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Bacterial community analysis of winter salad during fermentation, and its antimicrobial properties

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Abstract

The mixture of pickled vegetables with tomato juice, known as winter salad, is one of Iranian traditional fermented foods. The present work aimed to identify the predominant bacterial community in winter salad during fermentation, and to evaluate the antimicrobial activity of its cell-free supernatant (CFS) against *Aspergillus niger* IBRC-M 30095, *Botrytis cinerea* IBRC-M 30162, *Aspergillus flavus* IBRC-M 30092, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhi* PTCC 1609, *Bacillus cereus* ATCC 11778, and *Staphylococcus aureus* ATCC 25923 using disc diffusion agar and microdilution assays. The fermentation dynamics of winter salad, changes in pH, acidity, salt, ash, protein, and fat contents, and bacterial composition were analysed during spontaneous fermentation. A total of 120 bacterial isolates were identified using 16S rDNA sequencing. Results showed that the following genera were dominant in the early stage of fermentation: *Lactobacillus (Lb. brevis, Lb. japonicus, Lb. pentosus, Lb. senmaizukei, Lb. plantarum, Lb. acidifarinae, Lb. parabrevis,* and *Lb. alimentarius*) (44%); *Leuconostoc* (*Ln. mesenteroides* and *Ln. palmae*) (13%); *Pediococcus* (*Pc. pentosaceus, Pc. parvulus, Pc. cellicola, Pc. argentinicus,* and *Pc. stilesii*) (7%); *Acinetobacter* (*Ab. johnsonii*) (4%); *Enterobacter* (*E. soli*) (10%); and unclassified isolates (22%). All isolates were identified successively during fermentation for 40 days; however, the species count changed throughout the fermentation. The CFS of winter salad showed inhibitory activity against all tested fungal species. *Ps. aeruginosa* and *Sa. typhi* were the most sensitive bacteria, while the minimum inhibitory percentage (MIP) and minimum bactericidal percentage (MBP) showed that *St. aureus* (MIP_{CFS-c} 75; MBP_{CFS-c} 75) and *Ba. cereus* (MIP_{CFS-c} 50; MBP_{CFS-c} 75) were the most resistant bacteria.

Introduction

In modern societies, healthy people tend to consume organic, safe, and functional foods such as fermented foods. Some of the health benefits of fermented foods on humans include antihypertensive, antidiarrheal, blood glucose-lowering, and antithrombotic properties (Parvez *et al.,* 2006). Food fermentation by lactic acid bacteria (LAB) is one of the oldest food preservation methods (Siedler *et al.,* 2019).

Winter salad, which consists of fermented pickled vegetables and tomato juice, is widely consumed in Khorasan Razavi province, Iran. It is usually served as a salty and sour side dish at dinner, and mostly produced via spontaneous fermentation based on homemade recipes. The maturity of winter salad can be distinguished by its typical colour, flavour, taste, and texture. For winter salad preparation, fresh vegetables such as garlic, cabbage, cauliflower, tomato, marjoram, and carrot are spontaneously fermented in the presence of salt and other spices, under anaerobic conditions at 20 - 25°C for 40 days. Cabbage (one of the main ingredients of winter salad) is highly valued as a nutritional source, and contains high levels of bioactive compounds. The main constituents of white cabbage (g/100 g) include proteins (1.27 - 1.37), dietary fibre (1.9 - 2.9), fat (0.06 - 0.20), minerals (0.3 - 0.7), carbohydrates (4.18 - 5.51), and vitamin C (0.03 - 0.04) (Souci *et al.,* 2000).

There are various fermented vegetables such as sauerkraut, kimchi, and winter salad, and one of the main components of these products is cabbage. The beneficial effects of sauerkraut have been frequently investigated. According to previous studies, the health benefits of sauerkraut include antioxidant activities, anti-carcinogenic properties, protective effects against oxidative DNA damage, and anti-inflammatory effects. Sauerkraut can also be

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considered as a functional food containing probiotic bacteria (Frias *et al.,* 2016). Overall, the available bioactive compounds of fermented products are dependent on the environmental conditions, substrates, geographic areas of production, bacterial strains, and fermentation methods (Starr *et al.,* 2015).

Commonly, LAB are used to initiate the spontaneous fermentation of vegetables (Oguntoyinbo and Dodd, 2010). Spontaneous fermentation of most pickled vegetables is dependent on the indigenous LAB such as *Lactobacillus plantarum* and *Leuconostoc mesenteroides*, which are often present in plant materials (Barrangou *et al.,* 2002). The addition of salt to vegetables, lack of exposure to light, and physical air exclusion (anaerobic condition) facilitate the growth of indigenous LAB at the beginning of the spontaneous fermentation. Also, oxygen depletion by cellular respiration in plants and carbon dioxide production by heterofermentative LAB further enhance the anaerobic environment. The anaerobic condition and the production of antimicrobial metabolites by LAB can lead to the death of a large number of pathogenic and spoilage microbiota (Harris, 1998; Hutkins, 2006). Therefore, LAB can become more dominant than the other bacteria, such as Gram-negative aerobic, Enterobacteriaceae, yeasts, and moulds (Hutkins, 2006).

Fresh vegetables without fermentation have a short shelf-life due to the metabolic activities of undesirable microorganisms and plant cell enzymes (Harris, 1998).The shelf-life of vegetables can be extended through fermentation. Some LAB's have been found to produce growth-inhibitory effects against food-borne and spoilage microorganisms. The antimicrobial compounds derived from the metabolic activity of LAB against yeasts and moulds, and Gram-positive and Gram-negative bacteria include organic acids, diacetyl, acetaldehyde, acetoin, hydrogen peroxide, carbon dioxide, ethanol, reuterin/acrolein, 3-hydroxy fatty acids, reutericyclin, cyclic dipeptides, 3-phenyllactic acid, 4-hydroxyphenyllactic acid, 2-hydroxy-4-methylpentanoic acid, 2-hydroxy acids, methylhydantoin, mevalonolactone, d-Dodecalactone, and bacteriocins (Siedler *et al.,* 2019).

