III.　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻	ページ	出版年
Kanki M, Naruse H, Taguchi M, Kumeda Y.	Characterization of specific alleles in InlA and PrfA of *Listeria monocytogenes* isolated from foods in Osaka, Japan and their ability to invade Caco-2 cells.	Int J Food Microbiol.	211	18-22	2015
Masuda K, Yamamoto S, Kubota K, Kurazono H, Makino S, Kasuga F, Igimi S, Asakura H.	Evaluation of the dynamics of microbiological quality in lightly pickled napa cabbages during manufacture.	J Food Safety.	35	458-465	2015
Asakura H, Tachibana M, Taguchi M, Hiroi T, Kurazono H, Makino S, Kasuga F, Igimi S.	Seasonal and growth-dependent dynamics of bacterial community in radish sprouts.	J Food Safety.	In press	doi:10.1111/jfs.12256	2016
Momose Y, Asakura H, Kitamura M, Okada Y, Ueda Y, Hanabara Y, Sakamoto T, Matsumura T, Iwaki M, Kato H, Shibayama K, Igimi S.	Food-borne botulism in Japan in March 2012.	Int J Infect Dis.	24	20-22	2014
杉山広、荒川京子、柴田勝優、川上泰、森嶋康之、山崎浩、荒木潤、生野博、朝倉宏.	わが国における土壌媒介寄生中症，特に回虫症の発生とその汚染源の文献的および検査期間データに基づく調査.	食品衛生研究	65	37-41	2015
堀内朗子、荒川京子、秋庭達也、吉田建介、平田史子、松本奈保子、丸山弓美、奥津敬右、朝倉宏、杉山広.	ストマッカーを利用した野菜等の回虫卵検査法の検討.	食品衛生研究	65	45-50	2015

International Journal of Food Microbiology 211 (2015) 18–22

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Short communication

Characterization of specific alleles in InlA and PrfA of *Listeria monocytogenes* isolated from foods in Osaka, Japan and their ability to invade Caco-2 cells

Masashi Kanki *, Hisayo Naruse, Masumi Taguchi, Yuko Kumeda

Department of Infectious Diseases, Osaka Prefectural Institute of Public Health, 1-3-69 Nakamichi, Higashinari-ku, Osaka 537-0025, Japan

ARTICLE INFO

Article history:
Received 18 March 2015
Received in revised form 23 June 2015
Accepted 26 June 2015
Available online 2 July 2015

Keywords:
Listeria monocytogenes
Cellular invasion
InlA
PrfA
PMSC

ABSTRACT

Listeria monocytogenes expresses the surface protein internalin A (InlA), enabling the invasion of human intestinal epithelial cells to cause severe food-borne diseases. Full-length sequence analysis of *inlA* of 114 food isolates resulted in the detection of 29 isolates with a premature stop codon (PMSC) mutation and 6 isolates with 3-codon deletion mutations (aa 738 to 740) in *inlA*. The isolates with *inlA* PMSCs demonstrated a significantly lower level of invasion than the other food isolates in a Caco-2 cell invasion assay ($P < 0.01$), but the isolates with the 3-codon deletion exhibited invasion comparable to the isolates with non-truncated InlA ($P > 0.05$). According to analysis of the positive regulatory factor A (PrfA) sequences of 114 *L. monocytogenes* isolates, 7 isolates of serotype 1/2a from chicken samples contained a PrfA protein with a 5-nucleotide deletion from 712 to 716, including a stop codon. Although the isolates with a 5-nucleotide deletion in *prfA* demonstrated invasion comparable to the isolates with non-truncated InlA and PrfA after growth at 30 °C ($P > 0.05$), they exhibited a significantly higher level of invasion than the other isolates after growth at 20 °C ($P < 0.01$). To the authors' knowledge, this is the first report of *L. monocytogenes* isolates with the stop-codon deletion of PrfA.

1. Introduction

Listeria monocytogenes is a facultative intracellular, Gram-positive bacterium with a primary habitat that appears to consist of soil and decaying vegetation (Kathariou, 2002). This pathogen is transmitted to humans through ingestion of contaminated food, particularly ready-to-eat (RTE) meat, seafood, and dairy products (Lianou and Sofos, 2007). Although *L. monocytogenes* infections are rare in healthy individuals, these infections can cause meningitis and septicemia in neonates and the elderly, as well as miscarriage in pregnant women. Mortality rates for invasive listeriosis range from 20% to 40% (Kovacevic et al., 2013), and of an estimated 1591 listeriosis cases, 255 deaths occur each year in the United States (Scallan et al., 2011). Incidence of human listeriosis cases reportedly varies from 0.2 to 0.8 sporadic cases per 100,000 people per year in Europe and the United States (Vázquez-Boland et al., 2001). In contrast, a statistical probability was reported of 83 cases of listeriosis per year, on average, and an incidence of 0.065 cases per 100,000 people each year in Japan (Okutani et al., 2004a). To the authors' knowledge, Japan has only experienced one outbreak caused by *L. monocytogenes*, which was due to natural cheese in 2001 (Makino et al., 2005). Although Okutani et al. (2004b) initially hypothesized that reduced *L. monocytogenes* contamination of food in Japan might be related to the lower incidence of listeriosis, they found that the proportion of *L. monocytogenes* of food in Japan is similar to those in other countries, suggesting that other factors are at play. Other factors are likely to include the contamination level and type of food product, strain virulence, and host susceptibility in Japan (Lianou and Sofos, 2007; Okutani et al., 2004b), while this study focused on low-virulence strains in particular. For example, low-virulence strains are reportedly distributed among field strains of *L. monocytogenes* and exhibit mutations in genes associated with virulence, such as *prfA* and *inlA* (López et al., 2013; Roche et al., 2012). In Japan, although there is a previous study that low-virulence strains associated with *prfA* and *inlA* are not widely distributed among RTE seafood products (Handa-Miya et al., 2007), a distribution of low-virulence strains in foods remains unclear.

