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Effects of CFSs produced by lactic acid bacteria in combination with grape seed extract on the microbial quality of ready-to-eat baby leaf vegetables

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Abstract: The effects of the combined use of cell-free supernatant (CFS) and grape seed extract (GSE) in inhibiting the growth of pre-existing and inoculated food-borne bacteria on mixed, ready-to-eat (RTE) baby leaf vegetables were examined. *Lactobacillus brevis* WK12 and *Leuconostoc mesenteroides* WK32 produced significantly more acetic and lactic acid than citric, malic, succinic, or fumaric acids after 24 h of fermentation. The inhibition zone sizes observed when *Lactobacillus brevis* WK12 and *Leuconostoc mesenteroides* WK32 CFSs and 0.1% GSE were used in combination were much greater than those observed when either of the CFSs alone was used on agar plates. The application of 5% CFS from *Lactobacillus brevis* WK12 or *Leuconostoc mesenteroides* WK32 in combination with 0.1% GSE reduced the total counts of aerobic mesophilic bacteria, coliforms, and yeasts/molds in RTE baby leaf vegetable samples by 2.01, 1.74, and 1.87 log CFU/g, respectively, compared with those determined in the control. Combined CFS and GSE treatment reduced *Escherichia coli* O157:H7 and *Listeria monocytogenes* counts by 1.72 and 2.89 log CFU/g, respectively. These results suggest that the combined CFS and GSE treatment may act as a natural disinfectant, enhancing the safety of RTE baby leaf vegetables.

ABOUT THE AUTHORS

The authors have worked on the WIKIM Institutional Research Project (KE1603-2) through the World Institute of Kimchi funded by the Ministry of Science, ICT and Future Planning, Republic of Korea. The present paper is a part of the project work. In particular, Dr Ho Hyun Chun, the corresponding author, received his PhD from Chungnam National University in 2013. He joined World Institute of Kimchi, Gwanju, Republic of Korea in 2014. He is currently appointed as a senior researcher from Advanced Process Technology and Fermentation Research Group at the Institute. While working with a range of food and agricultural produce preservation-related projects, he has learned that non-thermal technologies such as natural sanitizer, UV-C, and electron beam irradiation can be used to improve food preservation and microbiological safety.

PUBLIC INTEREST STATEMENT

Chlorine is commonly used in the form of hypochlorous acid, and its use has already been prohibited in some European countries, due to the potential of toxic by product generation. Therefore, there is a need for a microbial decontamination method that is non-toxic, effective, and easily applicable. The results of this study provide clear evidence demonstrating that a natural sanitizer comprised of cell-free supernatant (CFS) and grape seed extract (GSE) can be useful for the reduction of native microbial flora and foodborne pathogens in mixed baby leaf vegetables. Particular importance are the results demonstrating that the greatest effects of *Lactobacillus brevis* WK12 or *Leuconostoc mesenteroides* WK32 CFSs on RTE baby leaf vegetables were achieved when CFSs were used in combination with GSE. Therefore, as an alternative to chlorine sanitation, combined washing with CFSs and GSE can improve the microbial safety of RTE baby leaves.

Subjects: Fruit & Vegetables; Food Microbiology; Food Biotechnology; Preservation

Keywords: antimicrobial activity; baby leaf vegetable; grape seed extract; lactic acid bacteria; sanitizer

1. Introduction

The consumption of fresh produce and ready-to-eat (RTE) vegetables has increased as they represent important natural sources of dietary fibre, phytochemicals, minerals, and vitamins (Zheng et al., 2013). However, consumers are also concerned about the safety of RTE leaf vegetables consumed raw in salads, burgers, and *Bibimbap* (a typical Korean dish) and increasingly demand foods free of toxins and harmful microorganisms (Shah et al., 2015). However, raw vegetables are expected to contain pathogenic bacteria, and minimal processing (washing, peeling, cutting, and packing) may increase the growth of spoilage microorganisms (Fröder et al., 2007; Santos, Martins, Pedroso, Sousa, & Ferreira, 2015). Levels of mesophilic and psychrotrophic microorganisms in the range of 10^3–10^8 CFU/g are commonly found in RTE vegetables (Siroli et al., 2015). In particular, data from previous studies have shown that the bacterial contaminants found in RTE vegetables include primarily *Escherichia coli* O157:H7 and *Listeria monocytogenes* strains (Alegre, Abadias, Anguera, Usall, & Viñas, 2010; Siroli et al., 2015). *E. coli* O157:H7 is one of the most prevalent enterohaemorrhagic strains of *E. coli*, causing approximately 30% of pathogenic infections in Korea, and it can cause serious diseases, such as haemolytic uremic syndrome (Jo et al., 2004). *Listeria monocytogenes* is a psychrotrophic pathogen that is able to grow under refrigeration, although at considerably lower growth rates (Dimitrijevic et al., 2006).

