Bactericidal Effect of a Combination of Food-Grade Antimicrobial Materials and its Application as an Alternative Sanitizer for Food Contact Surfaces

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Abstract: This study was designed to investigate the antimicrobial effects of combined sanitizers with food-grade antimicrobial agents, including Rosmarinus officinalis L., Camellia sinensis L. and citric acid. The minimal inhibitory concentrations of the individual agents ranged from 0.04%-0.16% (w/v) for R. officinalis L., 0.36%-1.25% (w/v) for C. sinensis L. and 0.25-1% (w/v) for citric acid against Staphylococcus aureus, Escherichia coli, Bacillus cereus, Salmonella Enteritidis and Listeria monocytogenes. All the agents tested showed the most effective antimicrobial activity against B. cereus. When the individual agents were combined, antibacterial effects were observed at lower concentrations than those used for treatments with the individual components. At a concentration of 0.25% (final concentrations: R. officinalis L., 0.000625%; C. sinensis L., 0.00125% and citric acid, 0.0625%) of the combined sanitizer, the viable levels of the five pathogens attached on stainless steel, cutting boards, knives and plastic baskets decreased by more than 5 log CFU/area and completely inactivated the growth of pathogens by 24 h after treatment. In particular, the formulated sanitizer more effectively inhibited the survival of B. cereus than the chemical sanitizer sodium hypochlorite. This study indicated that the combination of food-grade antimicrobial agents could inhibit both gram-positive and gram-negative foodborne pathogens by using lower concentrations of each individual agent in combination than those used for individual antimicrobial agent treatments and clearly showed potential applicability as an alternative sanitizer for food contact surfaces used in food processing environments.

Keywords: food-grade antimicrobial agent; antimicrobial activity; food contact surface; foodborne pathogen; sanitizer

1. Introduction

Foodborne diseases caused by pathogens present in foods are considered an emerging public health problem and encompass a wide spectrum of diseases [35]. These diseases are the result of the contamination of foods or foodstuffs with foodborne pathogens or toxic compounds derived from these pathogens, which is caused by inadequate hygiene practices and bacterial cross-contamination between foods and food surface materials in the food-processing industry [34]. Food contact surfaces can provide a suitable substrate for bacterial attachment and are potential sources of cross-contamination, which is a significant concern for the food industry [4, 15].

Many manufacturing facilities include materials such as stainless steel (SS) (e.g., washing tanks), rubber surfaces (e.g., belts, packing machines and liners) and utensils [28], which provide niches for bacterial cross-contamination between food and food contact surfaces. Although European and US
legislations have strict regulations concerning materials that come in contact with food, epidemic outbreaks caused by foodborne pathogens are frequently reported [18, 19]. Several studies have found that Escherichia coli, Staphylococcus aureus, Salmonella spp. and Listeria monocytogenes survive on hands, cloths, utensils and food-processing facility surfaces for hours or days after initial contact with the microorganisms. To prevent food poisoning outbreaks caused by foodborne pathogens, it is important to inhibit bacterial attachment to food contact surfaces and bacterial contamination of food sources [14]. The prevention of bacterial attachment and contamination can be approached by regularly sanitization and disinfection of surfaces. Sanitizing agents help inhibit and detach foodborne pathogens from food contact surfaces by killing the attached microorganisms and preventing further bacterial growth [42].

In the food industry, chemical sanitizers such as ethyl alcohol, organic acids, hydrogen peroxide, and quaternary ammonium are routinely used to eliminate foodborne pathogens [33]. However, ethyl alcohol and isopropyl alcohol, as disinfectants, exhibit slow action against nonenveloped viruses, lack of sporidical activity, reduced activity in the presence of organic matter and adverse effects on some types of medical equipment [5]. Chlorine has been associated with the formation of toxic compounds such as trihalomethanes [38], and organic acids may cause equipment damage and are toxic hazards to workers. The World Health Organization (WHO) is promoting the development of alternative methods for decontamination of food [47]. Plant extracts and food-grade additive antimicrobial agents have been increasingly reported as effective alternatives to current synthetic antimicrobial agents due to their safety and nontoxic status.