L. monocytogenes passing through the human stomach initially invades intestinal epithelial cells via expression of the 800-amino-acid surface protein internalin A (InlA), which is encoded by the key virulence gene *inlA*. InlA interacts with the E-cadherin receptor on the epithelial cell surface and facilitates the uptake of *L. monocytogenes* by host cells (Bonazzi et al., 2009; Van Stelten and Nightingale, 2008). Previous studies have demonstrated that natural mutations result in a premature stop codon (PMSC) in *inlA* and the expression of a truncated InlA (Nightingale et al., 2005), which produces virulence-attenuated phenotypes in animal models and significantly lowers invasion efficiency in human intestinal epithelial cells (Ward et al., 2010). A study in France

* Corresponding author.
E-mail address: kanki@iph.pref.osaka.jp (M. Kanki).

http://dx.doi.org/10.1016/j.ijfoodmicro.2015.06.023
0168-1605/

demonstrated that isolates carrying a PMSC mutation in *inlA* represented 35% of food isolates but only 4% of human clinical isolates (Jacquet et al., 2004). Another study in the United States also demonstrated that 45.0% of RTE food isolates and 5.1% of human clinical isolates are carrying a PMSC mutation (Van Stelten et al., 2010). As above, isolates with a PMSC mutation are dramatically underrepresented in listeriosis cases compared to food matrices, demonstrating that InlA has an important role in the bacterial pathogenesis.

Transcription of *inlA* is activated with other virulence factors such as *hlyA* and *actA* by the positive regulatory factor A (PrfA) encoded by *prfA* (Nightingale, 2010). In field strains, a truncated PrfA due to a seven-nucleotide insertion at codon 171 (PrfA Δ174–237) and a PrfA with a non-synonymous substitution at codon 220 (PrfA K220T) have been associated with attenuated virulence phenotypes (Roche et al., 2005; Velge et al., 2007). However, while some artificial PrfA-mutants with the substitution of a single amino acid residue such as PrfA G145S have been reported to enhance PrfA-dependent gene expression (Miner et al., 2008; Mueller and Freitag, 2005; Vega et al., 2004; Xayarath et al., 2011), high-level constitutive activation of PrfA in field strains has not been reported.

The Caco-2 cell invasion assay enables assessment of properties of InlA leading to the invasion of host epithelial cells (Kovacevic et al., 2013; Van Stelten et al., 2010). Nightingale et al. (2007) reported that *L. monocytogenes* strains exhibited significantly greater invasion efficiency in a Caco-2 cell invasion assay when grown at 30 °C than at 37 °C. In contrast, PrfA-mediated transcription is activated at temperatures over 30 °C and is terminated at approximately 20 °C or below (Ripio et al., 1997). As food processing and handling (temperature shifts) to which *L. monocytogenes* is exposed prior to ingestion can alter the virulence of this pathogen (Duodu et al., 2010), its internalization in intestinal cells is most likely influenced by growth at low temperature (20 °C).

In this study, to examine the possibility that the low-virulence strains are widely distributed among food isolates in Japan, *L. monocytogenes* isolates were collected from meat and fish samples, and the specific alleles in InlA and PrfA were identified. This study then investigated the invasion efficiencies of the isolates grown at 30 and 20 °C in a Caco-2 cell invasion assay to determine the temperature-dependent effects of PrfA on the regulation of InlA.

2. Materials and methods

2.1. Sampling of foods and isolation of L. monocytogenes

A total of 992 food samples was collected from retail shops and central kitchens in Osaka, Japan, between January 2011 and October 2013, as follows: beef (n = 125), pork (n = 117), chicken (n = 164), fresh seafood (n = 340), and seafood products (n = 246). Meat samples were all raw meat. Although the seafood samples were all non-heated, some samples were RTE foods such as "sashimi", sliced raw fish. A 25-g portion of each food sample was added to 225 ml of Half Fraser broth (Merck, Darmstadt, Germany) in a stomacher bag and processed for 30 s with the Pro-media SH-IIM Stomacher (Elmex Ltd., Tokyo, Japan), and homogenates were incubated at 30 °C for 24 h. Following this incubation, 0.1 ml of the cultivated broth was added to 10 ml of the Fraser broth (Merck) and incubated at 37 °C for 48 h. A loopful of culture was then streaked onto Agar *Listeria* according to Ottaviani and Agosti (ALOA; Merck Millipore, KGaA, Darmstadt, Germany), followed by incubation at 37 °C for 48 h. Up to 3 presumed colonies were streaked onto brain–heart infusion agar (BHI; Difco, BD, Sparks, MD) and incubated at 37 °C for 18 h. Presumed isolates were identified as *L. monocytogenes* by serotype identification with *Listeria* antisera (Denka Seiken Ltd., Tokyo, Japan) and PCR assays for DNA sequencing of the *inlA* and *prfA* genes. Since *Listeria* antisera cannot distinguish between serotypes 4b and 4e, isolates which belonged to serotypes 4b or 4e were designated as serotype 4b/4e. The isolates were kept in BHI (BHI; Difco, BD, Sparks, MD) broth with 10% glycerol at −80 °C.

2.2. DNA sequencing

Full-length sequences of the *inlA* and *prfA* genes from all isolates were determined by direct sequencing of PCR products using a BigDye Terminator v1.1 Cycle Sequencing Kit and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Previously published primer sets were used for PCR and sequencing of *inlA* (Ragon et al., 2008) and *prfA* (Handa-Miya et al., 2007). Nucleotide sequences were assembled and translated into protein sequences to analyze and compare InlA and PrfA between isolates using DNASIS software version 3.0 (Hitachi Solutions, Ltd., Tokyo, Japan).

2.3. Caco-2 invasion assays

The ability of test isolates to invade Caco-2 cells (no. 86010202; ECACC, Salisbury, UK) was determined as previously described (Ivy et al., 2010; Olier et al., 2003). Caco-2 cells used between passages 46 and 55 were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 10% inactivated fetal calf serum (HyClone, Thermo Scientific, Waltham, MA). Early confluent monolayers were obtained on 24-well tissue culture plates (Thermo Scientific), and medium was then replaced with 0.5 ml of fresh DMEM immediately before bacterial inoculation. Bacterial isolates were grown in BHI broth under two cultivation conditions of 30 °C for 18 h or 20 °C for 40 h. Caco-2 cells were incubated at 37 °C throughout the invasion assay. Cell monolayers were infected with 1.0 to 2.2×10^7 *L. monocytogenes* cells/well for 30 min, followed by three washes with Dulbecco's phosphate-buffered saline (DPBS; Gibco, Life Technologies, Grand Island, NY). After contact for 45 min, monolayers were overlaid with DMEM containing 150 μg/ml gentamycin to kill extracellular bacteria. After contact for 90 min, Caco-2 cells were washed three times with DPBS and lysed with 0.5 ml of ice-cold distilled water and vigorously pipetted. Viable intracellular bacteria were enumerated by plating appropriate dilutions of the cell lysate on BHI agar. Invasion assays for each isolate and cultivation conditions were conducted with duplicate wells in three independent experiments. Invasion efficiency was calculated as the percentage of the inoculum recovered from the infected Caco-2 cells by the enumeration of intracellular bacteria.