To improve the microbial safety of RTE vegetables, various chemical washing treatments, including sodium hypochlorite, ozone, sodium lactate, and electrolysed water have been used to reduce bacterial counts (Beuchat & Ryu, 1997; Chaidez, Lopez, Vidales, & Castro-Del Campo, 2007; Oh et al., 2014).

Chlorine is widely used in the fresh-cut industry due to its relatively low price, ease of application, and a wide spectrum of antimicrobial effects (Meireles, Giaouris, & Simões, 2016). However, the use of chlorine as a sanitizer in the RTE produce industry has been identified as a health concern, mainly due to the presence of trihalomethanes, haloacetic acids, and chlorophenols generated during chlorination (Bull et al., 2011). In fact, chlorine is commonly used in the form of hypochlorous acid, and its use has already been prohibited in some European countries, due to the potential of toxic by-product generation (Gil, Selma, López-Gálvez, & Allende, 2009). Ozone (O_3) is produced as a gas that can be dissolved in washing water. Ozone is unstable, rapidly decomposes, and can become very toxic as it can affect the respiratory tract and cause irritation to the eyes and throat (Meireles et al., 2016). Therefore, a need for a microbial decontamination method that is non-toxic, effective, and easily applicable exists, and many studies have been conducted in order to investigate potential disinfectants as effective alternatives to chlorine or ozone.

Lactic acid bacteria (LAB) have shown great potential as biocontrol agents that control the growth of pathogenic organisms in food products, because they have a long history of safe use (Carr, Chill, & Maida, 2002). Cell-free supernatants (CFSs) of LAB isolated from fermented vegetables and dairy products contain antimicrobial organic acids, such as acetic, lactic, and citric acids (Rattanachaikunsopon & Phumkhachorn, 2010; Salleh, Lani, & Ismail, 2014).

Additionally, grape seed extract (GSE) is a polyphenol-rich by-product derived from the seeds of grape, *Vitis vinifera*. Antioxidant and antimicrobial activity of GSE against foodborne pathogens is well documented (Baydar, Özkan, & Sağdiç, 2004; Jayaprakasha, Selvi, & Sakariah, 2003). Furthermore, the use of GSE at a concentration of 0.01–1% along with other growth retardants, such as low pH and temperature, showed synergistic antimicrobial activity in RTE vegetables and fruits (Xu et al., 2007). However, to the best of our knowledge, no previous reports demonstrated the effects of the combination of GSE and CFSs produced by LAB as a natural washing sanitizer for RTE vegetables.

Therefore, this study was conducted to investigate the inhibitory effects of CFSs produced by LAB isolated from cabbage kimchi in combination with GSE against pre-existing microorganisms and foodborne pathogens on RTE baby leaf vegetables.

2. Material and methods

2.1. RTE baby leaf vegetables

Mixed RTE baby leaf vegetables were purchased from a local market in Gwangju, Korea. RTE baby leaves were obtained from red beet (*Beta vulgaris* L.), amaranth (*Amaranthus caudatus*), and red radish (*Raphanus sativus* L.).

2.2. LAB growth conditions and CFS preparation

Selected *Lactobacillus brevis* WK12 and *Leuconostoc mesenteroides* WK32 strains, isolated from well-fermented cabbage kimchi (Gwak et al., 2015), were obtained from the Microorganism and Gene Bank at the World Institute of Kimchi. de Man, Rogosa & Sharpe Broth (MRS, Difco Co., Detroit, MI, USA) was used for *Lactobacillus brevis* WK12 and *Leuconostoc mesenteroides* WK32 seed culture. *Lactobacillus brevis* WK12 fermentation food-grade medium consisted of 4% lactose (Sung Poong Co. Ltd., Seoul, Korea), 2% yeast extract (Angest, Yichang, China), 0.5% sodium acetate (Qingdao Twell Sansino Import & Export Co., Qingdao, China), and 0.2% dipotassium hydrogen phosphate (Youngjin Inc., Yesan, Korea). *Leuconostoc mesenteroides* WK32 fermentation food-grade medium consisted of 5% lactose, 1% yeast extract, 0.5% sodium acetate, and 0.2% dipotassium hydrogen phosphate. Seed-cultivated solutions were added at 1% (v/v) to a 5 L jar fermenter (Bio CnS Co., Daejeon, Korea) containing 3 L of fermentation medium. *Lactobacillus brevis* WK12 and *Leuconostoc mesenteroides* WK32 cultures were fermented under aerobic and anaerobic conditions at 30°C and 500 rpm, respectively. During the fermentation, pH values of these cultures were determined using a digital pH meter (Inpro3030, Mettler Toledo, Columbus, OH, USA), and cell mass in each culture was measured using a UV spectrophotometer (UV-1800; Shimadzu, Tokyo, Japan) at 600 nm. Optical density (OD) values were converted to log CFU/mL values using a standard calibration curve.