Bacteriocins, as antimicrobial protein compounds, are produced by some bacteria during their growth. The LAB and bacteriocins as natural preservatives have been applied in processed foods to inhibit microbial spoilage. Today, lactobacilli are the most important producers of bacteriocins. Bacteriocins can be prepared in a purified form or in a bacteriocin-producing starter culture for food preservation (Zacharof and Lovitt, 2012). The concentrated cell-free supernatant (CFS) of fermented products containing antimicrobial compounds as food preservatives can be also recommended to food processing industries. Previous studies have indicated that CFS from several LAB have therapeutic properties (Arena *et al.,* 2016). It should be noted that some vegetables also possess antimicrobial compounds. For example, the extracts of green pepper, garlic, onion, and turnip inhibit the growth of pathogenic bacteria such as *Escherichia coli, Salmonella typhosa, Shigella dysenteriae*, and *Staphylococcus aureus* (Al-Delaimy and Ali, 1970). Sherman and Hodge (1936) also reported the bactericidal activity of cabbage.

So far, many strains of LAB isolated from spontaneously fermented products have been applied as starter cultures to monitor and optimise the fermentation process in laboratory-scale productions. The present work aimed to provide information about the bacterial community of winter salad during spontaneous fermentation, and to evaluate its antimicrobial properties. For the first time, we examined the dynamic changes of bacterial flora throughout the spontaneous fermentation. It seems that by identifying the bacterial diversity of winter salad during fermentation and evaluation of its antimicrobial properties, this process can be better understood, optimised, and controlled.

Materials and methods

Sample preparation

Three winter salad samples were prepared according to a traditional recipe from the northeast of Iran. Fresh garlic, cabbage, cauliflower, tomato, marjoram, and carrot purchased from a local supermarket in Mashhad, Khorasan Razavi Province, Iran were used to prepare the salad. Firstly, tomatoes (2,000 g) were rinsed and cut into four parts. The tomato juice was prepared by adding edible salt (60 g), followed by boiling at 100°C for 5 min, and cooling down to 25 - 30°C. Cabbage (250 g), cauliflower (250 g), and carrots (250 g) were washed, dried, sliced into small pieces (1 - 2 × 1 - 2 cm), and placed in 1,500-mL sterile glass jars along with garlic (125 g), angelica (1 g), chili pepper (6 g), and black pepper (1.25 g). Next, the cold salty tomato juice (1,000 mL) was added to the vegetables in the glass jars. After 24 h, the lids were tightly closed to exclude air, and the jars were stored at ambient temperature (20 - 25°C) for 40 d. The shredded vegetables were completely immersed to create anaerobic conditions, and prevent undesirable colour and flavour changes. The brine and mixed vegetables

were aseptically sampled every 5 d to analyse the bacterial community, and measure the pH, acidity, salt, protein, ash, and fat contents during fermentation.

Microbiological analysis

For this purpose, 25 g of the prepared sample was added to 225 mL of sterile peptone water, and homogenised with a vortex mixer; then, a serial dilution was prepared (10^{-7} dilution). For the LAB isolation, 0.1 mL of appropriate dilutions was plated onto the de Man, Rogosa, Sharpe (MRS), and M17 agar (Merck, Darmstadt, Germany). Next, the plates were incubated at 37°C under anaerobic conditions (Anaerocult® A; Merck, Darmstadt, Germany) for 48 h. To identify the bacteria, typical LAB colonies were selected, considering the differences in their morphology, and purified by the streaking method on MRS and M17 agar plates. All bacterial isolates were tested for Gram-staining reaction and catalase production. The catalase-negative and Gram-positive isolates were selected as presumptive LAB (Harrigan, 1998). To identify the members of the Enterobacteriaceae family, MacConkey agar (Merck, Darmstadt, Germany) was used to cultivate the bacteria at 37°C for 24 h (Mossel *et al.,* 1962).

Molecular identification of LAB isolates

For DNA extraction, the colonies of each isolate were suspended in 100 µL of sterile DNase-free water and 100 µL of chloroform: isoamyl alcohol (24:1 v/v), followed by vortexing for 5 s. Then, the mixture was centrifuged at 16,000 *g* for 5 min at 4°C. Next, the aqueous layer was used as a DNA template (Ruiz-Barba *et al.,* 2005). Polymerase chain reaction (PCR) for amplification of 16S rRNA gene was carried out in a 25-µL reaction tube, using the following primers: B27F (5′-AGAGTTT-GATCCTGGCTCAG-3′) and U1492R (5′-ACGTG-GTTTGAAGAGATTTTCG-3′) (Bioneer, Korea).

The PCR mixture consisted of 25 µL of 2X PCR Master Mix (SinaClon, Iran), 1 µL of reverse primer (10 pmol/µL), 1 µL of forward primer (10 pmol/µL), 18 µL of sterile PCR-grade water, and 5 µ L of each extracted DNA. Amplification was performed as follows: initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 1 min, at 56°C for 1 min, and at 72°C for 1 min. After a final extension at 72°C for 10 min, the reaction tubes were cooled down to 4°C (Davati *et al.,* 2015). The amplicons of the isolates were sent to Macrogen Company (Seoul, South Korea) for sequencing with 27F and 1492R primers. The partial sequence of 16S rRNA gene (approximately 1,500 bp) was analysed for all bacterial isolates by the BLAST tool available on the National Centre for Biotechnology Information (NCBI) website (http://www.nc-bi.nlm.nih.gov/).

Chemical analysis

The pH, fat, protein, salt, and ash contents were determined during fermentation according to the Association of Official Analytical Chemists (AOAC, 2000). Acidity was determined by titration of brine with 0.1 N sodium hydroxide, and phenolphthalein as the indicator (Xiong *et al.,* 2012). All tests were carried out in triplicate.