Green tea (Camellia sinensis L.) has also been reported to exhibit antimicrobial activity against various pathogenic bacteria via the action of catechins (epicatechin-3-gallate, ECG; epigallocatechin, EGC; EGC-3-gallate, EGGC) [43]. The extracts of C. sinensis L. have been shown to inhibit the growth of S. aureus, E. coli, Bacillus subtilis, Candida albicans and Bacillus cereus in many studies [2]. Rosmarinus officinalis L. is well known for its antimicrobial properties and has been widely commercialized as a preservation agent in the food industry [1]. R. officinalis itself contains a variety of polyphenolic compounds, such as carnosic acid and carnosol [21]. The plant-derived compounds exhibit significant antimicrobial activity against both gram-positive and gram-negative bacteria [32, 41]. The extracts of R. officinalis L. and C. sinensis L. have been approved by the European Union (EU) for use in food preservation and have been categorized by the FDA as Generally Recognized as Safe (GRAS). Organic acids are natural food additives and are generally considered GRAS by the United States Food and Drug Administration [29]. Citric acid can flow through cell membranes to lower the intracellular pH value. Low pH within cells causes damage to DNA, protein and extracellular membranes, leading to the death of bacteria such as E. coli O157: H7, Salmonella Typhimurium and Staphylococcus aureus [12, 36].

When antimicrobial agents are combined, different interactions may occur, showing various effects that may be synergistic, antagonistic or additive. These effects are not definite properties of plant extracts or food-grade antimicrobial agents [23]. However, combined antimicrobial agents show synergistic effects that enhance the antimicrobial activity, allowing the use of lower doses of each plant extract and food-grade antimicrobial agent. Studies on the individual antimicrobial properties of R. officinalis L., C. sinensis L. and organic acids have shown antimicrobial activity against foodborne pathogens. The synergistic effect of combined agents, leading to the ability to use low doses of each individual agent, in reducing foodborne pathogen levels has not been evaluated to date. The aim of the present study was to evaluate the antimicrobial properties of food-grade antimicrobial agents that exhibit synergistic effects, which may promote the use of natural and safe antimicrobial agents instead of synthetic sanitizers. In addition, this study evaluated the antimicrobial efficacy of combined sanitizers as an alternative approach to inactivating bacteria on food contact surfaces.

2. Materials and Methods

2.1. Food-grade antimicrobial material preparation
R. officinalis L. and C. sinensis L. extracts were powders obtained from Dyne Soze (Yongin, Korea). R. officinalis L. was extracted with ethanol, and the major compound in the R. officinalis L. extract was carnosic acid (including 20%). C. sinensis L. was extracted with methanol. Citric acid was obtained from Sigma-Aldrich (St Louis, MO, USA). The extracts of R. officinalis L. and C. sinensis L. were dissolved in ethanol and methanol, respectively, as surfactants to make 10% (w/v) stock solutions. The citric acid was dissolved in distilled water (DW) to prepare a 10% (w/v) stock solution. All disinfectant agents were prepared with a maximum pore diameter of 0.45 μm (Millipore Co., Billerica, Mass, USA).

2.2. Sanitizer formulation

The nonalcoholic sanitizer was composed of R. officinalis L. extract, C. sinensis L. extract and citric acid. An additional compound, glycerin fatty acid ester, was used to assist in the emulsification of the formulations, and polylysine was used as an antimicrobial preservative for the combined sanitizer. The suitable concentration for the sanitizer formulation for producing a 5 log CFU/mL reduction in the counts of E. coli and S. aureus in 5 min based on the time-kill rate was 0.005% (w/v) for R. officinalis L., 0.01% (w/v) for C. sinensis L., 0.5% (w/v) for citric acid, 0.01% (w/v) for polylysine and 0.5% (w/v) for glycerin fatty acid ester with DW. The combined sanitizer was enriched 50-fold.

The formulation process for this food-grade sanitizer was patented in 2018 [9].

2.3. Antimicrobial activity of food-grade antimicrobial materials

2.3.1. Bacterial strains

Five strains were used to evaluate the antimicrobial effects of individual disinfectant agents: E. coli ATCC 10536, B. cereus ATCC 14579, L. monocytogenes ATCC 15313, S. aureus ATCC 6538 and Salmonella Enteritidis ATCC 13076. All microbial strains were subcultured on tryptic soy broth (TSB, Merck, Germany) and incubated at 37 °C for Salmonella spp., E. coli and S. aureus and at 30 °C for B. cereus and L. monocytogenes. The cultures were washed twice in sterile isotonic saline solution (0.85% NaCl) by centrifugation at 1,570 x g for 20 min, and the pellets were resuspended in 1 mL of sterile phosphate-buffered saline (PBS) at approximately 8-9 log CFU/mL.