After fermentation, *Lactobacillus brevis* WK12 and *Leuconostoc mesenteroides* WK32 cultures were subjected to high-pressure saturated steam at 121°C for approximately 20 min. After sterilization, the cultures were centrifuged at 5,000 ×*g* (5810R centrifuge; Eppendorf, Hamburg, Germany) at 4°C for 15 min and then filtered through a 0.45 μm membrane filter to obtain CFSs.

2.3. High-performance liquid chromatography (HPLC)-based analysis of organic acid contents in CFSs

Organic acid contents in CFSs were analysed using a Waters Alliance e2695 HPLC system (Waters Co., Milford, MA, USA) according to the methods of Koo, Kim, and Kang (2015) and Özcelik, Kuley, and Özogul (2016), with some modifications. CFSs were filtered through a 0.45 μm nylon syringe filter (Whatman filters, GE healthcare, Chicago, IL, USA). Separations were performed using an Aminex HPX-87H reversed-phase column (7.8 × 300 mm, 9 μm particle size; BioRad, Berkeley, CA, USA) at 25°C, and 10 μL of each sample was injected. The mobile phase consisted of distilled 0.008 N H_2SO_4 (100%, v/v) with a flow rate of 0.4 mL/min. The organic acid contents in the CFSs were determined by monitoring the absorbance at 210 nm using a UV detector, and quantified by comparisons with the retention time and peak area of a mixture of 6 standard organic acids (lactic, acetic, fumaric, succinic, malic, and citric acids). The organic acid content was expressed in g/L.

2.4. Determination of antimicrobial activities

Agar disk diffusion assay (Chen et al., 2016) was used to determine the antibacterial activity of CFSs in combination with GSE against 2 target foodborne pathogens. Tryptic soy agar (Difco Co.) was cooled to 45°C after autoclaving, inoculated with 0.2 mL overnight cultures, and placed in sterile petri dishes. After solidification in a laminar flow biosafety hood for 60 min, sterile paper disks (6 mm diameter; Toyo Ltd., Tokyo, Japan) were placed on the agar and the plates were kept at room temperature for 30 min to allow liquid absorption into the disk. Ten microliters of CFS alone or CFS in

combination with GSE was pipetted onto the disks, and the plates were incubated at 37°C for 24 or 48 h. Following the incubation, antimicrobial activities were estimated by measuring the diameters (mm) of the zones of growth inhibition. The assays were performed in triplicate.

2.5. Foodborne pathogen strains and preparation of inocula

E. coli O157:H7 (NCTC 12079) and *Listeria monocytogenes* (ATCC 19111) strains, obtained from the American Type Culture Collection (ATCC) or from our own culture collection, were used for inoculation. *E. coli* O157:H7 and *Listeria monocytogenes* strains from frozen stock were streaked onto the tryptic soy agar (Difco Co.) and incubated at 37°C for 24 h. Single colonies of *E. coli* O157:H7 and *Listeria monocytogenes* were added to 50 mL tubes containing 30 mL of tryptic soy broth (Difco Co.), and incubated overnight at 37°C with shaking (150 rpm). From these overnight cultures, 1 mL each of *E. coli* O157:H7 and *Listeria monocytogenes* cultures were transferred to 500 mL of fresh medium and incubated for 24 h at 37°C with shaking (150 rpm). Harvested bacterial cell cultures were centrifuged at 4,000 ×*g* for 15 min, and the cultures were washed twice with sterile water containing 0.1% peptone. Inoculation solutions of *E. coli* O157:H7 and *Listeria monocytogenes* were prepared by diluting the bacteria in distilled water to a concentration of 7–8 log CFU/mL.

2.6. Inoculation of E. coli *O157:H7 and Listeria monocytogenes on RTE baby leaves*

Mixed RTE baby leaf vegetables were dipped in 70% ethyl alcohol and then exposed to UV-C irradiation for 20 min in a laminar flow hood equipped with UV-C lamps (40 W) to remove pre-existing microorganisms. The samples were dipped in a bacterial inoculum solution at a ratio of 1:10 (w/v) and agitated by stirring with a glove-covered hand for 15 min. The inoculated baby leaf samples were then air-dried in a laminar flow hood for 30 min to allow attachment of the pathogens to the surface.