Antibacterial activity

The antibacterial activity of metabolites produced by the microbiota of winter salad was evaluated against *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhi* PTCC1609, *Bacillus cereus* ATCC 11778, and *Staphylococcus aureus* ATCC 25923 by disc diffusion agar and broth microdilution assays. The bacterial strains were purchased from the Iranian Research Organization for Science and Technology (IROST, Iran). All pathogens were grown in tryptic soy broth (Merck, Darmstadt, Germany), and incubated at 37°C. The disc diffusion agar (in triplicate) and broth microdilution assays, including minimum inhibitory percentage (MIP) and minimum bactericidal percentage (MBP), were performed according to Herreros *et al.* (2005) and Mayrhofer *et al.* (2008).

Firstly, 50 g of winter salad was centrifuged at 8,000 *g* for 20 min, and the CFS was recovered and treated at 80°C for 10 min for the antimicrobial assays. To determine the growth-inhibitory effects of total microbial metabolites (CFS-a), bacteriocins (CFS-b), and other metabolites (CFS-c), the following treatments were applied. The CFS was sterilised by filtration through a 0.2-µm filter (Millipore, Billerica, MA, USA) (CFS-a). To overcome the inhibitory effects of hydrogen peroxide and organic acids, the pH of CFS-a was adjusted to 6.5 by using 2M NaOH, followed by catalase digestion (1 mg/mL; Sigma, USA) at 37°C for 1 h (CFS-b). Next, an aliquot of CFS-b was digested using proteinase K (1 mg/mL; Sigma, USA) at 37°C for 2 h, and heated at 80°C for 60 min to eliminate the possible inhibitory bacteriocins.

The surface of the plate containing tryptic soy agar was swabbed with tryptic soy broth containing the overnight cultures of pathogenic bacteria (1% v/v). Next, sterile paper discs (diameter, 6 mm) were impregnated with 10 µL of each CFSs (CFS-a, CFS-b, and CFS-c), and the control disc was

impregnated with 10 µL of sterile distilled water. Afterward, the discs were placed on the inoculated surface. The plates were incubated at 37°C for 24 h, and the inhibition zones around the discs were measured. For the broth microdilution assays, each well was loaded with 150 µL of tryptic soy broth, and inoculated with an overnight culture of 1% (v/v) pathogenic bacteria, and 150 µL of each CFS at different percentages (3.12, 6.25, 12.5, 25, 50, and 75%; $\frac{\text{CFS(mL)}}{\text{CFS(mL)} + \text{tryptic soy broth (mL)}} \times 100$ in a final volume of 300 µL.

The positive control was the well containing 150 µL of tryptic soy broth, inoculated with 1% (v/v) pathogenic bacteria and 150 µL of sterile distilled water. The negative control was the well containing 150 µL of CFSs and 150 µL of tryptic soy broth. The plates were incubated at 37°C for 24 h. The lowest percentage of CFS that inhibited any visible growth or turbidity in comparison with the positive control was recorded as the MIP of CFS. Treatments without any visible growth were cultured on tryptic soy broth, and incubated at 37°C for 24 h. The MBP of CFSs was recorded as the lowest percentage of CFSs inhibiting the colony-forming ability.

Antifungal activity

The antifungal activity of metabolites produced by the microbiota of winter salad was evaluated against *Aspergillus niger* IBRC-M 30095 (CCM 8155), *Aspergillus flavus* IBRC-M 30092 (CCM F-449), and *Botrytis cinerea* IBRC-M 30162 (CCM F-16). The fungal strains were purchased from the Iranian Biological Resource Centre (IBRC, Iran). To produce spore suspensions, the fungal strains were grown on potato dextrose agar (PDA; Sigma, USA) at 25 ± 2°C for 5 d, and washed with 10 mL of sterile distilled water. The fungal spore count was measured by direct microscopic examination using a haemocytometer.

The antifungal activity was evaluated using the agar disc diffusion assay in triplicate according to Kumar *et al.* (2016). For this purpose, 100 µL of the spore suspension (approximately 2×10^6 spores/mL) from each fungal strain were inoculated onto PDA, and spread to achieve a uniform fungal growth. Next, sterile paper discs (diameter, 6 mm) soaked in 10 µL of each CFSs and the control disc soaked in 10 µL of sterile distilled water were placed on the inoculated surface, followed by incubation at 25 ± 2°C for 7 d. The antifungal activity of CFSs was determined by measuring the diameter of the growth inhibition zone (mm) around each disc.

Statistical analysis

The effects of fermentation time on pH, titratable acidity, salt, fat, ash, and protein contents were analysed in SPSS Version 16, using repeated measures one-way ANOVA on the data obtained every five days in triplicate. The significance level was set at $p < 0.05$. Graphs were plotted using Microsoft Excel 2010. The results of the disc diffusion assay were analysed to calculate the mean and standard deviation (SD), also using Microsoft Excel 2010. The neighbour-joining tree was constructed for the phylogenetic analysis using MEGA-X software.

Results

Chemical analysis during fermentation of winter salad

The changes in the acidity, pH, ash, fat, protein, and salt contents during fermentation are shown in Table 1. Throughout the fermentation, pH decreased from approximately 4.34 ± 0.04 at the beginning of fermentation to 3.17 ± 0.02 at the end of fermentation. The total titratable acidity and salt percentage increased continuously during fermentation. The acidity increased from 0.69 ± 0.01 at the beginning of fermentation to 1.99 ± 0.01 at the end of fermentation. The salt content increased from 6 ± 0.02 at the beginning of fermentation to 11.12 ± 0.02 at the end of fermentation. Based on the statistical analysis, the fermentation time had significant effects on the pH, acidity, and salt content of fermented samples ($p < 0.05$), while it had no significant effects on the ash, fat, and protein contents ($p > 0.05$).

Changes in the dominant bacterial genera during the fermentation of winter salad

Based on Figure 1, the number of *Lactobacillus* genus increased significantly in the first 10 days of fermentation, followed by a slight increase until the 40th day (maximum count on the 40th day). On the other hand, a slow increase was observed in *Leuconostoc* and *Pediococcus* genera until the 5th day, followed by a slight reduction until the 40th day. Based on the results, the number of *Acinetobacter* and *Enterobacter* spp. decreased in the initial stage of fermentation until the 5th and 10th days, respectively. The number of these bacteria remained almost constant in the middle stage, and decreased in the final stage of fermentation (Figure 1).