2.4. Statistical analysis

Student's *t* test was used to compare differences in invasion efficiencies between different phenotypes. Wilcoxon's rank sum test was used to examine the influence of the growth temperature of *L. monocytogenes* at 30 and 20 °C on invasion efficiency. *P* values < 0.01 were considered statistically significant.

2.5. Nucleotide sequence accession numbers

Nucleotide sequence data for all 114 isolates were submitted to the DDBJ database and assigned accession nos. LC005855 to LC005968 for *inlA* and LC006131 to LC006244 for *prfA*.

3. Results and discussion

3.1. Incidence of L. monocytogenes in food

A total of 114 *L. monocytogenes* isolates were isolated from 113 food samples, including samples of beef (n = 16), pork (n = 19), chicken (n = 55), fresh seafood (n = 13), and seafood products (n = 10) (Table 1). The most common serotype was 1/2a (47 strains), followed by 4b/4e (24 strains), 1/2b (22 strains), and 1/2c (16 strains). Chicken exhibited a high degree of contamination with *L. monocytogenes* (33.5%), with nearly half being of serotype 1/2a, whereas seafood exhibited contamination as low as 4%. The isolates exhibited a higher prevalence in raw meat samples, with more isolates found in chicken

Table 1
Incidence and serotypes of *L. monocytogenes* in food samples.

	Number of positive samples/number of samples tested (% positive samples)	Serotypes (number of strains)
Beef	16/125 (12.8%)	1/2a (5), 1/2b (1), 1/2c (4), 4b/4e (5), 4ab (1)
Pork	19/117 (16.2%)	1/2a (4), 1/2b (5), 1/2c (7), 4b/4e (3)
Chicken	55/164 (33.5%)	1/2a (27), 1/2b (13), 1/2c (4), 4b/4e (9), 3a (1), 3b (1), 3c (1)
Fresh seafood	13/340 (3.8%)	1/2a (4), 1/2b (2), 4b/4e (7)
Seafood products	10/246 (4.1%)	1/2a (7), 1/2b (1), 1/2c (1), 3b (1)

samples than those of seafood samples, consistent with a previous review (Lianou and Sofos, 2007).

3.2. Detection of PMSC and 3-codon deletion mutations in *inlA* genes of *L. monocytogenes* isolates

In this study, 29 isolates with 7 different PMSC types and 6 isolates with 9-nucleotide deletion mutation due to the full-length DNA sequences of the *inlA* were detected from 114 isolates (Table 2). Although the rest of the isolates had InlA protein that exhibited up to a 23 amino acid (aa) difference, their InlA was a full-length protein (800 aa). Genotyping of *inlA* PMSC mutations was performed as described by Van Stelten et al. (2010). Although they identified 18 different *inlA* PMSC mutations among 1009 isolates (PMSC types 1 to 18), type 1, 3, 4, 6, 11, 12, and 13 PMSC mutations were identified in this study. Type 1, 3, 6, 11, 12, and 13 PMSC mutations were each constituted by one distinct serotype, 1/2a, 1/2b, or 1/2c. In contrast, a type 4 PMSC mutation was constituted by the isolates of serotypes 1/2a, 1/2c, and 3c. Serotype 4b/4e isolates possessed no PMSC mutations in the *inlA* genes, whereas all 1/2c isolates possessed PMSC mutations, which were divided into 4 different types. Although a type 13 PMSC mutation had only been observed in an isolate of serotype 1/2a from a RTE seafood sample in Japan (Handa-Miya et al., 2007), six isolates of serotype 1/2c with the type 13 PMSC mutation were isolated from beef and pork samples in this study. This type of mutation has not been previously isolated from other countries, and might reach widespread distribution only in Japan (Handa-Miya et al., 2007; Van Stelten et al., 2010). In the current study, 25.4% of food isolates had *inlA* PMSC mutations. According to previous reports, *inlA* PMSC mutations account for 48.5% of RTE isolates in the United States (Ward et al., 2010), 35% of isolates from various types of food in France (Jacquet et al., 2004), and 35% of isolates from the Canadian food supply (Kovacevic et al., 2013). Given a previous finding that only 1 of 59 isolates (1.7%) from RTE seafood samples had *inlA* PMSCs in Japan (Handa-Miya et al., 2007), it appears likely that food isolates with *inlA* PMSCs may be less widely distributed in Japan than those in the United States, France or Canada.

It was observed that 1 isolate of serotype 1/2a and 5 isolates of serotype 4b/4e had an in-frame deletion of InlA aa positions 738 to 740, which was previously referred to as the 3-codon deletion by Kovacevic et al. (2013). Of the 6 strains with the 3-codon deletion, InlA protein of 4 isolates of serotype 4b/4e exhibited 100% aa sequence identity, and that of another isolate of serotype 4b/4e and an isolate of serotype 1/2a showed a 6 and a 24 aa difference from that of the former 4 isolates of serotype 4b/4e. This mutation has been reported in isolates of serotype 4b from RTE seafood samples in Japan (Handa-Miya et al., 2007); in an isolate of serogroup D, including serotypes 4b, 4d, and 4e, from a meat facility in Portugal (Ferreira et al., 2011); in isolates of serotype 4b from food products in France (Roche et al., 2012); and in isolates of serotype 4b and 1/2a from foods and food-processing environments in Canada (Kovacevic et al., 2013). Furthermore, this mutation has been found in a clinical isolate of serogroup 4 including 4a, 4ab, 4b, 4c 4d, and 4e, from materno-fetal fluid in Denmark (Holch et al., 2010). It is therefore considered that serotype 4b/4e isolates with the 3-codon deletion of InlA are relatively common and easy to identify. In contrast, a serotype 1/2a isolate with the 3-codon deletion has previously been observed only in Canada (Kovacevic et al., 2013).