2.3.2. Antibacterial activity of food-grade antimicrobial materials

The antibacterial activity testing procedure was modified from Prabuseenivasan et al. [37]. Briefly, the bacterial suspension was adjusted to McFarland standard 0.5 (approximately 10⁶ CFU/mL) and spread over Muller Hinton Agar (MHA; Merck, Germany) plates using a sterile cotton swab. Each antimicrobial material (R. officinalis L., C. sinensis L. and citric acid) was prepared at a 0.5% (w/v) concentration and sterilized by filtration. Sterilized disks (Whatman No. 5, 6 mm diameter) were impregnated with 20 μL of antimicrobial materials and placed on the MHA surface; 10% DMSO was used as a negative control. After incubation at 37 °C or 30 °C for 20 h, the inhibition zone was measured. All experiments were performed independently in triplicate, and the mean value was calculated.

2.3.3. Minimum inhibitory concentration (MIC) determination

The antimicrobial activity of R. officinalis L., C. sinensis L., citric acid and the combined sanitizer against foodborne pathogens was tested by determining the minimum inhibitory concentration (MIC) in 96-well flat-bottom plates (BD Biosciences, Basel, Switzerland) according to the CLSI method [10]. Overnight cultures were prepared by incubating inocula and adjusting to the 0.5 McFarland standard. In each well of a 96-well plate, 100 μL of TSB was dispensed, followed by addition of 100 μL of antimicrobial material (stock solution) into the first well and serial dilution to achieve final concentrations ranging from 5% to 0.0192% (w/v) for R. officinalis L., C. sinensis L. and the formulated sanitizer. Citric acid solutions were prepared at concentrations ranging from 8% to 0.03125% (w/v) for MIC determination. Subsequently, 10 μL of the bacterial suspension (0.5
McFarland) was added to each well, and the microtiter plates were incubated at the optimal temperature for each pathogen for 18-24 h. The last well, as the negative control, contained 200 µL of TSB alone without any antimicrobial material. The lowest concentration that completely inhibited visible growth was established as the MIC.

2.4. Antimicrobial activity of the formulated sanitizer

2.4.1. Surface material preparation.

Cutting boards (polypropylene, PP), knives (SS), plastic baskets (PP), SS (ASI type 304) and PP were selected as representative food contact surfaces used in the food industry. The cutting boards were divided into surfaces of specific dimensions (10 x 10 cm, 100 cm²) to be used for the experiments. The blades of the knives had surfaces that were 4 cm in width and 7.5 cm in length (i.e., a total surface area of 30 cm²), and the plastic baskets were cut to obtain a total surface area of 100 cm² (10 x 10 cm).

The SS and PP were cut into coupons with surfaces of 2 x 7 x 0.2 cm. These coupons were then washed with 70% ethyl alcohol for 10 min, followed by two rinses in sterile DW. The cleaned and washed surface materials were air-dried in a laminar flow biosafety cabinet for 2 h and then sterilized by autoclaving (121 °C, 15 min).

2.4.2. Bacterial survival on stainless steel (SS) and polypropylene (PP).

For assessment of antimicrobial efficacy for SS and PP, we used modified versions of the techniques described by Kim et al. [25]. Single colonies of S. aureus, E. coli, B. cereus, S. Enteritidis and L. monocytogenes were selected and inoculated into 10 mL of sterile TSB. The cultures were incubated at 37 °C for Salmonella Enteritidis, E. coli and S. aureus and at 30 °C for B. cereus and L. monocytogenes. A 100-µl suspension was transferred to 10 mL of fresh TSB and incubated in the same manner before bacterial inoculation on the surface. The bacterial suspension was pelleted by centrifugation at 3,000 x g for 20 min at 4 °C, and the pellet was washed twice with sterilized PBS before final resuspension in 10 mL of sterile 0.85% NaCl. The bacterial suspensions were adjusted to a concentration between 6 and 7 log CFU/mL. The prepared SS and PP coupons were placed in Petri dishes, and 100 µL of bacterial suspension (7 log CFU/g) was inoculated on the surface. The coupons were dipped in 12 mL of combined sanitizer at 0.25%, 0.5% and 1% (w/v) and incubated for 5 min. The coupons were then removed from the Petri dish using flame-sterilized forceps and washed in sterile DW to remove the remaining unattached sanitizer on the surface. The coupons were incubated at 37 °C for Salmonella Enteritidis, E. coli and S. aureus and at 30 °C for B. cereus and L. monocytogenes for 1, 4, 12 and 24 h.