The sharp rise in LAB count in the early stage of fermentation was probably related to the presence of salt, temperature (20 - 25°C), and

Table 1. Changes in pH, acidity, fat, protein, ash, and salt contents of winter salad during fermentation.

	Fermentation time (day)								
	1	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**
pH	4.34 ± 0.04^{a}	4.07 ± 0.06^{b}	3.83 ± 0.02^{c}	3.62 ± 0.03^{d}	3.52 ± 0.02^{e}	3.32 ± 0.02^{f}	3.22 ± 0.02^{g}	3.20 ± 0.03^{g}	3.17 ± 0.02^{i}
Acidity (%)	0.69 ± 0.01^{a}	0.74 ± 0.01^{a}	1.07 ± 0.06^{c}	1.24 ± 0.02^{d}	1.30 ± 0.01^{d}	1.41 ± 0.01^{f}	1.76 ± 0.01^{g}	1.94 ± 0.01^{h}	1.99 ± 0.01^{h}
Salt (g/100 g)	6 ± 0.02^{a}	6.11 ± 0.01^{ab}	6.22 ± 0.01^{be}	6.34 ± 0.01^{e}	8.28 ± 0.01^{d}	9.53 ± 0.03^{c}	10.05 ± 0.05^{g}	10.29 ± 0.08^{g}	11.12 ± 0.02^{f}
Protein (g/100 g)	6.11 ± 0.78^{a}	6.12 ± 0.26^{a}	6.12 ± 0.07^{a}	6.12 ± 0.54^{a}	6.12 ± 0.31^{a}	6.12 ± 0.13^{a}	6.12 ± 0.05^{a}	6.12 ± 0.18^{a}	6.12 ± 0.24^{a}
Ash (g/100 g)	2.11 ± 0.72^{b}	2.11 ± 0.33^{b}	2.11 ± 0.41^{b}	2.10 ± 0.87^{b}	2.10 ± 0.72^{b}	2.10 ± 0.61^{b}	2.10 ± 0.52^{b}	2.10 ± 0.43^{b}	2.10 ± 0.21^{b}
Fat (g/100 g)	0.24 ± 0.03^{c}	0.23 ± 0.04^{c}	0.23 ± 0.01^{c}	0.23 ± 0.04^{c}	0.22 ± 0.07^{c}	0.22 ± 0.02^{c}	0.22 ± 0.04^{c}	0.22 ± 0.02^{c}	0.22 ± 0.01^{c}

Within a row, means sharing similar lowercase superscript are not significantly different ($p < 0.05$).

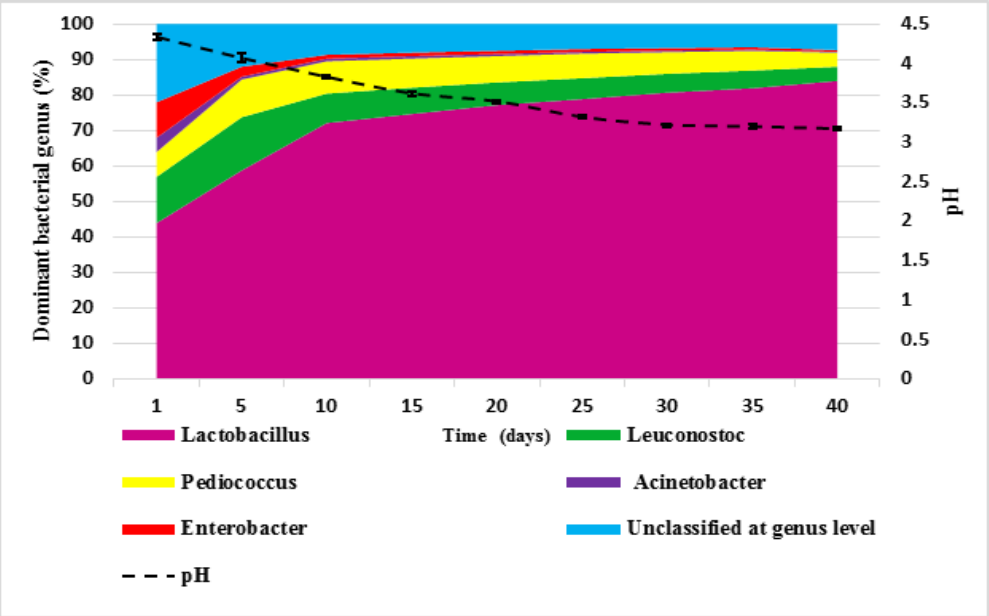

Figure 1. Dynamics of bacterial community composition (at the genus level) and changes in pH (n = 3) during fermentation. Error bars represent standard error of the mean for pH.

anaerobic environment (available oxygen removal in the glass jar by the resident heterofermentative LAB on vegetables). On the other hand, the inhibitory effects of salt, low pH, anaerobic conditions, high content of organic acids (*e.g.,* lactic acid), and other antimicrobial metabolites produced by LAB led to the considerable reduction of non-LAB species during fermentation. Therefore, *Lactobacillus, Leuconostoc*, and *Pediococcus* spp. became the dominant genera, responsible for the ripening of winter salad during fermentation.

Changes in the dominant bacterial species during the fermentation of winter salad

A total of 120 strains were isolated from winter salad. Using 16S rRNA gene sequencing, bacterial isolates were identified as *Lactobacillus brevis, Lactobacillus japonicus, Lactobacillus pentosus, Lactobacillus senmaizukei, Lactobacillus plantarum, Lactobacillus acidifarinae, Lactobacillus parabrevis, Lactobacillus alimentarius, Leuconostoc. mesenteroides, Leuconostoc palmae, Pediococcus pentosaceus, Pediococcus cellicola, Pediococcus parvulus, Pediococcus argentinicus, Pediococcus stilesii, Acinetobacter johnsonii, Enterobacter soli,* and unclassified isolates. All these species were identified successively during fermentation, although their counts changed throughout the fermentation. The results showed that *Lactobacillus* spp. (*Lb. brevis, Lb. japonicus, Lb. pentosus, Lb. senmaizukei, Lb. plantarum, Lb. acidifarinae, Lb. parabrevis,* and *Lb. alimentarius*) (44%), *Leuconostoc* spp. (*Ln. mesenteroides* and *Ln. palmae*) (13%), *Pediococcus* spp. (*Pc. pentosaceus, Pc. cellicola, Pc. parvulus, Pc. argentinicus*, and *Pc. stilesii*) (7%), *Acinetobacter* spp. (*Ab. johnsonii*) (4%), *Enterobacter* spp. (*E. soli*) (10%), and unclassified isolates (22%) were dominant in the early stage of fermentation.

