.16%) (Sigma-Aldrich, USA). Besides NDS, green tea extract was also used (GTE, 1 mg/mL) (Nante Chemistry, Turkey). A combination approach was tested by incorporating acetic acid (0.7 M) (Sigma-Aldrich, USA, 99.8%) into both the NDS and GTE solutions. NaOCl and sterile tap water evaluated as a control group.

Inoculation Process

The spore suspension of *C. difficile* ATCC 9689 (Microbiologics, USA), was obtained by the method given by Tosun et al. (2022), and was then diluted to 10^6 CFU/mL in a sterile beaker. Spinach leaves were submerged in the prepared spore suspension after disinfection and allowed to stand for 10 minutes. After immersion, they were maintained for 2 hours at room temperature under a laminar flow cabinet for microbial adhesion.

Application of Washing Solutions

In our previous study (Tosun et al., 2023), the most effective concentration for both washing solutions (NDS, GTE-AA) was determined as 2×MIC for *C. difficile*, TAMB and *Enterobactericeae*. Even when applied for extended durations, this concentration was found to be optimal for preserving the physical and chemical properties of spinach. For NaOCl, this value was determined as 50 ppm (v/v). Spinach leaves were washed using the immersion method for 3 and 6 minutes with washing solutions at 2×MIC concentration and NaOCl at 50 ppm. Following the washing process, the spinach leaves were rinsed with sterile water to eliminate residual antimicrobial substances and subsequently dried under a laminar flow cabinet.