2.7. RTE baby leaf vegetable washing

To prepare sanitizer solutions containing CFSs and GSE, *Lactobacillus brevis* WK12 and *Leuconostoc mesenteroides* WK32 CFSs were dissolved in sterile distilled water to a concentration of 5% and pH values of 4.08 and 4.50, respectively. GSE was dissolved in these solutions to a concentration of 0.1%. Mixed baby leaf samples were submerged in a bath containing distilled water, 100 ppm sodium hypochlorite, 5% CFS, or 5% CFS plus 0.1% GSE (1:10, baby leaf: washing solution, w/v), with gentle agitation for 5 min. Untreated baby leaf samples were used as controls.

2.8. Microbiological analysis

After washing, 20 g of baby leaf samples were placed in a sterile stomacher bag with a polyethylene filter layer containing 180 mL of peptone water (0.1% sterile peptone, w/v). Samples were then homogenised using a stomacher blender (MIX 2, AES Laboratoire, France) for 3 min and diluted with peptone water for microbial counting. Serial dilutions were performed in triplicate. For total aerobic mesophilic bacterial counts, samples were plated onto 3 M Petrifilm Aerobic Count Plates (Petrifilm AC; 3 M Co., St. Paul, MN, USA) and incubated at 37°C for 48 h. Coliforms were counted using 3 M Petrifilm *E. coli*/Coliform Count Plates incubated at 37°C for 24 h. Yeast/molds were plated on 3 M Petrifilm Yeast and Mold Count Plates (Petrifilm YM; 3 M Co.) and incubated at 25°C for 72 h. *E. coli* O157:H7 and *Listeria monocytogenes* counts were determined by plating diluted samples onto Sorbitol-MacConkey agar (Difco Co.) and Modified Oxford agar (Difco Co.) and incubating the plates at 37°C for 24 and 48 h, respectively. Each microbial count was determined as the mean of 3 measurement results and was expressed as log CFU/g.

2.9. Statistical analysis

Analysis of variance and Duncan's multiple range tests were performed using the SPSS software package (Version 19, SPSS Inc., Chicago, IL, USA), and differences were considered significant at $p < 0.05$. Each microbial count represents the average of 3 determinations.

3. Results and discussion

3.1. Growth characteristics of Lactobacillus brevis *WK12 and* Leuconostoc mesenteroides *WK32 during fermentation*

The growth rates and pH changes in the cultures of *Lactobacillus brevis* WK12 and *Leuconostoc mesenteroides* WK32 during fermentation are shown in Figure 1. Under aerobic conditions, the cell mass of *Lactobacillus brevis* WK12 gradually increased in a time-dependent manner during 24 h of fermentation (Figure 1(a)). The maximum cell mass of 10.02 log CFU/mL was measured at 24 h. The pH of *Lactobacillus brevis* WK12 culture gradually decreased until it reached the minimum at 3.85. For *Leuconostoc mesenteroides* WK32, the cell mass rapidly increased to 9.99 log CFU/mL up during the first 9 h of fermentation and it remained stable under anaerobic conditions thereafter. The pH of the sample was shown to be 4.2 after 9 h of fermentation (Figure 1(b)). The pH markedly decreased with *Leuconostoc mesenteroides* WK32 cell mass increase.