3.3. Characterization of PrfA alleles of *L. monocytogenes* isolates

On analysis of PrfA protein sequences of the 114 *L. monocytogenes* isolates, PrfA sequences of 106 isolates corresponded perfectly and this sequence was referred to as "wild-type PrfA". Only 8 strains with PrfA alleles different from those of wild-type PrfA were observed (Table S1). Seven isolates of serotype 1/2a from chicken samples contained a PrfA protein with a 5-nucleotide deletion at nucleotide positions 712 to 716 including a stop codon, which was referred to as the stop-codon deletion. An exact amino-acid length (>285 aa) of PrfA with the stop-codon deletion failed to be detected, as PCR products contained no stop codons. To the authors' knowledge, this is the first report of *L. monocytogenes* isolates with the stop-codon deletion of PrfA. On the other hand, there was no isolate with a truncated PrfA due to a seven-nucleotide insertion at codon 171 (PrfA Δ174–237) found among field strains in France and Spain (López et al., 2013; Roche et al., 2005). With regard to a single aa substitution in PrfA, it was found that only 1 isolate that had a phenylalanine to isoleucine substitution at aa position 8 (PrfA F8I; Table 1). Artificial PrfA-mutants such as PrfA Y63C, G145S, P219S and Y83S have been known as single aa substitutions in PrfA that enhance or suppress bacterial virulence, while a single-aa substitution affecting bacterial virulence in PrfA from field isolates has not been reported other than PrfA K220T (Miner et al., 2008; Mueller and

Table 2
Distribution of PMSCs and the 3-codon deletion in *inlA* among the 114 isolates of *L. monocytogenes* from food samples.

Serotype	*inlA* PMSC								Number of isolates with the 3-codon deletion (aa 738–740)
	Number of isolates with PMSCs/number of isolates from food samples (% of isolates with PMSCs)	Type[a] 1 (aa 606[b])	Type 3 (aa 700)	Type 4 (aa 9)	Type 6 (aa 492)	Type 11 (aa 685)	Type 12 (aa 577)	Type 13 (aa 527)	
1/2a	9/47 (19.1%)	0	5	1	3	0	0	0	1
1/2b	3/22 (14.3%)	3	0	0	0	0	0	0	0
1/2c	16/16 (100.0%)	0	0	3	0	5	2	6	0
4b/4e	0/24 (0.0%)	0	0	0	0	0	0	0	5
Others	1[c]/5 (20.0%)	0	0	1	0	0	0	0	0
Total	29/114 (25.4%)	3	5	5	3	5	2	6	6

[a] *inlA* PMSC mutation type described by Van Stelten et al. (2010).
[b] Amino acid position corresponding to *inlA* PMSC.
[c] Serotype 3c.

Freitag, 2005; Vega et al., 2004; Velge et al., 2007; Xayarath et al., 2011). In the present study, this study did not identify any of the already-known PrfA mutations which affect the activity of the virulence proteins.

3.4. Invasion of Caco-2 cells by *L. monocytogenes* grown at 30 and 20 °C

The invasion efficiencies (%; the mean ± standard deviation) of 114 isolates after growth at 30 and 20 °C are shown in Table 1. When isolates were grown in BHI broth at 30 °C, the average invasion efficiencies of 4 groups were as follows: (i) 72 isolates with full-length InlA and PrfA (an invasion efficiency of 0.94% ± 0.32%); (ii) 29 isolates with *inlA* PMSCs (0.0064% ± 0.0021%); (iii) 6 isolates with a 3-codon deletion of InlA (0.72% ± 0.37%); and (iv) 7 isolates with a stop-codon deletion of PrfA (1.11% ± 0.14%) (Fig. 1). Isolates with *inlA* PMSCs exhibited 147 times lower average invasion efficiency than isolates with full-length InlA ($P < 0.01$; *t* test), while isolates with a 3-codon deletion of InlA and with a stop-codon deletion of PrfA exhibited invasion ability comparable to isolates with full-length InlA and PrfA ($P > 0.05$) after growth at 30 °C. Ferreira et al. (2011) and Roche et al. (2012) characterized isolates with the 3-codon deletion as low-virulence strains; however, isolates with this mutation exhibited invasive activity equivalent to or surpassing isolates with full-length InlA in previous reports (Handa-Miya et al., 2007; Kovacevic et al., 2013), and this mutation has also been found in a clinical isolate from materno-fetal fluid in Denmark (Holch et al., 2010). It seems likely that both high-virulence isolates and low-virulence isolates are present among the isolates with the 3-codon deletion of InlA.

When the isolates were grown at 20 °C, the average invasion efficiencies of 3 groups were as follows: (i) 72 isolates with full-length InlA and PrfA (0.23% ± 0.09%); (ii) 6 isolates with a 3-codon deletion of InlA (0.20% ± 0.07%); and (iii) 7 strains with the stop-codon deletion of PrfA (0.72% ± 0.06%) (Fig. 1). As isolates with *inlA* PMSC mutations exhibited attenuated invasion when grown at 30 °C, invasion assays using these isolates with growth at 20 °C were not tested. The majority of *L. monocytogenes* isolates exhibited significantly decreased invasive ability after growth at 20 °C compared with that after growth at 30 °C ($P < 0.01$; Wilcoxon rank sum test; Table S1). However, the invasion efficiency of isolates grown at 20 °C maintained over 20% of that at 30 °C. Unlike most human pathogens, *L. monocytogenes* can grow in refrigerators, and virtually all human listeriosis cases are caused by RTE foods that permit *L. monocytogenes* growth (e.g., deli meats, smoked fish, cheese, and salads) (Kathariou, 2002; López et al., 2013). These RTE foods are generally consumed soon after purchase or refrigerated storage without reheating. Given the previous suggestion that invasion efficiency of a highly virulent strain into Caco-2 cells does not markedly differ by incubation temperature between 4 and 20 °C (Duodu et al., 2010), growth of *L. monocytogenes* at 20 °C might reflect a growth condition in RTE foods. Isolates with the stop-codon deletion of PrfA exhibited significantly greater average invasion efficiencies than isolates with full-length InlA and PrfA after growth at 20 °C ($P < 0.01$). Given that isolates with the stop-codon deletion of PrfA showed invasion ability comparable to isolates with full-length InlA and PrfA after growth at 30 °C, it appears that the invasive expression of the isolates with the stop-codon deletion of PrfA may remain high after exposure to low temperature (20 °C), as observed in the strains possessing PrfA I45S, L140F, and G145S mutations (Ripio et al., 1997; Vega et al., 2004). It is noteworthy that some food isolates preserved a high invasive ability after growth at low temperature. Although the only 1 isolate with a single amino acid substitution (PrfA F8I) was detected, this isolate exhibited attenuated invasion on Caco-2 cells because it also had an *inlA* PMSC. Finally, two phenotypes affecting invasion efficiency, namely *inlA* PMSCs and the stop-codon deletion of PrfA, were identified in this study.