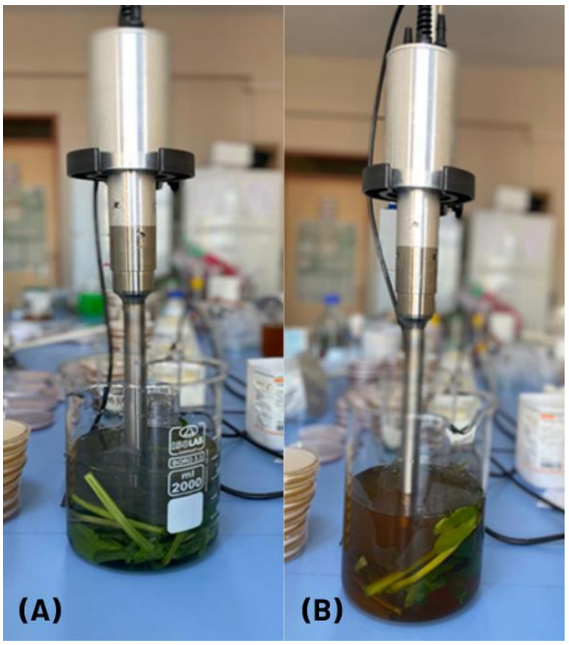

Figure 1. Low-frequency ultrasound (A) NDS (B) GTE-AA

NDS: Natural Disinfection Solution GTE: Green Tea Extract AA: Acetic acid

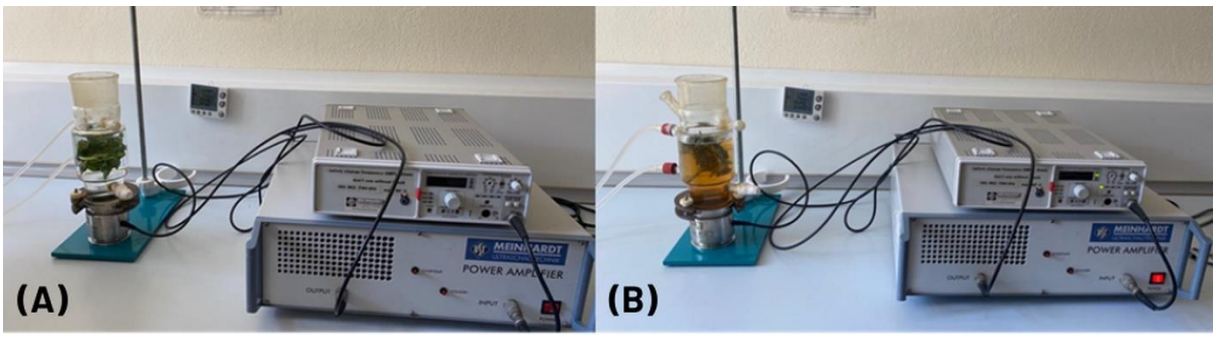

Figure 2. High-frequency ultrasound (A) NDS (B) GTE-AA
NDS: Natural Disinfection Solution GTE: Green Tea Extract AA: Acetic acid

Ultrasound Application

Low-Frequency Ultrasound

The low-frequency ultrasound (LFU) treatment was applied at 100 watts (20 kHz; Vibra-Cell 505, Sonics & Materials, Inc., Newtown, CT), with 60% amplitude and 5-second intervals (Özcan & Zorba, 2016; Sagong et al., 2013), for durations of 3 and 6 minutes. For the ultrasound application, autoclavable containers were filled with washing solutions (1.5 L), and inoculated spinach (25 g) was weighed and placed on top of mesh (Figure 1). In this way, the direct contact of the spinach with the ultrasound probe was prevented, allowing the cavitation effect to target microorganisms through the washing solution.

High-Frequency Ultrasound

The high-frequency ultrasound system consists of a frequency adjustable unit (582, 864 or 1144 kHz), a titanium transducer with a diameter of 75 mm, both of which are connected to a power supply of 120 W (Meinhardt Ultraschalltechnik K80-5, Germany). The high-frequency ultrasound (HFU) treatment was applied at 582 kHz with 100% amplitude power setting using NDS and GTE-AA washing solutions on spinach leaves (Figure 2). The actual amount of ultrasonic power delivered into the liquid at the maximum amplitude (%100) was measured around 30 W using calorimetry test (Mason et al., 1992). The ultrasound treatment was applied for 3 and 6 minutes. The washing solutions were prepared in 1.5 L glass beakers.

HFU treatment was not applied to spinach samples inoculated with *C. difficile* due to biosafety concerns and the risk of contaminating the HFU equipment. The design of the device does not allow for thorough sterilization after use with pathogenic spores, and applying *C. difficile* to this system could have posed a cross-contamination risk for subsequent experiments. Therefore, HFU was only used to evaluate its effects on TAMB and *Enterobacteriaceae*

Microbiological Analyses

The effects of washing treatment and washing followed by ultrasound application on *C. difficile*, TAMB, and *Enterobacteriaceae* loads on spinach samples were investigated.

Enumeration of C. difficile

To enumerate *C. difficile*, 25 grams of spinach were placed into phosphate-buffered saline (PBS) and homogenized using a stomacher for 30 seconds to ensure adequate microbial release. Subsequently, serial dilutions were prepared, and appropriate volumes from each dilution were spread onto Brain Heart Infusion (BHI) agar plates (Himedia, India). Inoculated plates were incubated anaerobically at 35–36°C for 24 hours. After incubation, colony-forming units (CFUs) were counted to determine the microbial load (Sorg & Dineen, 2009). Control of pre-inoculation disinfection effectiveness was carried out with the same method.

Enumeration of TAMB and Enterobacteriaceae

Spinach samples (10 g) were homogenized with 90 mL of phosphate-buffered saline (PBS) to prepare microbial suspensions. Serial dilutions were prepared and plated onto appropriate agar media for microbial enumeration. For TAMB, Tryptone Soy Agar (TSA; Merck, Germany) was used, and plates were incubated at 37°C for 24 hours (Uhlig et al., 2017). For *Enterobacteriaceae*, dilutions were spread onto Violet Red Bile Glucose (VRBG; Merck, Germany) agar and incubated under the same conditions. Characteristic pink, mucoid colonies were biochemically (IMVIC, oxidase) confirmed and recorded (Richter et al., 2022; Balki et al., 2023).