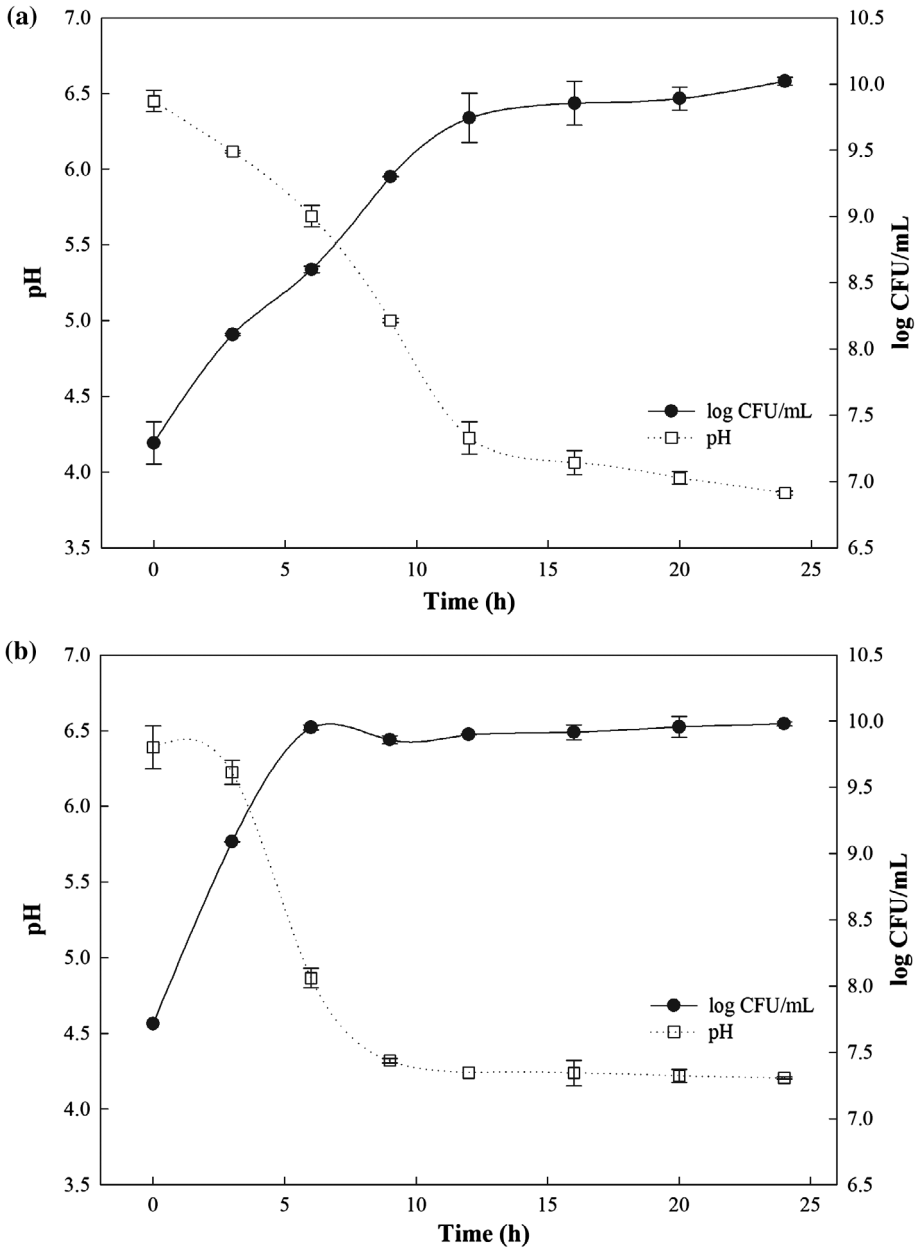

Figure 1. Growth profile and pH changes of lactic acid bacterial culture during fermentation of (a) *Lactobacillus brevis* WK12 or (b) *Leuconostoc mesenteroides* WK32.

Note: Values are shown as mean and standard error.

3.2. Organic acid content analysis following the fermentation

The organic acids produced by *Lactobacillus brevis* WK12 and *Leuconostoc mesenteroides* WK32 after fermentation were analysed by HPLC (Figure 2). Among 6 organic acids, the major components found in *Lactobacillus brevis* WK12 and *Leuconostoc mesenteroides* WK32 CFSs were lactic and acetic acids. In particular, higher acetic and lactic acid contents were detected in the *Lactobacillus brevis* WK12 CFS, with concentrations of 12 and 14 g/L, respectively, than in CFSs of *Leuconostoc mesenteroides* WK32. Gerez, Torino, Rollán, and Font de Valdez (2009) reported that the production levels of lactic and acetic acids during *Lactobacillus brevis* fermentation were 1 and 3.8 g/L, respectively. A previous study showed that lactic (8.4 g/L) and acetic (6.6 g/L) acids were produced by *Leuconostoc mesenteroides* at higher levels than other organic acids, such as citric, succinic, and malic acid (Lee & Chang, 2016). The levels and types of organic acids produced during fermentation were shown to depend on LAB species, culture composition, and growth conditions (Özcelik et al., 2016).