The coupons were then placed in sterile 50-mL conical centrifuge tubes containing 30 mL of PBS and 2 g of sterile glass beads (Sigma-Aldrich, St. Louis, MO, USA) and then agitated for 5 min with a bench-top vortex mixer set at maximum speed to detach the cells from the coupons. The cell suspensions in the tubes were serially diluted tenfold in 0.85% NaCl and examined in duplicate using 3M™ Petrifilm™ Aerobic Count Plates (3M Petrifilm, St. Paul, MN). The plates were incubated at 37 °C for Salmonella Enteritidis, E. coli and S. aureus and at 30 °C for B. cereus and L. monocytogenes for 24-48 h, and then, the mean viable bacterial counts were determined as log CFU/coupon.

2.4.3. Bacterial survival on cutting boards, knives and plastic baskets.

The bacterial suspensions were prepared by the method described in section 2.3.1 and adjusted to concentrations ranging from 6 to 7 log CFU/mL. Then, 1.0 mL (cutting board and plastic basket) and 0.5 mL (knife) of each bacterial suspension (6-7 log CFU/mL) was used for inoculation, providing a final population of 5-6 log CFU/cm². After 1 h of rest for bacterial attachment, the cutting boards, knives and plastic baskets were entirely dipped in 200 ppm sodium hypochlorite (10%, Sigma-Aldrich) and 0.25% of combined sanitizer for 5 min. The viable bacterial counts were determined at 1, 2, 4, 8 and 24 h. Each area was swabbed using separate sterile wet cotton swabs that were previously prepared in 10 mL of sterile D/E Neutralizing Broth (Difco Laboratories, USA). The swabs with the broth were vortexed for 5 min, and 1 mL of the homogenized suspension was serially diluted in 9 mL of 0.85% NaCl. Samples were examined in duplicate using a 3M™ Petrifilm™ Aerobic Count
Plate (3M Petrifilm, St. Paul, MN). The plates were then incubated at the optimal temperature for 48 h. The mean viable bacterial counts were determined as log CFU/100 cm² for cutting boards and plastic baskets and log CFU/30 cm² for knives. Surfaces that were not treated with disinfectant solutions served as controls. All tests were carried out in duplicate.

2.5. Statistical analysis.

All assays were performed in triplicate in two independent experiments, and the results were expressed as average and log-transformed values. SPSS statistical software was used to evaluate the results by analysis of variance (ANOVA). To compare the means, Duncan’s test was used with a significance level of p=0.05.

3. Results and Discussion

3.1. Antimicrobial effect of food-grade antimicrobial materials

Food-grade antimicrobial materials were investigated to evaluate their antimicrobial activity against foodborne pathogens, including three strains of gram-positive bacteria (B. cereus, S. aureus and L. monocytogenes) and two strains of gram-negative bacteria (E. coli and S. enteritidis) using the disk diffusion method. The evaluation of the antimicrobial activity is recorded in Table 1. The results revealed that the tested antimicrobial materials were potentially effective at suppressing the growth of foodborne microbial pathogens with variable potency. The extract of R. officinalis L. was the most effective material, retarding the growth of both gram-positive and gram-negative microbial pathogens at a concentration of 0.5%, while the extract of C. sinensis L. was effective against only B. cereus and S. enteritidis. Citric acid exhibited inhibitory effects against B. cereus, S. aureus and S. Enteritidis. The observed antimicrobial activity of the food-grade antimicrobial materials suggests that R. officinalis L. was the most effective antimicrobial material against both gram-positive and gram-negative pathogens. Hence, experiments were conducted to determine the MIC of each antimicrobial material against foodborne pathogens.