Statistical Analysis

IBM SPSS (v21) and Minitab (v17) software were used for statistical analyses. Analysis was performed in 3 replicates. The inhibitory effects among the different treatments on the tested samples were compared using ANOVA Where $P \leq 0.05$, Tukey's test was employed to identify significant differences between treatments.

Results and Discussion

In a prior study (Tosun et al., 2023), acetic acid was added to both washing solutions, and the synergistic activities of the combinations were determined. The fractional inhibitory concentration index (FICI) of NDS-AA was 1.5, indicating an additive effect with no interaction. Conversely, the FICI of GTE-AA was 0.36, reflecting a synergistic relationship. Based on these outcomes, NDS was applied alone in the current study, while GTE was used in combination with acetic acid.

The minimum inhibitory concentration (MIC) of NDS was found to be 0.125%, while the MIC of GTE-AA was determined as 1.5 mg/mL green tea extract and 0.125% acetic acid. It was observed that the optimum concentration for both washing solutions, which achieved the highest antimicrobial activity while preserving the physical and chemical properties of the spinach, was 2xMIC (Tosun et al., 2023).

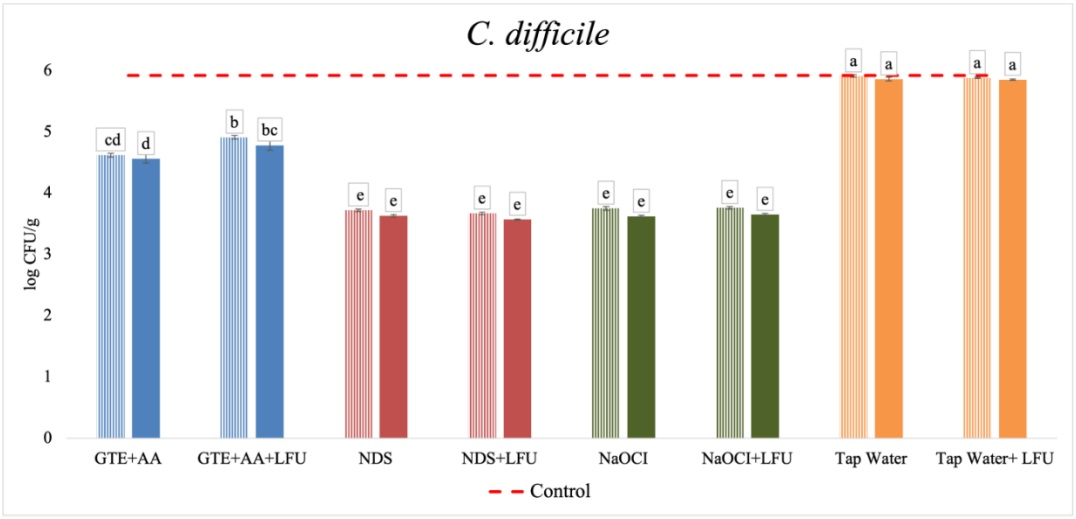

Figure 3. Effect of LFU and washing on the *C. difficile* inhibition

LFU: Low-Frequency Ultrasound NDS: Natural Disinfection Solution, GTE: Green Tea Extract AA: Acetic acid. Striped bars represent 3-minute treatments, while solid bars indicate 6-minute treatments. Different lowercase characters represent significant differences at P<0.05 by Tukey's test. The control group represents the initial C. difficile load in the samples before treatment