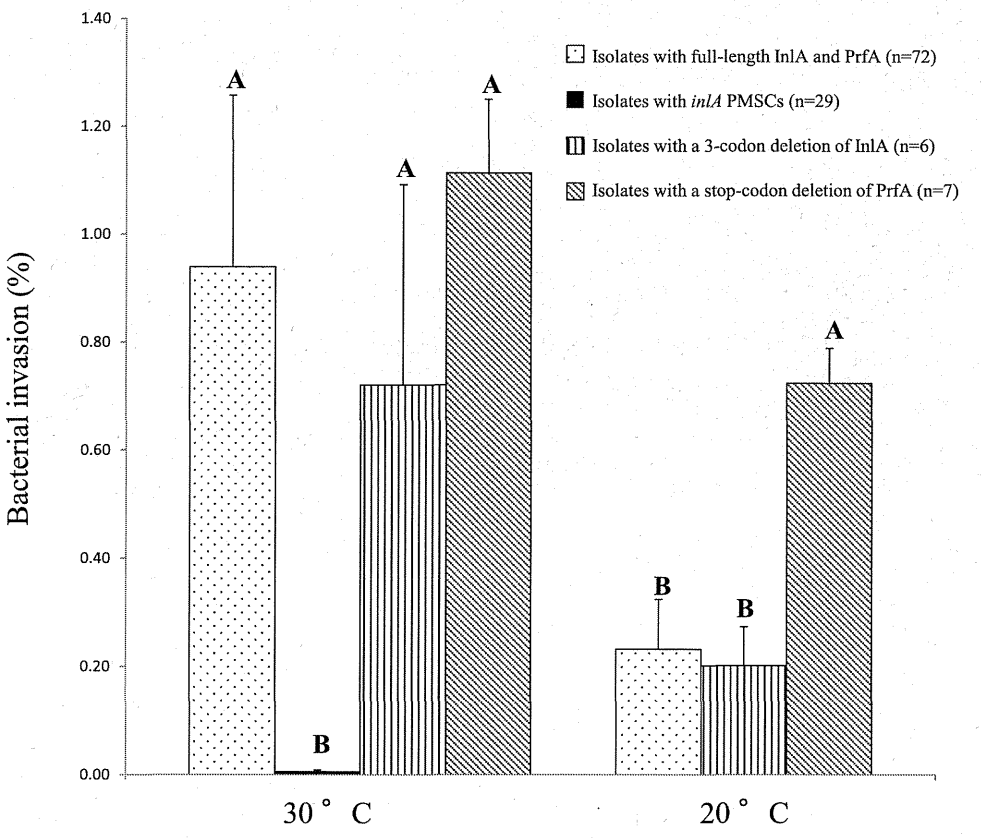

Fig. 1. Caco-2 invasion efficiencies of *L. monocytogenes* isolates after growth at 30 and 20 °C. Data represent the mean of 4 or 3 allelic groups. Invasion assays using isolates with PMSCs with growth at 20 °C were not conducted. Error bars represent standard deviations of the mean. Different letters above the bars indicate statistically differences of invasion efficiencies at a given growth temperature ($P < 0.01$; *t* test).

3.5. Conclusions

Regarding the specific alleles in InlA and PrfA, it was observed that no low-virulence phenotypes other than *inlA* PMSC mutations among food isolates in Osaka, Japan. Further, the isolates with *inlA* PMSCs were distributed less predominantly in Japan than those in other countries. These results are consistent with a previous study that nonsense-mutated *inlA* and *prfA* are not widely distributed among RTE seafood products in Japan (Handa-Miya et al., 2007), even though isolates from meat samples in addition to fish samples were collected. Contrary to findings with low-virulence isolates, this study detected isolates possessing a novel PrfA mutation with the stop-codon deletion that showed high invasion efficiency in Caco-2 cells after growth at 20 °C. Since this mutation exercises an invasive ability at low temperature, further studies are required to confirm whether it is of clinical importance in consideration of the current supply of RTE foods.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2015.06.023.

Acknowledgments

This study was supported by a grant-in-aid from the Ministry of Health, Labour and Welfare, Japan (H25-Shokuhin-Ippan-010).

References

Bonazzi, M., Lecuit, M., Cossart, P., 2009. *Listeria monocytogenes* internalin and E-cadherin: from bench to bedside. Cold Spring Harb. Perspect. Biol. 1, a003087.

Duodu, S., Holst-Jensen, A., Skjerdal, T., Cappelier, J.M., Pilet, M.F., Loncarevic, S., 2010. Influence of storage temperature on gene expression and virulence potential of *Listeria monocytogenes* strains grown in a salmon matrix. Food Microbiol. 27, 795–801.

Ferreira, V., Barbosa, J., Stasiewicz, M., Vongkamjan, K., Moreno Switt, A., Hogg, T., Gibbs, P., Teixeira, P., Wiedmann, M., 2011. Diverse geno- and phenotypes of persistent *Listeria monocytogenes* isolates from fermented meat sausage production facilities in Portugal. Appl. Environ. Microbiol. 77, 2071–2715.

Handa-Miya, S., Kimura, B., Takahashi, H., Sato, M., Ishikawa, T., Igarashi, K., Fujii, T., 2007. Nonsense-mutated *inlA* and *prfA* not widely distributed in *Listeria monocytogenes* isolates from ready-to-eat seafood products in Japan. Int. J. Food Microbiol. 117, 312–318.