Table 1. Antimicrobial screening of food-grade antimicrobial materials (0.5%, w/v) against foodborne pathogens.

<table>
<thead>
<tr>
<th>Disinfectant agent</th>
<th>Inhibition zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gram-positive bacteria</td>
</tr>
<tr>
<td></td>
<td>B. cereus</td>
</tr>
<tr>
<td>R. officinalis L.</td>
<td>23.51±0.08</td>
</tr>
<tr>
<td>C. sinensis L.</td>
<td>10.22±0.17</td>
</tr>
<tr>
<td>Citric acid</td>
<td>9.42±0.27</td>
</tr>
</tbody>
</table>

1 Data are means of two replicates (n=2) ± standard error, 2 (-) indicates no growth inhibition zone.

3.2. MICs of food-grade antimicrobial materials

The inhibitory effect of R. officinalis L. started at 0.04% against B. cereus, S. aureus and L. monocytogenes, followed by 0.08% against S. Enteritidis and 0.16% against E. coli (Table 2), while C. sinensis L. and citric acid suppressed the growth of these bacterial pathogens at a concentration of approximately 0.3%. These results indicate that gram-positive bacteria were more susceptible to antimicrobial agents than gram-negative bacteria, which is consistent with the results of other studies [2, 3, 24, 26]. According to Archana and Abraham et al. [2], the MIC values of a methanolic C. sinensis L. extract were 0.8 mg/mL against E. coli, 0.8 mg/mL against S. aureus and 1.2 mg/mL against Salmonella Typhi. An ethanolic extract of C. sinensis L. leaves generated a larger inhibition zone against E. coli (13 mm) than against S. aureus (12 mm) [35]. An alcoholic extract of R. officinalis L.
showed higher antimicrobial activity against gram-positive bacteria (MIC 0.20-0.48 mg/mL) than against gram-negative bacteria (MIC 1.16-1.72 mg/mL) [24]. The MIC of R. officinalis L. was 5 mg/mL against L. monocytogenes and E. coli and 10 mg/mL against Salmonella Enteritidis. Generally, different crude extracts show different antimicrobial levels against the same microbes tested [3]. These inconsistencies might be due to differences in the antimicrobial activity of the polyphenolic compounds present in the extracts [11]. Nevertheless, these results indicated that gram-positive bacteria, especially B. cereus, were more susceptible to antimicrobial materials than gram-negative bacteria. Gram-negative bacteria have a unique outer membrane that can act as a barrier, so they are more resistant to disinfectant agents than gram-positive bacteria [22, 30].

Table 2. MIC values of food-grade antimicrobial materials against foodborne pathogens.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Gram-positive bacteria</th>
<th>Gram-negative bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B. cereus</td>
<td>S. aureus</td>
</tr>
<tr>
<td>R. officinalis L.</td>
<td>0.04(^1)</td>
<td>0.04</td>
</tr>
<tr>
<td>C. sinensis L.</td>
<td>0.30</td>
<td>1.25</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.25</td>
<td>0.25</td>
</tr>
</tbody>
</table>

\(^1\) Data are the means of three replicates (n = 3).