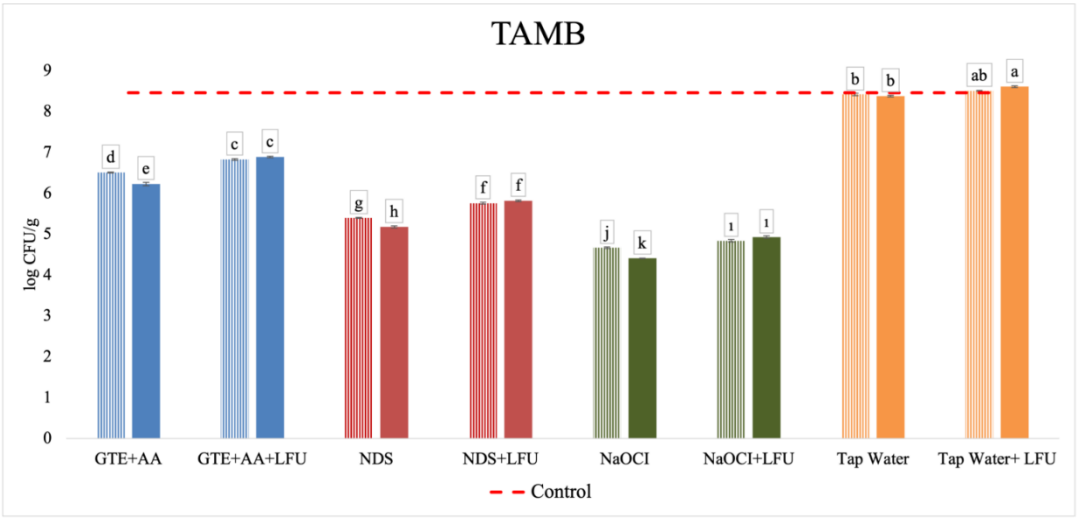

Figure 4. Effect of LFU and washing on the TAMB inhibition

LFU: Low-Frequency Ultrasound NDS: Natural Disinfection Solution, GTE: Green Tea Extract AA: Acetic acid TAMB: Total Aerobic Mesophilic Bacteria. Striped bars represent 3-minute treatments, while solid bars indicate 6-minute treatments. Different lowercase characters represent significant differences at P<0.05 by Tukey's test. The control group represents the initial TAMB load in the samples before treatment

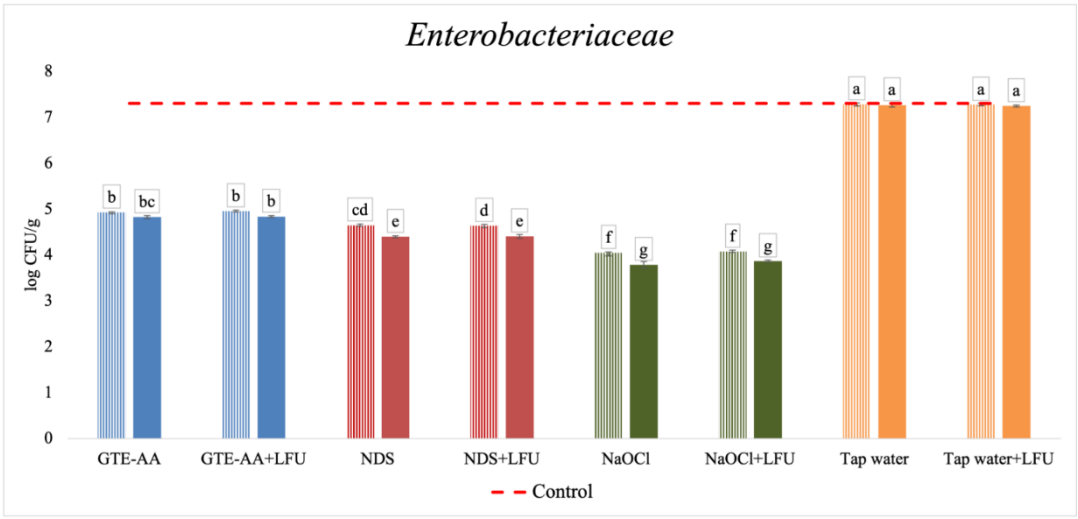

Figure 5. Effect of LFU and washing on the *Enterobacteriaceae* inhibition

LFU: Low-Frequency Ultrasound NDS: Natural Disinfection Solution, GTE: Green Tea Extract AA: Acetic acid. Striped bars represent 3-minute treatments, while solid bars indicate 6-minute treatments. Different lowercase characters represent significant differences at P<0.05 by Tukey's test. The control group represents the initial Enterobacteriaceae load in the samples before treatment

For NaOCl, the optimum concentration was found to be 50 ppm (v/v); thus, the washing solutions for spinach were prepared at this concentration.