Moreover, *Lactobacillus* spp. (*Lb. brevis, Lb. japonicus, Lb. pentosus, Lb. senmaizukei, Lb. plantarum, Lb. acidifarinae, Lb. parabrevis*, and *Lb. alimentarius*) (84%), *Leuconostoc* spp. (*Ln. mesenteroides* and *Ln. palmae*) (4%), *Pediococcus* spp. (*Pc. pentosaceus, Pc. cellicola, Pc. parvulus, Pc. argentinicus*, and *Pc. stilesii*) (4%), *Acinetobacter* spp. (*Ab. johnsonii*) (0.2%), *Enterobacter* spp. (*E. soli*) (0.5%), and unclassified isolates (7.3%) were dominant in the final stage of fermentation. Changes in the dominant LAB and non-LAB bacterial species, along with titratable acidity during fermentation, are shown in Figure 2. The major contributors to the ripening of winter salad were *Lb. brevis, Lb. japonicus, Lb. pentosus, Lb. senmaizukei, Lb. plantarum*, and *Ln. mesenteroides* in the tested samples.

During fermentation, the number of *Lb. japonicus* increased and reached its maximum level on the 10th day (23%), followed by a slight decrease until the 40th day. Moreover, the load of *Lb. brevis* gradually increased and reached its maximum on the 40th day (23%). We found a nearly linear increase in the number of *Lb. pentosus* and *Lb. senmaizukei*, with maximum increase observed on the 40th day

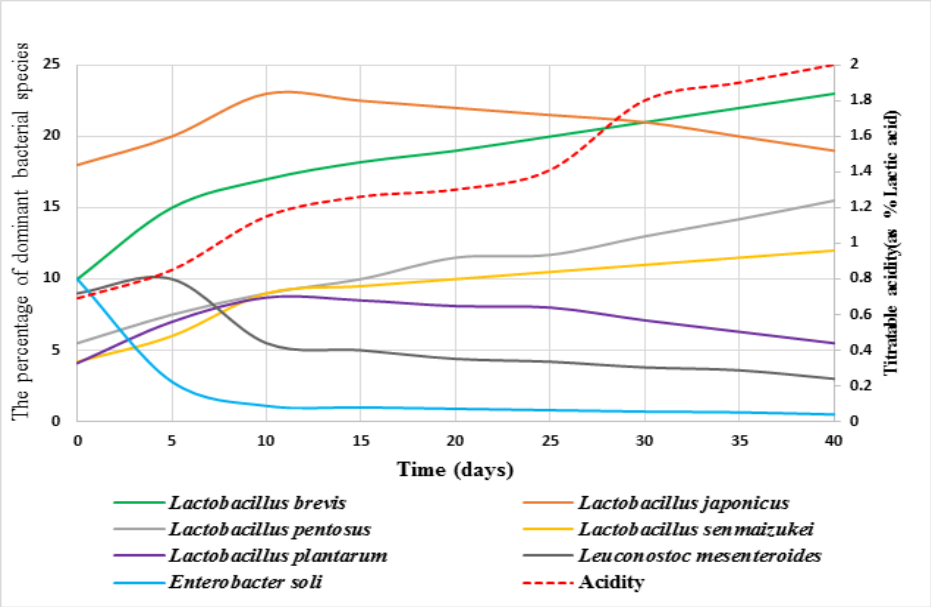

Figure 2. Changes in the percentage of dominant LAB and non-LAB species along with acidity during fermentation.

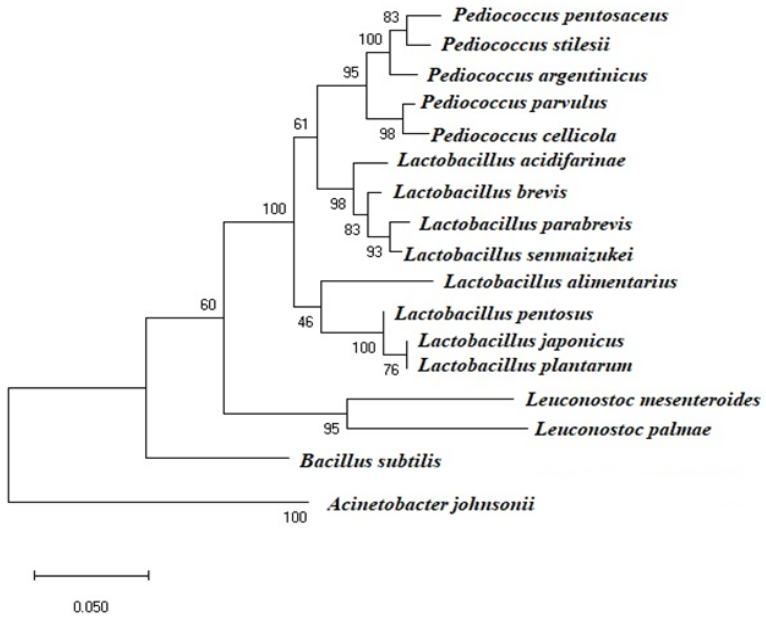

Figure 3. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence analysis showing the phylogenetic positions of isolates from the winter salad. The tree was constructed by the Maximum Likelihood statistical method, tested by bootstrap method of 500 replicates, and *B. subtilis* was used as the out group.