3.3. Determination of antimicrobial activity

The antimicrobial activities of CFSs alone or in combination with GSE, against *E. coli* O157:H7 or *Listeria monocytogenes* were examined using the agar diffusion test and the obtained results are presented in Table 1. *Lactobacillus brevis* WK12 CFSs exhibited inhibitory effects, producing zones of inhibition for *E. coli* O157:H7 and *Listeria monocytogenes*. Combination treatment with *Lactobacillus brevis* WK12 CFS and GSE showed better overall antimicrobial activities than GFS treatment alone against *E. coli* O157:H7 and *Listeria monocytogenes*. The antimicrobial effects were shown to increase with increased GSE concentrations in CFSs. Similarly, the inhibition zone sizes obtained when *Leuconostoc mesenteroides* WK32 CFSs and GSE were used were much greater than those observed when either CFS was used alone. Tejero-Sariñena, Barlow, Costabile, Gibson, and Rowland (2012) reported a strong correlation between the organic acid concentration, organic acid species, pH, and microbial inactivity. Low pH makes organic acids liposoluble, allowing them to traverse the cell membrane and reach the cytoplasm of microorganisms (Djadouni & Kihal, 2012). Acetic acid was reported to have greater antimicrobial activity than lactic acid due to its higher pKa value (acetic acid, 4.75; lactic acid, 3.08) (Tejero-Sariñena et al., 2012). Neutralised CFSs (pH-adjusted using 0.1 N NaOH) did not lead to the generation of inhibition zones for *E. coli* O157:H7 and *Listeria monocytogenes* (data not shown). These results indicate that growth inhibition of foodborne pathogens may be attributed to the decreased pH values and antibacterial activities of organic acids in CFSs produced by LAB.

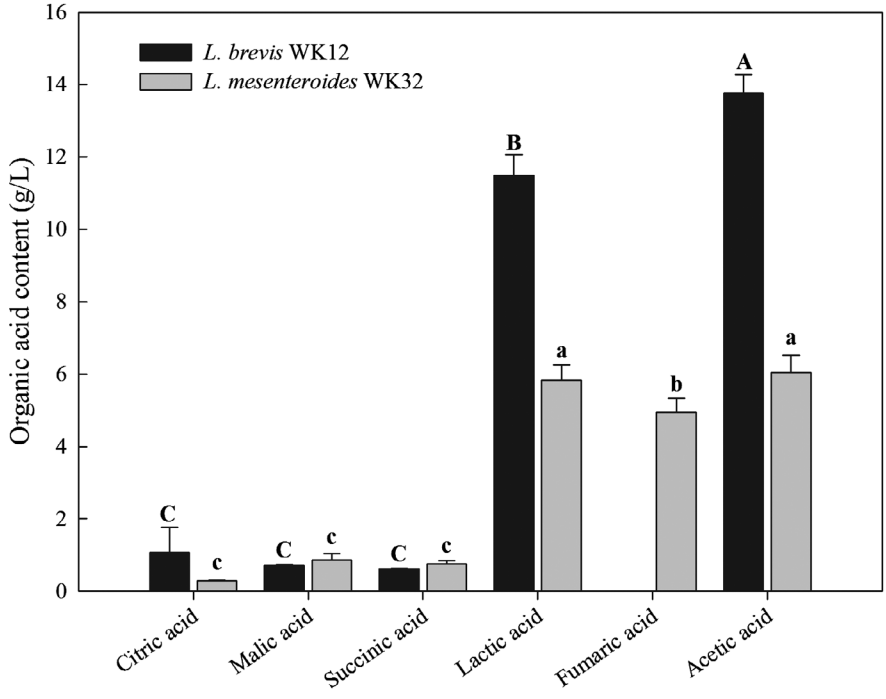

Figure 2. Organic acid content (g/L) in CFSs produced by *Lactobacillus brevis* WK12 and *Leuconostoc mesenteroides* WK32 after fermentation.

Notes: The means are followed by different letters (A–C or a–c), which reflects significant differences ($p < 0.05$), as determined using Duncan's multiple-range test.

Table 1. Antimicrobial activity of CFSs in combination with GSE against *Escherichia coli* O157:H7 and *Listeria monocytogenes*, as determined by agar-diffusion testing

Treatment	Inhibition zone (mm)	
	***Escherichia coli* O157:H7**	***Listeria monocytogenes***
Control (DW)	–	–
Lactobacillus brevis WK12 CFS	2.66 ± 0.13DE (+)	4.87 ± 0.32DE (++)
Lactobacillus brevis WK12 CFS + 0.01% GSE	3.55 ± 0.21C (++)	5.37 ± 0.11BC (+++)
Lactobacillus brevis WK12 CFS + 0.05% GSE	4.95 ± 0.11B (++)	5.81 ± 0.15AB (+++)
Lactobacillus brevis WK12 CFS + 0.1% GSE	5.66 ± 0.37A (+++)	5.96 ± 0.46A (+++)
Leuconostoc mesenteroides WK32 CFS	2.21 ± 0.17F (+)	4.37 ± 0.23F (++)
Leuconostoc mesenteroides WK32 CFS + 0.01% GSE	2.42 ± 0.20EF (+)	4.57 ± 0.11EF (++)
Leuconostoc mesenteroides WK32 CFS + 0.05% GSE	2.87 ± 0.13D (+)	4.92 ± 0.23CDE (++)
Leuconostoc mesenteroides WK32 CFS + 0.1% GSE	3.26 ± 0.25C (++)	5.09 ± 0.28CD (+++)

Notes: CFS: cell-free supernatant. The means are followed by different letters (A–F) that reflect significant differences ($p < 0.05$), as determined using Duncan's multiple-range test.