In all washing applications, *C. difficile* log reductions were similar at 3- and 6-minute treatments (P>0.05). The bacterial inhibition rates ranged from 1.3 to 2.3 log CFU/g. The highest *C. difficile* inhibition (2.3 log CFU/g) was achieved using NaOCl (50 ppm, v/v) for 6 minutes, whereas the weakest effect (1.3 log CFU/g) was noted for the GTE-AA treatment, indicating its lower effectiveness compared to other disinfectants (Figure 3). No statistically significant difference was determined between NDS and NaOCl in terms of *C. difficile* reduction for either treatment duration.

The removal of TAMB from spinach significantly differed in NDS, GTE-AA, and NaOCl washing solutions (P<0.05). Furthermore, comparing the 3- and 6-minute applications revealed significant differences, suggesting that longer contact times may potentially enhance TAMB inactivation for each washing solution. The TAMB inhibition ranged from 2.23 to 4.05 log CFU/g, with the maximum removal recorded in spinach samples treated with NaOCl for 6 minutes. (Figure 4).

The inhibitory effect of washing treatments on *Enterobacteriaceae* was within the range of 2.38 to 3.52 log CFU/g. Among the treatments, the highest antimicrobial efficacy was observed in the samples washed with NaOCl for 6 minutes, while the lowest was recorded in the samples subjected to GTE-AA for 3 minutes. (Figure 5). The treatment duration (3 or 6 minutes) did not result in significant changes in *Enterobacteriaceae* reduction for the samples washing with GTE-AA solution (P>0.05). However, the reduction in bacterial load significantly improved with increased contact time (from 3 to 6 minutes) in the other washing solution groups. The findings indicated that rinsing with only tap water did not substantially decrease the counts of *C. difficile*, TAMB, or *Enterobacteriaceae* on spinach. This suggests that, in the absence of antimicrobials, microbial load is not effectively removed from the surface. Furthermore, the reduction observed following antimicrobial washing cannot be solely explained by the transfer of microorganisms into the washing solution. In our analysis, no microorganisms were recovered from the wash water, indicating that they were either absent or present at levels below the detection limit. Therefore, the ineffectiveness of water-only washing in significantly removing the microbial load, along with the absence of substantial microbial transfer into the wash water, supports the notion that the effectiveness of the antimicrobial treatment is primarily due to the direct inactivation of microorganisms or cellular-level damage, rather than their physical detachment from the surface.

In this study, ultrasound was applied in combination with NDS, GTE-AA, or NaOCl to minimize the microbial contamination in spinach. Low-frequency ultrasound treatment alone did not significantly inactivate *C. difficile* on spinach (Figure 3). There is no prior report on the effect of low-frequency ultrasound on *C. difficile* inactivation. But the ultrasonic inactivation of some other spore bacteria, such as *B. subtilis* or *B. cereus*, has been investigated. Joyce et al. (2003) found that *B. subtilis* spores were quite resistant to ultrasonic treatment performed at 20 and 38 kHz frequencies for 15 minutes.

Similarly, Lv et al. (2018) found that ultrasound at 20 kHz, used alone or with pressure and heat, resulted in only a 0.3 log reduction in *B. cereus* spores after 30 minutes. These results indicate that low-frequency ultrasound has limited efficacy against spore-forming bacteria. *C. difficile* spores share a structural resemblance with those of *Bacillus subtilis* and other spore-formers, but a key difference is the presence of an exosporium (Edwards et al., 2016). These structural differences can enhance the spores' resilience against extreme environmental conditions and significantly influence the effectiveness of various decontamination methods.