(15.5 and 12%, respectively). Besides, the number of *Lb. plantarum* increased and reached its maximum on the 10th day (8.7%), which decreased until the 40th day. Also, the number of *Ln. mesenteroides* increased and reached its maximum on the 5th day (10%), followed by a sharp decrease by the 10th day, and a gradual decrease until the 40th day. On the other hand, a sharp decrease was observed in *E. soli*, as the dominant non-LAB species, at the beginning of fermentation (5th day), which reached its minimum level (0.5%) in the final stage of fermentation. The phylogenetic analysis showed a great biodiversity of bacterial species in the winter salad samples (Figure 3).

Determination of the antimicrobial properties of CFSs

To investigate the antimicrobial metabolites produced by the microbial community of winter salad, the CFSs were subjected to three treatments (organic acid neutralisation, hydrogen peroxide

elimination, and bacteriocin digestion). The CFSs of winter salad showed inhibitory effects against four food pathogenic bacteria due to the presence of antibacterial metabolites produced by LAB, as shown by the disc diffusion assay (Table 2); these effects were further assessed by the broth microdilution assay. In the CFS-a tests, the largest (15 ± 0.3 mm) and smallest (11 ± 0.7 mm) inhibition zone diameters, based on the disc diffusion assay, were observed against *Salmonella typhi* and *Staphylococcus aureus*, respectively.

The MIPs of CFS-a for *Pseudomonas aeruginosa, Salmonella typhi, Bacillus cereus*, and *Staphylococcus aureus* were 6.25, 6.25, 25, and 25%, respectively. The MBPs of CFS-a for *Pseudomonas aeruginosa, Salmonella typhi, Bacillus cereus*, and *Staphylococcus aureus* were 12.5, 50, 50, and 50%, respectively. Besides, the MIP of CFS-b was estimated at 25% for *Pseudomonas aeruginosa, Salmonella typhi, Bacillus cereus*, and *Staphylococcus aureus*. Also, the MBP of CFS-b was 50% for *Pseudomonas aeruginosa, Salmonella typhi, Bacillus cereus*, and *Staphylococcus aureus*. In addition, the MIPs of CFS-c for *Pseudomonas aeruginosa, Salmonella typhi, Bacillus cereus*, and *Staphylococcus aureus* were 25, 25, 50, and 75%, respectively, and the MBPs of CFS-c for *Pseudomonas aeruginosa, Salmonella typhi, Bacillus cereus*, and *Staphylococcus aureus* were 50, 75, 75, and 75%, respectively.

Based on Table 2, the CFS of winter salad lost its antibacterial potential against *Salmonella typhi* and *Pseudomonas aeruginosa* as Gram-negative bacteria after pH neutralisation and catalase treatment (CFS-b). However, the antibacterial potential of CFS reduced against *Bacillus cereus* and *Staphylococcus aureus* as Gram-positive bacteria after bacteriocin digestion (CFS-c). The CFSs of winter salad showed antifungal activities against three food-borne fungal species due to the presence of growth-inhibitory metabolites produced by LAB, as shown by the disc diffusion assay (Table 2). The CFSs completely inhibited the growth of fungal species, and no growth was observed after seven days in comparison with the control disc.

Discussion

The decrease in pH and the increase in acidity may be related to the fermentation of reducing sugar in winter salad to produce lactic acid by indigenous bacteria. The salt content of winter salad, which was determined by chloride ion concentration, increased during fermentation. The increase in the salt concentration may be due to the release of chloride ions during the degradation process of organic compounds and the metabolic activities of the microbial community in winter salad. *Lactobacillus brevis, Lb. japonicus, Lb. pentosus, Lb. senmaizukei, Lb. plantarum, Lb. acidifarinae, Lb. parabrevis, Lb. alimentarius, Leuconostoc. mesenteroides, Ln. palmae, Pc. pentosaceus, Pc. cellicola, Pc. parvulus, Pc. argentinicus, Pc. stilesii, Ab. johnsonii*, and *E. soli* were found in winter salad on day 0; in other words, these species were present in the raw vegetables and transferred to the fermentation jar. Results showed that *Lb. brevis* was the dominant LAB during the fermentation of winter salad until the 40th day, probably due to the high tolerance of *Lb. brevis* against nitrite and salt (Xia *et al.,* 2017).

The high content of nitrite in white cabbage, carrot, and cauliflower has been previously confirmed (Ding *et al.,* 2018). Our findings are in agreement with the results reported by Xiong *et al.* (2012) and Xia *et al.* (2017) who showed that *Lb. brevis* played an important role in the fermentation of Chinese pickles. Moreover, in a study by Oh *et al.*

Table 2. Antimicrobial properties of CFSs.

	Minimum inhibitory percentage of CFSs			**Minimum bactericidal percentage of CFSs**			**The diameter of growth inhibition zone (mm, mean ± SD)**		
	CFS-a	**CFS-b**	**CFS-c**	**CFS-a**	**CFS-b**	**CFS-c**	**CFS-a**	**CFS-b**	**CFS-c**
Pseudomonas aeruginosa	6.25	25	25	12.5	50	50	14 ± 0.8	9 ± 0.4	8 ± 0.6
Salmonella typhi	6.25	25	25	50	50	75	15 ± 0.3	8 ± 0.5	7 ± 0.7
Bacillus cereus	25	25	50	50	50	75	12 ± 0.2	10 ± 0.5	2 ± 0.2
Staphylococcus aureus	25	25	75	50	50	75	11 ± 0.7	8 ± 0.4	1 ± 0.5
Aspergillus niger							No growth	No growth	No growth
Botrytis cinerea							No growth	No growth	No growth
Aspergillus flavus							No growth	No growth	No growth

(2004), the ability to metabolise nitrite was confirmed in LAB other than *Lb. brevis*, such as *Lb. plantarum* and *Ln. mesenteroides*, which were also isolated from the winter salad assessed in the present work; this might have been due to the similarity in the composition of winter salad and Chinese pickles. Meanwhile, *Lb. pentosus* and *Lb. plantarum* were isolated from Sichuan, Thai fermented foods, and different varieties of pickled vegetables such as Chinese sauerkraut (Yu *et al.,* 2012; Xiong *et al.,* 2012).