Symbols: – No inhibition zone; + small inhibition zone (1–3 mm); ++ medium inhibition zone (3–5 mm); +++ larger inhibition zone (>5 mm).

3.4. Antimicrobial effects of CFSs and GSE in the treatment of mixed RTE baby leaf vegetables

The effects of CFSs alone or in combination with GSE on the inactivation of natural microflora and foodborne pathogens found on mixed RTE baby leaf vegetables were examined (Table 2). Prior to washing, the initial counts of total aerobic mesophilic bacteria, coliforms, and yeast/molds on baby leaf vegetables ranged from 5.93 to 8.57 log CFU/g. These results demonstrate the presence of high levels of natural microflora in commercial baby leaves. Azizkhani, Elizaquível, Sánchez, Selma, and Aznar (2013) reported that baby leaf vegetables can be contaminated by various microorganisms, and high microbial counts in baby leaf vegetables can lead to the development of vegetable-related illnesses. Therefore, sanitization is required for the reduction of microorganism count and to ensure the microbial safety of baby leaf vegetables.

The initial counts of total aerobic mesophilic bacteria were determined to be 8.57 log CFU/g in the control (untreated) group, while the bacterial counts in samples treated with distilled water were 8.14 log CFU/g, indicating only a 0.43 log CFU/g reduction. Treatment with a 100 ppm NaOCl solution resulted in a decrease in the total aerobic mesophilic bacteria count to 7.21 log CFU/g, which is a reduction of 1.36 log CFU/g relative to the control. A single 0.1% GSE, 5% *Lactobacillus brevis* WK12 CFS, or *Leuconostoc mesenteroides* WK32 CFS treatment reduced the count of total aerobic mesophilic bacteria in the baby leaf vegetables by 1.60 log CFU/g. The application of CFSs in combination with 0.1% GSE significantly reduced total aerobic mesophilic bacteria counts, in comparison with those determined after the treatment with GSE or either CFS alone.

The presence of coliforms in leafy vegetables is undesirable because it indicates poor hygiene conditions due to contamination or inadequate washing (Choi, Park, Choi, Kim, & Chun, 2015). The control was shown to contain 7.00 log CFU/g coliforms, and washing the leafy samples with distilled water led to a reduction in coliform levels to 6.32 log CFU/g, corresponding to the reduction of 0.68 log CFU/g in coliform count (Table 2). Similar to the coliform count reductions observed following NaOCl treatment, the coliform counts observed after washing with GSE or either CFS decreased

Table 2. Inactivation of the natural microflora and inoculated foodborne pathogens by washing with CFSs in combination with GSE (log CFU/g)

Treatment	pH	Non-inoculated			Inoculated	
		Total aerobic mesophilic bacteria	Coliforms	Yeast/molds	*Escherichia coli* O157:H7	*Listeria monocytogenes*
Control	–	8.57 ± 0.13A	7.00 ± 0.03A	5.93 ± 0.09A	7.36 ± 0.09A	7.97 ± 0.07A
Distilled water	7.05	8.14 ± 0.14B	6.32 ± 0.06B	5.60 ± 0.25B	6.85 ± 0.08B	6.95 ± 0.07B
100 ppm NaClO	9.55	7.21 ± 0.05D	5.92 ± 0.08C	4.57 ± 0.12D	6.44 ± 0.05C	6.20 ± 0.08CD
0.1% GSE	3.96	7.40 ± 0.11CD	5.97 ± 0.09C	4.81 ± 0.18D	6.29 ± 0.09CD	6.26 ± 0.05CD
5% *Lactobacillus brevis* WK12 CFS	4.08	6.97 ± 0.19E	5.98 ± 0.04C	5.31 ± 0.12C	6.21 ± 0.09D	6.12 ± 0.06D
5% *Leuconostoc mesenteroides* WK32 CFS	4.50	7.54 ± 0.13C	5.87 ± 0.06C	5.14 ± 0.08C	6.14 ± 0.22D	6.57 ± 0.16BC
5% *Lactobacillus brevis* WK12 CFS + 0.1% GSE	4.03	6.56 ± 0.15F	5.26 ± 0.10D	4.24 ± 0.19E	5.64 ± 0.15F	5.08 ± 0.58E
5% *Leuconostoc mesenteroides* WK32 CFS + 0.1% GSE	4.28	6.74 ± 0.12F	5.36 ± 0.21D	4.06 ± 0.14E	5.88 ± 0.12E	6.01 ± 0.11D

Notes: The means are followed by different letters (A–F) that reflect significant differences ($p < 0.05$), as determined using Duncan's multiple-range test.

to 5.87–5.97 log CFU/g. Combination treatment with *Lactobacillus brevis* WK12 or *Leuconostoc mesenteroides* WK32 CFS and GSE resulted in coliform levels of 5.26 and 5.36 log CFU/g, representing the reduction of 1.74 and 1.64 log CFU/g, respectively, in comparison with that determined in the control sample.