The acoustic cavitation caused by ultrasonic irradiation of water leads to extremely high temperatures (3000-5000 K) and pressures (500-1000 atm) in and around cavitation bubbles. High frequency ultrasound (f > 100 kHz) is more involved with chemical effects when extremely powerful hydroxyl (•OH) and hydrogen (•H) radicals are produced during bubble collapse (Suslick, 1990). LFU exerts relatively weak chemical effects but can generate strong physical effects. Its high shear forces can easily erode solid surfaces, including metals, plastics, and both organic and inorganic tissues. According to Zhou et al. (2022), the use of ultrasound at low frequencies and elevated intensities may physically disrupt biological structures. In this study, physical disruptions and breakages on spinach leaves were observed after 6 minutes of ultrasonic treatment. To prevent such tissue damage, ultrasonic treatment should be conducted at low power intensities. These structural deformations may alter enzymatic activities, potentially reducing product shelf life and negatively affecting overall quality. Furthermore, extending ultrasound treatment beyond 5 minutes is considered impractical due to increased operational costs (Zhou et al., 2023).

The ultrasound did not contribute much to the inactivation of TAMB (Figure 4) in washing solutions. Similarly, with regard to *Enterobacteriaceae* load (Figure 5), ultrasound application did not have any additional effect beyond the inhibition provided by the washing process. A direct ultrasonic inactivation of microorganisms is not as effective as the activation made by chemical disinfectants such as chlorine, chlorine dioxide or ozone (Ayyıldız et al., 2011). At low power intensities, the resistance mechanisms that bacteria develop in response to ultrasonic stress can prevent complete inactivation (Dai et al., 2025). Low-intensity or short-duration sonication can induce sublethal stress on bacteria, triggering DNA repair and metabolism, which can accelerate cell proliferation. The bactericidal efficiency of ultrasound can be increased by increasing ultrasonic power, but excessive power uses have its negative consequences of high operational costs. For food disinfection, the applied power of ultrasound should not exceed a critical level above which food materials become damaged. Moreover, ultrasound may unintentionally enhance bacterial survival by disrupting surface structures of food and releasing intracellular nutrients that support microbial growth. Pitt and Ross (2003) observed that exposing bacteria like *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis* to low-frequency (70 kHz) and low-power (<2 W/cm²) ultrasound enhanced their access to oxygen and nutrients, thereby accelerating their growth.