Our results are also in accordance with the findings reported by Di Cagno *et al.* (2013) who isolated *Lb. plantarum* from tomato, carrot, and cabbage; *Lb. brevis* from tomato and cabbage; and *Ln. mesenteroides* from cabbage, carrot, and pepper. In comparison with previous studies, *Ln. palmae*, which was found during the fermentation of winter salad in the present work, was not detected in other fermented vegetables. Besides, *Lb. senmaizukei* has been previously reported as a novel species isolated from Japanese pickles (Hiraga *et al.,* 2008). So far, the isolation of *Lb. japonicus, Pc. cellicola, Pc. parvulus, Pc. argentinicus*, and *Pc. stilesii* from spontaneously fermented vegetables has been rarely reported. The presence of these rare species in the present work suggested that the ingredients and environmental conditions of winter salad have led to a unique fermentation process.

Similar to other spontaneous fermentation processes of vegetables, fermentation of winter salad is initiated by *Leuconostoc* spp. (*Ln. mesenteroides* and *Ln. palmae*). Overall, the production of carbon dioxide and organic acids reduces pH and limits the activity of undesirable microorganisms. The created anaerobic conditions also suppress the chemical oxidation of vitamins, and lead to colour changes in vegetables. The reduced cell number of *Leuconostoc* spp. after five days could be attributable to lower pH (Harris *et al.,* 1992). It seems that *Lb. brevis* and *Lb. acidifarinae* as obligately heterofermentative bacteria, *Lb. pentosus, Lb. alimentarius*, and *Lb. plantarum* as facultatively heterofermentative bacteria, and *Ln. mesenteroides* contribute to the production of more anaerobic conditions with a lower pH.

During the fermentation of winter salad, the growth of *Pediococcus* spp. as homofermentative bacteria continued to a final pH of 3.17; a similar finding has been reported in other fermented pickled vegetables, such as sauerkraut (Xiong *et al.,* 2012). *Acinetobacter johnsonii* (formerly *Ab. calcoaceticus* var. *lwoffii*) was previously isolated from the digestive tract of a prawn for the first time in Australia (Domingues *et al.,* 2011), as well as in vegetables (Berlau *et al.,* 1999). Therefore, the presence of *Ab. johnsonii* in winter salad on day 0 indicated its attachment to raw vegetables and its transfer to the jars.

The presence of *Enterococcus* spp. in the present work (*E. soli*) revealed that winter salad was produced under poor hygienic conditions or that faecal contamination of vegetables occurred for a long time, as this genus was resistant to environmental conditions (Hamilton-Miller and Shah, 2001). The sharp decrease in *Ab. johnsonii* and *E. soli* populations until the 5th and 10th days, respectively, could probably be attributed to the presence of antibacterial metabolites produced by LAB, low pH, and the anaerobic environment of winter salad (Hamilton-Miller and Shah, 2001; Xiong *et al.,* 2012).

The dynamics of the microbial community in other traditional pickled vegetables have been investigated in previous studies. The bacteria detected in pickled cucumbers during fermentation were often *Ln. mesenteroides, Lb. plantarum, Lb. brevis*, and *Pc. cerevisiae*. Based on previous studies, spontaneous fermentation of sauerkraut was initiated by *Ln. mesenteroides*, followed by *Pc. cerevisiae* and *Lb. brevis*, and ended with *Lb. plantarum* (Banwart, 2012). On the other hand, in fermented green olives, the microbial flora responsible for ripening included *Lb. plantarum, Lb. paraplantarum, Lb. pentosus, Ln. pseudomesenteroides, Pediococcus* spp., *Pseudomonas* spp., and *Raoultella* spp. (Blana *et al.,* 2014). Moreover, Hong *et al.* (2016) investigated the microbial population of Korean cabbage kimchi and reported that *Ln. gelidum, Ln. citreum, Ln. mesenteroides,* and *Pc. pentosaceus* were the dominant bacterial species.

Based on the present findings, there were differences in the microbial community structure of winter salad and other similar fermented samples. This could be due to the variations in the geographic region of production, raw materials, vegetable varieties, edible salt concentrations, spices, seasons, procedures, cultivation patterns, and harvesting conditions. These factors may be important for selecting the number and type of microorganisms attaching to vegetables and the fermentation environment (Cho *et al.,* 2006; Xiong *et al.,* 2012; Montet *et al.,* 2014).

According to Cho *et al.* (2006), the microbial community structure may vary between fermented products depending on the environment temperature. Lee *et al.* (2008) detected *Weissella koreensis* as a psychrophilic species in kimjang kimchi, which was

probably related to the low temperature of the fermentation environment (5 - 9°C) and storage (-2°C). The optimal temperature for winter salad fermentation was 20 - 25°C. At this temperature, the ripening of this product was achieved in four weeks. Therefore, another reason for the unique microbial community of winter salad might be the fermentation temperature.

The dynamic changes of the microflora during fermentation have been studied more extensively in pickled cucumbers, sauerkraut, and kimchi. However, in the present work, for the first time, we identified the major contributors to the fermentation of winter salad. By evaluating the bacterial diversity of winter salad, its microbial ecology can be better understood and technologically controlled. Moreover, the spontaneous fermentation process of winter salad did not indicate distinct phases, and the most dominant LAB species, isolated during fermentation, were heterofermentative bacteria.

According to previous studies, the identified LAB in the present work may produce different antimicrobial compounds including lactic acid, diacetyl, acetaldehyde, acetoin, and hydrogen peroxide derived from the LAB isolates (against yeasts, Gram-positive, and Gram-negative bacteria); acetic acid, carbon dioxide, and ethanol from *Lb. brevis, Lb. acidifarinae, Lb. pentosus, Lb. alimentarius, Lb. plantarum*, and *Ln. mesenteroides* (against yeasts, Gram-positive, and Gram-negative bacteria); benzoic acid from *Lb. plantarum* (against moulds and Gram-negative bacteria); cyclic dipeptides from *Lb. plantarum* and *Lb. pentosus* (against moulds); 3-phenyllactic acid from *Lb. alimentarius* and *Lb. plantarum*; 4-hydroxyphenyl-lactic acid from *Lb. brevis* and *Ln. mesenteroides* (against moulds); 2-hydroxy-4-methypentanoic acid from *Lb. plantarum* (against moulds); mevalonolactone and d-Dodecalactone from *Lb. plantarum* (against moulds and Gram-negative bacteria); and bacteriocins, such as pediocin, plantaricin, pentocin, and leucocin from *Pediococcus* spp., *Lactobacillus* spp., and *Leuconostoc* spp. (Siedler *et al.,* 2019).