Holch, A., Gottlieb, C.T., Larsen, M.H., Ingmer, H., Gram, L., 2010. Poor invasion of trophoblastic cells but normal plaque formation in fibroblastic cells despite *actA* deletion in a group of *Listeria monocytogenes* strains persisting in some food processing environments. Appl. Environ. Microbiol. 76, 3391–3397.

Ivy, R.A., Chan, Y.C., Bowen, B.M., Boor, K.J., Wiedmann, M., 2010. Growth temperature-dependent contributions of response regulators, σ^B, PrfA, and motility factors to *Listeria monocytogenes* invasion of Caco-2 cells. Foodborne Pathog. Dis. 7, 1337–1349.

Jacquet, C., Doumith, M., Gordon, J.I., Martin, P.M.V., Cossart, P., Lecuit, M., 2004. A molecular marker for evaluating the pathogenic potential of foodborne *Listeria monocytogenes*. J. Infect. Dis. 189, 2094–2100.

Kathariou, S., 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. J. Food Prot. 65, 1811–1829.

Kovacevic, J., Arguedas-Villa, C., Wozniak, A., Tasara, T., Allen, K.J., 2013. Examination of food chain-derived *Listeria monocytogenes* strains of different serotypes reveals considerable diversity in *inlA* genotypes, mutability, and adaptation to cold temperatures. Appl. Environ. Microbiol. 79, 1915–1922.

Lianou, A., Sofos, J.N., 2007. A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. J. Food Prot. 70, 2172–2198.

López, V., Navas, J., Martínez-Suárez, J.V., 2013. Low potential virulence associated with mutations in the *inlA* and *prfA* genes in *Listeria monocytogenes* isolated from raw retail poultry meat. J. Food Prot. 76, 129–132.

Makino, S.I., Kawamoto, K., Takeshi, K., Okada, Y., Yamasaki, M., Yamamoto, S., Igimi, S., 2005. An outbreak of food-borne listeriosis due to cheese in Japan, during 2001. Int. J. Food Microbiol. 104, 189–196.

Miner, M.D., Port, G.C., Freitag, N.E., 2008. Functional impact of mutational activation on the *Listeria monocytogenes* central virulence regulator PrfA. Microbiology 154, 3579–3589.

Mueller, K.J., Freitag, N.E., 2005. Pleiotropic enhancement of bacterial pathogenesis resulting from the constitutive activation of the *Listeria monocytogenes* regulatory factor PrfA. Infect. Immun. 73, 1917–1926.

Nightingale, K., 2010. *Listeria monocytogenes*: knowledge gained through DNA sequence-based subtyping, implications, and future considerations. J. AOAC Int. 93, 1275–1286.

Nightingale, K.K., Windham, K., Martin, K.E., Yeung, M., Wiedmann, M., 2005. Select *Listeria monocytogenes* subtypes commonly found in foods carry distinct nonsense mutations in *inlA*, leading to expression of truncated and secreted internalin A, and are associated with a reduced invasion phenotype for human intestinal epithelial cells. Appl. Environ. Microbiol. 71, 8764–8772.

Nightingale, K.K., Milillo, S.R., Ivy, R.A., Ho, A.J., Oliver, H.F., Wiedmann, M., 2007. *Listeria monocytogenes* F2365 carries several authentic mutations potentially leading to truncated gene products, including InlB, and demonstrates atypical phenotypic characteristics. J. Food Prot. 70, 482–488.

Okutani, A., Okada, Y., Yamamoto, S., Igimi, S., 2004a. Nationwide survey of human *Listeria monocytogenes* infection in Japan. Epidemiol. Infect. 132, 769–772.

Okutani, A., Okada, Y., Yamamoto, S., Igimi, S., 2004b. Overview of *Listeria monocytogenes* contamination in Japan. Int. J. Food Microbiol. 93, 131–140.

Olier, M., Pierre, F., Rousseaux, S., Lemaître, J.P., Rousset, A., Piveteau, P., Guzzo, J., 2003. Expression of truncated Internalin A is involved in impaired internalization of some *Listeria monocytogenes* isolates carried asymptomatically by humans. Infect. Immun. 71, 1217–1224.

Ragon, M., Wirth, T., Hollandt, F., Lavenir, R., Lecuit, M., Le Monnier, A., Brisse, S., 2008. A new perspective on *Listeria monocytogenes* evolution. PLoS Pathog. 4, e1000146.

Ripio, M.T., Domínguez-Bernal, G., Lara, M., Suárez, M., Vázquez-Boland, J.A., 1997. A Gly145Ser substitution in the transcriptional activator PrfA causes constitutive overexpression of virulence factors in *Listeria monocytogenes*. J. Bacteriol. 179, 1533–1540.

Roche, S.M., Gracieux, P., Milohanic, E., Albert, I., Virlogeux-Payant, I., Témoin, S., Grépinet, O., Kerouanton, A., Jacquet, C., Cossart, P., Velge, P., 2005. Investigation of specific substitutions in virulence genes characterizing phenotypic groups of low-virulence field strains of *Listeria monocytogenes*. Appl. Environ. Microbiol. 71, 6039–6048.

Roche, S.M., Grépinet, O., Kerouanton, A., Ragon, M., Leclercq, A., Témoin, S., Schaeffer, B., Skoski, G., Mereghetti, L., Le Monnier, A., Velge, P., 2012. Polyphasic characterization and genetic relatedness of low-virulent *Listeria monocytogenes* isolates. BMC Microbiol. 12, 304.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States—major pathogens. Emerg. Infect. Dis. 17, 7–15.

Van Stelten, A., Nightingale, K.K., 2008. Development and implementation of a multiplex single-nucleotide polymorphism genotyping assay for detection of virulence-associated gene *inlA*. Appl. Environ. Microbiol. 74, 7365–7375.

Van Stelten, A., Simpson, J.M., Ward, T.J., Nightingale, K.K., 2010. Revelation by single-nucleotide polymorphism genotyping that mutations leading to a premature stop codon in *inlA* are common among *Listeria monocytogenes* isolates from ready-to-eat foods but not human listeriosis cases. Appl. Environ. Microbiol. 76, 2783–2790.

Vázquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., Kreft, J., 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. 14, 584–640.

Vega, Y., Rauch, M., Banfield, M.J., Ermolaeva, S., Scortti, M., Goebel, W., Vázquez-Boland, J.A., 2004. New *Listeria monocytogenes prfA** mutants, transcriptional properties of PrfA* proteins and structure–function of the virulence regulator PrfA. Mol. Microbiol. 52, 1553–1565.

Velge, P., Herler, M., Johansson, J., Roche, S.M., Témoin, S., Fedorov, A.A., Gracieux, P., Almo, S.C., Goebel, W., Cossart, P., 2007. A naturally occurring mutation K220T in the pleiotropic activator PrfA of *Listeria monocytogenes* results in a loss of virulence due to decreasing DNA-binding affinity. Microbiology 153, 995–1005.

Ward, T.J., Evans, P., Wiedmann, M., Usgaard, T., Roof, S.E., Stroika, S.G., Hise, K., 2010. Molecular and phenotypic characterization of *Listeria monocytogenes* from U.S. Department of Agriculture Food Safety and Inspection Service surveillance of ready-to-eat foods and processing facilities. J. Food Prot. 73, 861–869.

Xayarath, B., Smart, J.I., Mueller, K.J., Freitag, N.E., 2011. A novel C-terminal resulting in constitutive activation of the *Listeria monocytogenes* central virulence regulatory factor PrfA. Microbiology 157, 3138–3149.

Journal of
Food Safety

Journal of Food Safety ISSN 1745-4565

EVALUATION OF THE DYNAMICS OF MICROBIOLOGICAL QUALITY IN LIGHTLY PICKLED NAPA CABBAGES DURING MANUFACTURE

KAZUYA MASUDA[1], SHIORI YAMAMOTO[1], KUNIHIRO KUBOTA[2], HISAO KURAZONO[3], SOU-ICHI MAKINO[4], FUMIKO KASUGA[2], SHIZUNOBU IGIMI[1] and HIROSHI ASAKURA[1,5]

Divisions of [1]Biomedical Food Research and [2]Safety Information on Drugs, Foods and Chemicals, National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158-8501, Japan[3]Department of Animal and Food Hygiene, Obihiro University of Agriculture & Veterinary Medicine, Obihiro, Hokkaido, Japan[4]Department of Domestic Science, Kyoto Seibo College, Fushimi-ku, Kyoto, Japan

[5]Corresponding author.
TEL: +81-3-3700-9169;
FAX: +81-3-3700-9246;
EMAIL: hasakura@nihs.go.jp

Received for Publication September 23, 2014
Accepted for Publication March 21, 2015

doi: 10.1111/jfs.12195

ABSTRACT

Following a large outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O157 infection in 2012, the prerequisite program for the production of pickles in Japan was revised to include disinfection with sodium hypochlorite (NaClO). Here, we examined the indicator bacterial counts, incidence of STEC and *Salmonella* spp. (by polymerase chain reaction [PCR] screening), and bacterial community composition (by 16S rRNA pyrosequencing analysis) from the intermediate products and the related facilities at a collaborative factory to evaluate the dynamics of microbial quality in lightly pickled vegetables during the manufacturing process following the revised program. Plate counts showed a significant reduction in coliform counts throughout processing, whereas the reduction in total viable counts was relatively less than that of coliforms. No STEC and *Salmonella* spp. were recovered from any of the samples. 16S rRNA pyrosequencing analysis revealed the process-by-process alteration of bacterial community composition in which the yields of *Pseudomonas* spp. were drastically affected by soaking in high concentration of NaCl. In summary, we demonstrate that the revised prerequisite program is indeed functional to reduce the microbial risks.

PRACTICAL APPLICATIONS

Fresh or minimally processed produce are common vehicles of foodborne pathogens that have been responsible for several outbreaks worldwide. Through the use of indicator bacterial counts in combination with a metagenomic approach, we could trace the process-by-process dynamics of bacterial community composition. Information and relief provided to manufacturers/consumers could help improve public health and food safety.

INTRODUCTION

Fresh or minimally processed vegetables are consumed worldwide, and the consumption of these vegetables has recently risen due to their high nutritional value and ability to improve human health (Hanif *et al.* 2006). However, corresponding to their increased consumption, a number of foodborne outbreaks have been reported (Lynch *et al.* 2009). Bacterial pathogens have continued to be a leading cause of these outbreaks (Gould *et al.* 2013); *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* (STEC) are recognized as some of the most common pathogens and account for nearly half of the produce-associated outbreaks (Sivapalasingam *et al.* 2004; Wendel *et al.* 2009; Launders *et al.* 2013; Slayton *et al.* 2013). In Japan, a large outbreak of STEC O157 caused by the consumption of contaminated lightly pickled napa cabbage occurred in 2012 (NIID 2013), which led us to consider the microbial quality of such

minimally processed vegetables. Following this outbreak, the Japan Ministry of Health, Labour and Welfare (MHLW) attempted to trace the route of STEC O157 contamination, specifying that the possible contamination point occurred during manufacturing. The MHLW thereafter revised the prerequisite program for the production of pickles to include disinfection with sodium hypochlorite (NaClO) (0.02% for >5 min or 0.01% for >10 min) during manufacturing (MHLW 2013).

Here, we focused on how and whether the revised prerequisite program truly reduced the risk of microbial contamination in the lightly pickled vegetables. To address this issue, the microbial quality of the lightly pickled napa cabbages was examined by testing intermediate products of the lightly pickled napa cabbages at one collaborative factory. Indicator bacterial counts and detection of STEC and *Salmonella* spp. clearly indicated a reduction of these microbial risks throughout processing. Metagenomic approaches further revealed changes in the bacterial community in response to each processing step during manufacturing. The possible association of the salt concentration used for soaking of the fresh materials with the dynamics of *Pseudomonas* spp. was examined by experimentally soaking these materials in different concentrations of NaCl.