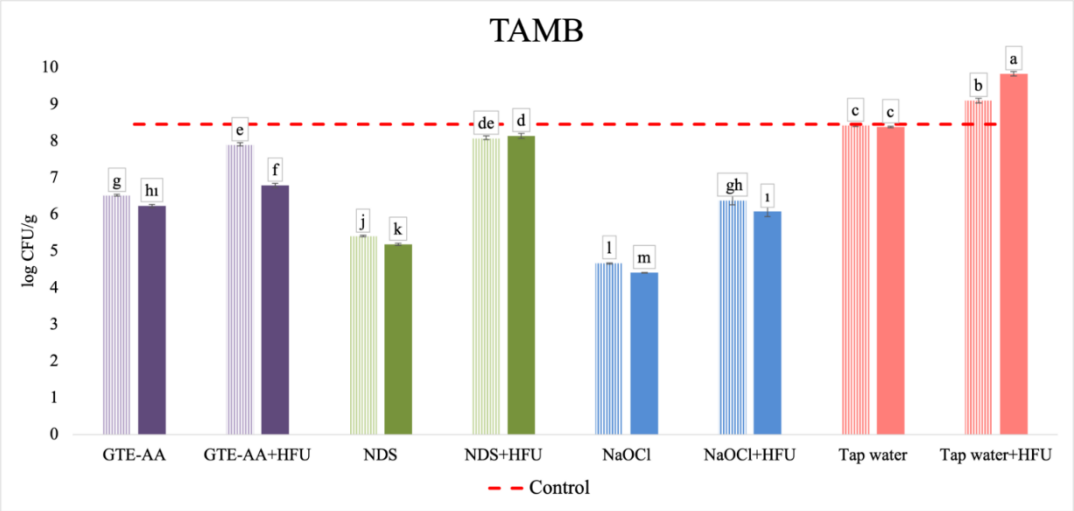

Figure 6. Effect of HFU and washing on the TAMB inhibition

HFU: High-Frequency Ultrasound NDS: Natural Disinfection Solution, GTE: Green Tea Extract AA: Acetic acid TAMB: Total Aerobic Mesophilic Bacteria. Striped bars represent 3-minute treatments, while solid bars indicate 6-minute treatments. Different lowercase characters represent significant differences at P<0.05 by Tukey's test. The control group represents the initial TAMB load in the samples before treatment

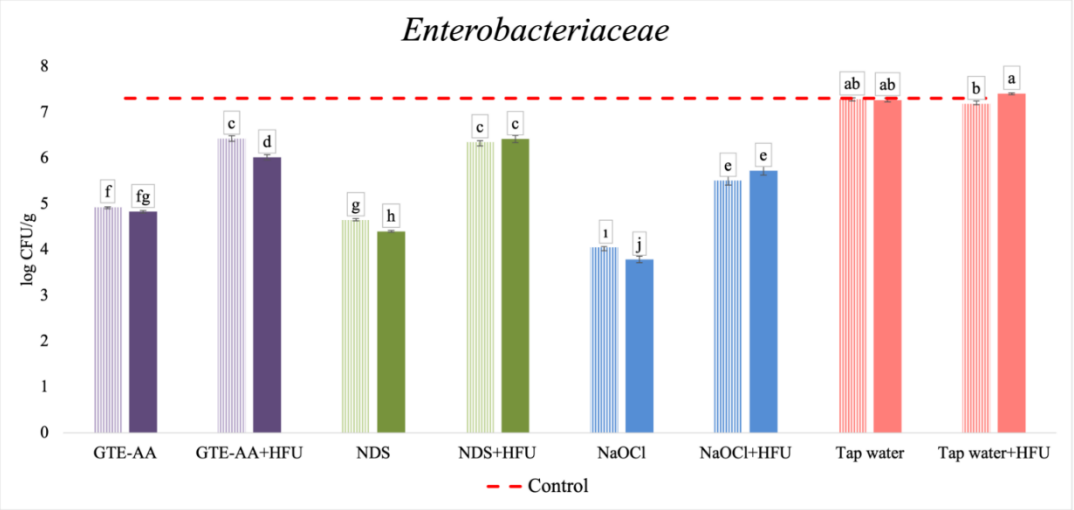

Figure 7. Effect of HFU and washing on the *Enterobacteriaceae* inhibition

HFU: High-Frequency Ultrasound NDS: Natural Disinfection Solution, GTE: Green Tea Extract AA: Acetic acid. Different lowercase characters represent significant differences at P<0.05 by Tukey's test. The control group represents the initial Enterobacteriaceae load in the samples before treatment

In addition, ultrasound can have a biofilm-disrupting effect and may release bacteria tightly adhered to food surfaces. This could lead to a higher bacterial count in plaque method analyses (Huang et al., 2017). Joyce et al. (2003) suggested that the breakup of bacterial aggregates at low frequencies could mask actual inactivation.

Biofilms on plant surfaces can support the colonization of microorganisms, providing a protective environment for pathogens. This may reduce the effectiveness of disinfectants and other inhibitory agents (Beuchat, 2002). In particular, leafy vegetables like spinach have a complex surface structure that provides a natural habitat for microorganisms. The leaf surface may have indentations and protrusions, as well as epidermal pores (stomata) and microcracks. While washing solutions can provide decontamination by direct contact with bacteria, the mechanical forces generated by sonication may push bacteria into these protected areas. This could reduce the exposure time to antimicrobial agents, limiting the effectiveness of inactivation.