Based on the present results, the CFSs strongly inhibited the growth of *As. flavus, As. niger*, and *Bo. cinerea*. As mentioned earlier, this can be attributed to the possible presence of 2-hydroxy-4-methypentanoic acid, cyclic dipeptides, mevalonolactone, cyclic dipeptides, benzoic acid, 4-hydroxyphenyllactic acid, and d-Dodecalactone produced by the LAB in winter salad. The results showed that CFS-a (probably containing organic acids, hydrogen peroxide, bacteriocins, and other antimicrobial metabolites) was more effective than CFS-b against *Ps. aeruginosa* and *Sa. typhi* as Gram-negative bacteria (probably containing bacteriocins and other antimicrobial metabolites). Also, our results showed that CFS-b had a greater growth-inhibitory potential than CFS-c against *Ba. cereus* and *St. aureus* due to the possible presence of bacteriocins. Since bacteriocins are antimicrobial substances produced by numerous strains of LAB to prevent the growth of closely related Gram-positive strains such as *Ba. cereus* and *St. aureus*, they are less effective against Gram-negative bacteria (Nespolo and Brandelli, 2010).

Some LAB isolated from winter salad with bacteriocin-producing capacities include *Lb. brevis* (Banerjee *et al.,* 2013), *Lb. pentosus* (pentocin), *Lb. plantarum* (plantaricin EF, W, S, and JK), *Leuconostoc* spp. (leucocin) (Zacharof and Lovitt, 2012), and *Pediococcus* spp. (pediocin) (Yusuf and Hamid, 2013). In this regard, Banerjee *et al.* (2013) showed that *Lb. brevis* isolated from freshwater fish could produce bacteriocins and exhibited a broad spectrum of inhibitory effects against *Lb sakei, E. faecalis, E. coli, Lb. casei*, and *St. aureus*. In another study, the bacteriocin produced by *Lb. brevis*, isolated from dongchimi exhibited growth-inhibitory effects against *Listeria monocytogenes* and *E. faecalis* (Lim, 2011). Moreover, Todorov and Dicks (2007) showed that the bacteriocin produced by *Lb. pentosus* inhibited the growth of *E. coli, E. faecalis, Ps. aeruginosa, Klebsiella pneumonia, Lb. casei*, and *Lb. curvatus*.

Previous studies have shown that *Lb. plantarum* strains produce different antimicrobial compounds such as diacetyl, organic acids, bacteriocins, hydrogen peroxide, and antimicrobial peptides against *L. monocytogenes* and *E. coli* (Arena *et al.,* 2016). Bennik *et al.* (1997) reported two bacteriocin-producing strains of *Pc. parvulus* isolated from minimally processed vegetables. Also, some studies revealed that Pc. pentosaceus inhibited the growth of *Lactococcus, Pediococcus, Leuconostoc, Enterococcus, Staphylococcus, Listeria, Lactobacillus*, and *Clostridium* spp. (Piva and Headon, 1994).

According to several studies, the CFS of *Ln. mesenteroides* strains exhibit antimicrobial activities against the growth of *Carnobacterium* spp., *E. faecalis, Ba. subtilis,* some *Lactobacillus* spp., *Listeria innocua, Lactococcus lactis* subsp. *Cremoris, Pc. pentosaceus, Streptococcus thermophilus, St. aureus, Clostridium* spp., *and L. monocytogenes*. However, in these studies, the growth of Gram-negative bacteria was not inhibited (Daba *et al.,* 1991;

Todorov and Dicks, 2004). The low resistance of Gram-negative bacteria, such as Salmonella spp. to the CFS of winter salad can be attributed to their thin peptidoglycan of cell wall and sensitivity to organic acids (Sharma et al., 2017). Venkadesan and Sumathi (2015) isolated *Lb. fermentum, Lb. delbrueckii* subsp. *bulgaricus, Lb. acidophilus, Lb. plantarum, Leuconostoc* spp., *St. thermophilus, Pc. acidilactici, Pc. pentosaceus*, and *Lc. lactis* from curd, cheese, yogurt, butter, and buttermilk. Similar to our study, they showed that the culture filtrates of the LAB isolated from the dairy products exhibited growth-inhibitory effects against *St. aureus* and *Salmonella* species.

Conclusion

Results of the present work provide valuable information and fundamental knowledge for future studies on the dynamics and monitoring of winter salad fermentation. Also, the identification of microbial community of winter salad can prevent the loss of its biodiversity. It was found that *L. brevis, L. japonicus, L. pentosus, L. senmaizukei, L. plantarum, L. acidifarinae*, and *Ln. mesenteroides* were suitable as starter cultures for the controlled production of winter salad. Overall, preventive care can be achieved by the intake of functional foods such as fermented vegetables, which can promote various aspects of human health. Since some of the major players of mature winter salad such *as L. brevis, L. pentosus, L. plantarum*, and *Ln. mesenteroides* were previously known as probiotic bacteria, winter salad can be recommended as a functional food. However, the probiotic properties of these isolates should be further investigated. Overall, the CFSs of winter salad exhibited growth-inhibitory activities against food-borne bacteria and fungi due to the presence of antimicrobial compounds produced by the LAB during the fermentation of winter salad. Bacteriocins are effective and safe alternatives for chemical preservatives which can pose health risks to consumers. The dehydrated CFS of winter salad, which possibly contains bacteriocins, can be a suitable food preservative to inhibit the growth of spoilage microorganisms and increase the shelf-life of processed foods.

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