3.3. MICs of the formulated sanitizer

Plant extracts, including R. officinalis L. and C. sinensis L. extracts, are good antimicrobial agents, but naturally derived agents tend to be more expensive than synthetic disinfectant agents. It can be difficult to attain the necessary levels of naturally derived antimicrobial agents without them becoming cost-prohibitive. Thus, we determined the effective combination concentration for reduction in foodborne pathogen levels and formulated a novel sanitizer (stock solution, 5%) using R. officinalis L. extract (0.005%, w/v), C. sinensis L. extract (0.01%, w/v) and citric acid (0.5%, w/v) with glycerin fatty acid ester (0.5%, w/v) and polylysine (0.01%, w/v) in a previous study [9]. As shown in Table 3, the MIC value of the formulated sanitizer was 1% against S. aureus and 0.5% against L. monocytogenes, E. coli and Salmonella Enteritidis. The MIC against B. cereus was the lowest, with a value of 0.25%. According to the results shown in Table 2, B. cereus was susceptible to all the antimicrobial materials tested, and the formulated sanitizer showed high antimicrobial efficacy against B. cereus. In addition, the formulated sanitizer also exhibited the best antibacterial activity against gram-negative bacteria such as E. coli and S. Enteritidis as well as against gram-positive bacteria. Gram-negative bacteria are generally less susceptible to antibacterial plant extracts than gram-positive bacteria as their outer membrane consisting of lipoproteins and lipopolysaccharides acts as a barrier to antimicrobial agents [13]. Combining food-grade antimicrobial materials had a positive synergistic effect against gram-negative as well as gram-positive bacteria and could help reduce the necessary concentrations of R. officinalis L. extract, C. sinensis L. extract and citric acid for antimicrobial activity compared with the use of individual antimicrobial agents. In some studies, researchers have suggested that various active components in combined antimicrobial agents have stronger antimicrobial effects than the individual components, suggesting that the minor components in the plant extracts are also crucial for the observed activity [23]. The inhibitory effect of an R. officinalis L. extract was higher at low pH and high NaCl concentrations than under other conditions [12]. Antimicrobial agents such as citric acid on the outer membrane of gram-negative bacteria induce changes in the intracellular pH. Low-pH conditions within cells caused by citric acid treatment induce damage to the extracellular membrane and suppress NADH oxidation, eventually leading to cell death [12]. Thus, inclusion of citric acid in sanitizer formulation may be an effective strategy against gram-negative bacteria and may accentuate the antimicrobial effect of R. officinalis L. extract.
Table 3. MIC values of the formulated novel sanitizer (RGC) against foodborne pathogens.

<table>
<thead>
<tr>
<th>Strain</th>
<th>0.25%</th>
<th>0.5%</th>
<th>1%</th>
<th>MIC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. monocytogenes ATCC 15313</td>
<td>-</td>
<td>9.12</td>
<td>10.41</td>
<td>0.5</td>
</tr>
<tr>
<td>B. cereus ATCC 14579</td>
<td>8.62</td>
<td>11.04</td>
<td>12.71</td>
<td>0.25</td>
</tr>
<tr>
<td>S. aureus ATCC 6538</td>
<td>-</td>
<td>-</td>
<td>9.97</td>
<td>1</td>
</tr>
<tr>
<td>E. coli ATCC 10536</td>
<td>-</td>
<td>11.07</td>
<td>11.34</td>
<td>0.5</td>
</tr>
<tr>
<td>S. Enteritidis ATCC 13076</td>
<td>-</td>
<td>11.04</td>
<td>12.14</td>
<td>0.5</td>
</tr>
</tbody>
</table>

1% formulated sanitizer: R. officinalis L. extract, 0.005%; C. sinensis L. extract, 0.01%; citric acid, 0.5%; glycerin fatty acid esters, 0.5%; polylysine, 0.01%.

3.4. Evaluation of microbial survival on SS and PP treated with the formulated sanitizer

Studies evaluating plant extracts and organic acids for bacterial reduction on food contact surfaces remain somewhat limited. Based on the antimicrobial effect of the formulated sanitizer against foodborne pathogens, the combined sanitizer could potentially be used as an alternative sanitizer for food contact surfaces. We performed an antimicrobial activity test against foodborne pathogens attached on SS and PP, and different concentrations of the formulated sanitizer (1%, 0.5% and 0.25%) were used to determine the effective concentration to inactivate viable cells (Figure 1). Against all foodborne pathogens attached on the SS surface, the formulated sanitizer showed a reduction of more than 5 log CFU/coupon at the tested concentrations. The formulated sanitizer at the lowest concentration of 0.25% immediately inhibited the survival of E. coli and L. monocytogenes after treatment, and the antimicrobial activity was maintained for 24 h. The reduction rates for B. cereus and S. Enteritidis were 73% and 65% after treatment with a concentration of 0.25% but decreased to less than 1 log CFU/coupon within 1 h. Thereafter, these bacteria were not detected on the SS surface for 24 h. The levels of all the bacteria tested decreased to less than 1 log CFU/coupon within 1 h when the concentrations of the formulated sanitizer were 0.5% and 1%. On PP surfaces, treatment with 0.25% was effective for inactivation of the tested pathogens, as this concentration completely inactivated the initially inoculated E. coli, B. cereus, S. Enteritidis and L. monocytogenes within 1 h. When a concentration of 0.25% was used, S. aureus survived on the PP surface for 24 h, but the level of S. aureus rapidly decreased to 1.0 log CFU/coupon from the initial inoculum level of 7.2 log CFU/coupon within 1 h, and the level was maintained at 1 log CFU/coupon for 24 h. The formulated sanitizer may have excellent ability to reduce surface colonization and cross-contamination of foodstuff by foodborne pathogens, thereby preventing foodborne illness. A similar observation was made in a previous study regarding the ability of plant extracts to prevent attachment of E. coli, B. cereus and S. aureus [40]. The efficacy of plant extracts against microbial attachment was reported by Vazquez-Sanchez et al. [46], who examined the antibiofilm activity of R. officinalis L. extract against S. aureus on SS surfaces. Another study examined the efficacy of sanitizer formulations based on plant extracts against attachment of different bacterial species, such as Staphylococcus simulans, Lactobacillus fermentum, Pseudomonas putida, Salmonella enterica and L. monocytogenes [8, 45].