Contrary to this view, intense sound waves directly detach bacteria from the outer surfaces and pores of solid materials into the aqueous solution. The results given in Figure 6 and Figure 7 show that the number of TAMB and *Enterobacteriaceae* on spinach increased after the washing solutions were irradiated with ultrasound. Particularly in samples treated with sterile tap water, the TAMB count exceeded that of the control group following ultrasound application. It seems that high-frequency sound waves help bacteria detach from the spinach leaves rather than achieving bacterial inactivation. This might be interpreted as if the ultrasound treatment reduced the antimicrobial effectiveness of the washing solutions. It is also possible that high-frequency ultrasound may have effectively separated bacterial cell clusters, making initially undetectable cells measurable.

Therefore, this increase can be associated with an increase in the visibility of existing bacteria rather than bacterial growth. According to Joyce et al. (2003), high-frequency ultrasound primarily causes declumping of bacterial aggregates, while actual microbial inactivation remains minimal. In the same study, an abrupt increase in *B. subtilis* cell numbers was observed after 5 minutes of ultrasound application at 512 and 850 kHz frequencies; after 15 minutes of treatment, bacterial viability exceeded the concentration of the initial bacterial suspension.

Although this study did not find a significant disinfection effect of ultrasound on TAMB and *Enterobacteriaceae* across different washing treatments, other research has reported microbial suppression using low- and high-frequency sonication techniques (Pelissari et al., 2021; Zhou et al., 2009) and high frequency (Gao et al., 2014; Koda et al., 2009). This variation may stem from multiple influencing parameters, including applied power and frequency of ultrasound, sonication time, system volume, pressure amplitude, transducer type, probe or plate diameter, liquid height, and temperature (Costello et al., 2021). In addition, certain bacterial characteristics can influence the effectiveness of ultrasonic inactivation. Rod-shaped bacteria, for instance, tend to be more susceptible to ultrasonic treatment than coccoid forms. Furthermore, literature indicates that aerobic microorganisms tend to exhibit higher resistance to ultrasound compared to anaerobic ones, and spores are generally more resilient than their vegetative cells (Beitia et al., 2023).

Conclusion

The inactivation of *C. difficile*, TAMB, and *Enterobacteriaceae* was evaluated in various washing solutions, both with and without the application of ultrasound. The addition of ultrasonic treatment to the washing process did not significantly enhance antimicrobial efficacy against the tested microorganisms. This suggests that the tested bacterial strains exhibit limited susceptibility to ultrasonic cavitation effects, thereby reducing the overall effectiveness of ultrasound when used in combination with antimicrobial agents. This may be attributed to the inherent resistance of the target microorganisms particularly *C. difficile* spores as well as limitations related to device design and operational parameters. Additionally, the structural properties of spinach leaves could have influenced treatment outcomes. Current findings in the literature suggest that the effectiveness of sonication on bacterial inactivation can vary depending on the applied frequency, power, duration, and other processing parameters. Furthermore, bacterial characteristics and environmental factors can also influence the results of ultrasonic treatments. These findings suggest that sonication, whether applied independently or alongside chemical washing, may offer limited contribution to microbial decontamination under the tested conditions. Future research should aim to optimize ultrasound parameters such as frequency, intensity, and duration to enhance its efficacy for targeted microbial inactivation. Additionally, combinations of different methods (e.g., mild heat, pressure, or biofilm disruptors) and alternative approaches could be explored to enhance the efficiency of sonication for food safety and microbial inactivation.

Declarations

Author Contribution Statement

M.N.T.D.: Writing – original draft, Methodology, Data curation,

G.T.Y.: Writing – original draft, Methodology, Data curation,

G.K.: Data curation,

Ö.A.: Writing – review & editing, Methodology, Supervision,

N.N.D.Z.: Writing – review & editing, Methodology, Supervision, Project administration

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Conflict of Interest

The authors report no conflicts of interest with any individuals or institutions in relation to this work.

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