Yeast/mold counts determined in this study showed patterns similar to those of the total aerobic mesophilic bacteria (Table 2). Washing with solution that contained 100 ppm NaOCl reduced the counts of yeast/molds in the leaf samples by approximately 1.36 log CFU/g. The hygienic capacity of 100 ppm NaOCl treatment against yeast/molds did not differ significantly from that of 0.1% GSE. In contrast, CFEs from *Lactobacillus brevis* WK12 and *Leuconostoc mesenteroides* WK32 in combination with GSE were more effective in reducing the yeast/mold levels, resulting in 1.69 and 1.87 log CFU/g reductions, respectively, relative to the control.

For inoculated RTE baby leaf vegetables, the initial counts of *E. coli* O157:H7 and *Listeria monocytogenes* inoculated on the samples were 7.36 and 7.97 log CFU/g, respectively. Washing with 100 ppm NaOCl resulted in *E. coli* O157:H7 and *L Listeria monocytogenes* counts of 6.44 and 6.20 log CFU/g, respectively, which represented the reduction of 0.92 and 1.77 log CFU/g, respectively. The combination of *Lactobacillus brevis* WK12 CFS and GSE reduced the levels of *E. coli* O157:H7 and *Listeria monocytogenes* by 5.64 and 5.08 log CFU/g, respectively. Our results indicate that washing with a binary solution of 5% CFS containing 0.1% GSE was more effective than treatments with either 100 ppm NaOCl or 5% CFS alone. Koo et al. (2015) reported that *Leuconostoc* isolates inhibited both the growth of pathogenic bacteria by producing antimicrobial agents (including organic acids) and the growth of meat spoilage bacteria, and that *E. coli* O157:H7 growth in ground beef was inhibited by *Leuconostoc* CFS. Pujato, del L Quiberoni, Candioti, Reinheimer, and Guglielmotti (2014) showed that *Leuconostoc citreum* CFS significantly reduced *Listeria monocytogenes* growth in milk. Although the antimicrobial activity of LAB has been previously reported, the present investigation is the first to evaluate the antimicrobial efficacy of combined CFS and GSE treatment. The pH of 5%

CFSs, 0.1% GSE, and 5% CFSs in combination with 0.1% GSE ranged from 3.96 to 4.50. We demonstrated that the effectiveness of single or binary sanitizer solutions against microorganisms found in RTE baby leaf vegetable samples is pH-dependent. This is probably due to the fact that the molecular structure, size, and pKa value of acids are important for the effects of these solutions as well. Furthermore, the use of chemical sanitizers with fresh produce is strictly regulated. For example, a maximum of 4 ppm residual chlorine in wastewater discharge is allowed by the U.S. National Organic Program Standard (Zhang, Critzer, Davidson, & Zhong, 2014), which can limit the effectiveness of sanitization. In contrast, organic acids are generally recognised as safe, and acetic, citric, and lactic acids are the most common acids used in the food industry for sanitization purposes (Meireles et al., 2016). No visible changes of the leaf vegetables were observed after washing them with CFSs alone or in combination with GSE (data not shown). Therefore, our findings provide evidence for the improved antimicrobial activity of *Lactobacillus brevis* WK12 and *Leuconostoc mesenteroides* WK32 CFSs in combination with GSE as natural sanitizers of baby leaf vegetables.

4. Conclusions

In summary, the results of this study provide clear evidence demonstrating that a natural sanitizer comprised of CFS and GSE can be useful for the reduction of native microbial flora and foodborne pathogens in mixed baby leaf vegetables. In particular, the greatest inactivation effects by *L. brevis* WK12 or *L. mesenteroides* WK32 CFSs on RTE baby leaf vegetables were achieved when used in combination with GSE. Therefore, as an alternative to chlorine sanitation, combined washing with CFSs and GSE can improve the microbial safety of RTE baby leaves. Additionally, combining this treatment with others, such as irradiation, modified atmosphere packaging, and low storage temperature, should be further investigated.

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Competing Interests
The authors declare no competing interest.

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