Bacterial survival after sanitization represents a potential risk to the food industry and the consumer [39]. It must be emphasized that an appropriate and efficient hygiene protocol is of fundamental importance; the American Public Health Association [20] recommends a maximum limit of 2 CFU/cm² for a food contact surface to be deemed appropriate, whereas the WHO suggests a limit of 30 CFU/cm² [47]. Based on the results obtained, the combined sanitizer with food-grade antimicrobial materials used in this study inactivated almost all viable cells of S. aureus, E. coli, B. cereus, S. Enteritidis and L. monocytogenes attached onto SS and PP surfaces. Thus, the present study evaluated the possibility of using the formulated sanitizer from this study as an alternative sanitizer for utensils used in the food-processing industry.
3.5. Evaluation of microbial survival on cutting boards, knives and plastic baskets treated with the formulated sanitizer

Instruments and materials used in the food-processing industry can be vehicles for pathogenic contamination. Our study selected cutting boards, knives and plastic baskets to evaluate the use of alternative sanitizers for food contact surfaces. As shown in Figure 2, nontreatment (NT) was complementary to adhesion on the tested utensil surfaces because pathogens inoculated on the surfaces of cutting boards, knives and plastic baskets survived for 24 h, except for S. Enteritidis on the cutting board and E. coli on the knife. Several studies have indicated that E. coli, S. aureus and S. Enteritidis survive on hands, sponges/cloths, utensils and currency for hours or days after initial contact with these materials [27]. Montville et al. [31] reported the quantification of cross-contamination between hands and foods or various kitchen surfaces and foods. Infection with Salmonella or Campylobacter in the Netherlands was caused by cross-contamination directly or indirectly from raw poultry via contaminated surfaces or niches in food processing for ready-to-eat products [16]. These results suggest the importance of hygiene procedures for surfaces that come in contact with food because foodborne pathogens can survive for long periods of time.

The present study compared the antibacterial effects of a formulated sanitizer (0.25%) and sodium hypochlorite (200 ppm) against foodborne pathogens attached on food contact surfaces to evaluate the applicability of this sanitizer as an alternative to chemical antimicrobial agents. Based on the antimicrobial activity, Table 4 shows the time required for 99.999% foodborne pathogen reduction ($T_{99.999}$) by treatment with sodium hypochlorite and the formulated sanitizer. Both sodium hypochlorite and the formulated sanitizer immediately reduced the initially attached S. aureus, E. coli and L. monocytogenes counts to below the level of detection (< 1 log CFU/area) after treatment (0 h), and these bacteria were no longer detected after 24 h. The formulated sanitizer was more effective.
against *B. cereus* on all tested food contact surfaces than sodium hypochlorite at 200 ppm. A 99.999% reduction in *B. cereus* levels was observed immediately after treatment on the knife and plastic basket and within 2 h after treatment on the cutting board. The antimicrobial efficacies of the chemical and formulated sanitizers on the knife and plastic basket against *S. Enteritidis* were similar (immediate 99.999% reduction after treatment), but the formulated sanitizer more effectively inhibited the attachment of *S. Enteritidis* (0 h) on the cutting board than sodium hypochlorite (1 h). The growth of foodborne pathogens was inhibited after treatment with the formulated sanitizer for 24 h. Sodium hypochlorite (NaOCl) is the most widely used disinfectant in the food industry due to its strong oxidizing capacity [44]. NaOCl disrupts the plasma membranes of bacterial cells and disables the enzymatic active site [17]. However, chemical disinfectants based on chlorine, chloramines and chlorine dioxide produce unwanted disinfection byproducts (DBPs) when reacting with natural organic matter, anthropogenic contaminants, bromide, or iodide present in the source water [38]. These DBPs may themselves be harmful and have carcinogenic, mutagenic or genotoxic properties [6]. The alternative antimicrobial agents studied here exhibit disinfection qualities comparable to those of traditional disinfectants and sanitizers. The antimicrobial properties of the extracts of plants, including *R. officinalis* L., have been known for centuries, but the strong flavors of these extracts have limited their use in food [12]. In the nontreatment group, the pathogens survived on cutting boards, knives and plastic baskets for 24 h, but the formulated sanitizer completely inactivated the pathogens on the food contact surfaces after treatment. In Korea, utensils used during food processing are typically sanitized by immersion (5 min) in a ‘utensil sterilizer’ containing 200 ppm sodium hypochlorite solution for microbial reduction. In the present study, the formulated sanitizer effectively inhibited bacterial attachment to the food contact surfaces. This might be the first investigation of the antimicrobial effectiveness of formulated sanitizers using food-grade antimicrobial materials against major foodborne pathogens, including spore-forming bacteria, on laboratory-inoculated utensil surfaces such as cutting boards, knives and plastic baskets. We suggest that the formulated sanitizer in the present study could be used as an alternative to chemical sanitizers for killing and growth inhibition of foodborne bacteria on food contact surfaces.

![Figure 2](image-url)

**Figure 2.** Growth kinetics curve for foodborne pathogens on the cutting board (A), knife (B) and plastic basket (C) surfaces for 24 h without formulated sanitizer treatment. The average of 3 repetitions is represented on the graph. Error bars depict standard error.
Table 4. Time required for a 5 log CFU/area reduction in foodborne pathogens on a cutting board, knife and plastic basket via the use of sodium hypochlorite (200 ppm) and the combined sanitizer (0.25%).

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Cutting board</th>
<th>Plastic basket</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaOCl</td>
<td>Sanitizer</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td>0h</td>
<td>0h</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>0h</td>
<td>0h</td>
</tr>
<tr>
<td><strong>B. cereus</strong></td>
<td>24.0</td>
<td>6.0</td>
</tr>
<tr>
<td><strong>S. Enteritidis</strong></td>
<td>1.0</td>
<td>0h</td>
</tr>
<tr>
<td><strong>L. monocytogenes</strong></td>
<td>0h</td>
<td>0h</td>
</tr>
</tbody>
</table>

1. 0h means the initial time after treatment during 5 min.

4. Conclusions

The main aim of this study was to assess the antimicrobial effects of combined sanitizers with food-grade antimicrobial materials as alternative sanitizers against foodborne pathogens attached to food contact surfaces. The low concentrations used in the combination of *R. officinalis* L. extract, *C. sinensis* L. extract and citric acid markedly improved the antimicrobial effect against foodborne pathogens compared with the individual concentrations. It was further found that formulated sanitizers containing plant extracts and organic acids could exhibit application potential as novel alternatives to chemical sanitizers. Compared to sodium hypochlorite, which is generally used in the food-processing industry, the formulated sanitizer exhibited powerful antimicrobial activity against foodborne pathogens. The sanitizer exhibited better killing effects against *B. cereus* attached to food contact surfaces than chemical sanitizers. Formulated sanitizers for inhibiting bacterial growth are novel tools for reducing microbial colonization of food contact surfaces in the food industry. The use of combined sanitizers in this study could provide an alternative or supplemental method for efficient sanitization to inhibit microbial attachment and adhesion on surfaces and cross-contamination between food contact surfaces and food products.


Funding: This study was conducted with the support of the Korea Food Research Institute (E0192101-01) and the High Value-added Food Technology Development Program of the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries, Republic of Korea (No. 316068-3).

Acknowledgments: The authors would like to thank the financial support provided by KFRI and IPET.

Conflicts of Interest: The authors declare no conflict of interest.

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