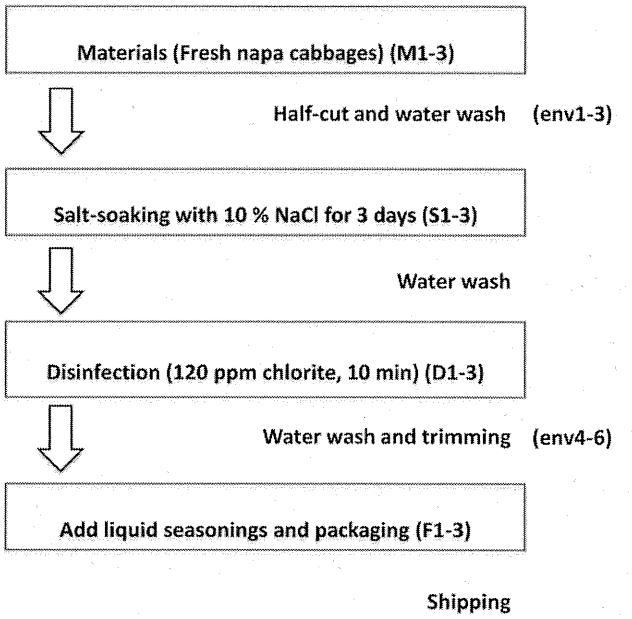

FIG. 1. MANUFACTURING PROCESS OF LIGHTLY PICKLED NAPA CABBAGES

Samples were collected from the starting materials (M), after salt-soaking (S), disinfection (D) and final products (F), respectively. Simultaneously, cutting boards used for cutting of materials (env.1–3) or for disinfected intermediate products (env.4–6) were also collected to verify the microbial quality of the facility.

MATERIALS AND METHODS

Sample Collection

In collaboration with a factory that manufactures lightly pickled vegetables in Japan, we collected materials, intermediate and final products of napa cabbages. A brief workflow for this process is presented in Fig. 1. Fresh napa cabbages ("materials") were half-cut, washed with groundwater and immediately soaked in 10-fold weight/volume of groundwater containing 10% NaCl. After soaking for 3 days at ambient temperature (15–18C), the intermediate products were obtained as "salt-soaked samples." The intermediates were then desalted with water and disinfected in a chiller containing 0.02% of NaClO for 10 min. These samples were considered to be "disinfected samples." After washing with water, the vegetables were trimmed and packaged with liquid seasoning containing 2% NaCl ("final products"). In parallel to the collection of these products, we also sampled a total of nine swab samples (10 cm × 10 cm square) from facilities or equipment used for half-cutting the materials (designated as "env1-3") or for the final cutting processes ("env. 4–6") (Table 1).

Enumeration of Indicator Bacteria

Next, 25 g of each of the collected samples was aseptically cut with sterile scissors to approximately 3 cm × 3 cm pieces. These samples were then homogenized in 225 mL of buffered peptone water (BPW) (Oxoid, Hampshire, U.K.) at 6.0 strokes/s for 1 min using a Lab blender (IUL instruments, Barcelona, Spain). Duplicates of each 100 μL aliquot of the homogenates were then inoculated onto standard agar (Becton Dickinson, Franklin Lakes, NJ), violet red bile lactose agar (Becton Dickinson) and tryptone bile X-glucuronide agar (Oxoid) to determine total viable counts (TVCs), coliform counts and β-glucuronidase positive *E. coli* counts, respectively. Simultaneously, the swab samples were soaked in 10 mL of sterile phosphate buffered saline (Becton Dickinson), and duplicates of each 100 μL aliquot were then spread onto the above agar plates in duplicate.

DNA Extraction and 16S rRNA Pyrosequencing of the Intermediate Products

Two representative samples were selected randomly from each process (material, salt-soaked, disinfected and final product samples), and 10 mL of homogenates was centrifuged at 21,500 × g for 10 min at 4C. The pellets were then subjected to DNA extraction using the PowerFood DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA). 16S

TABLE 1. SUMMARY OF INDICATOR BACTERIAL COUNTS IN NAPA CABBAGES THROUGHOUT THE MANUFACTURING PROCESSES

Sample	Process*	Description	Indicator bacterial counts (log cfu/g or 100 cm^2)† TVCs	Coliforms
Materials, intermediate or final products (/g)				
M1	M	Fresh napa cabbages (materials)	4.68 ± 4.64	3.48 ± 3.58
M2				
M3				
M4				
S1	S	Salt-soaked napa cabbages (10% NaCl for 3 days)	3.60 ± 3.33	1.70 ± 2.00
S2				
S3				
S4				
D1	D	Disinfected samples (200 ppm, 10 min)	3.95 ± 3.67	1.94 ± 2.24
D2				
D3				
D4				
F1	F	Final products (2% NaCl)	3.76 ± 2.92	ND
F2				
F3				
F4				
Swab samples (/100 cm^2)				
env1	M to S	Cutting boards	1.18 ± 1.24	0.52 ± 0.76
env2				
env3				
env4	D to F	Cutting boards	2.68 ± 2.34	ND
env5				
env6				

* M, material; S, salt-soaking; D, disinfection; F, final product; M to S, between M and S; D to F, between D and F.

† The data represent standard means (SM) ± standard deviations (SD) from at least three samples at each process.

ND, not detected (<100 cfu/g); TVCs, total viable counts.

rRNA gene sequences were analyzed via barcoded pyrosequencing to estimate composition of the bacterial communities as described previously (Leff and Fierer 2013). Briefly, the 16S rRNA sequences were PCR-amplified using the primer pairs 799f and 1115r (Supporting Information Table S1), followed by purification using E-gel Size Select 2% (Life Technologies, Carlsbad, CA) and Agencourt AMPure XP (Beckman Coulter, Brea, CA). After the measurement of DNA concentrations using the Ion Library Quantification kit (Ion Torrent, Carlsbad, CA), equal quantities of DNA from the eight samples were combined together. The pooled DNA sample was then subjected to the Ion Torrent PGM (400 bases) sequencing platform using 318v2 chip (Ion Torrent). The pyrosequencing data were deposited to the DNA Data Bank of Japan (DDBJ) under accession number DRA002513.

Detection of STEC and *Salmonella* spp.

The remaining homogenates were incubated at 37C for 20 h, and the prevalence of STEC or *Salmonella* spp. in the pickled samples was examined as described previously (Asakura *et al.* 2012) (for STEC) or according to the guideline of ISO6579:2002 (for *Salmonella* spp.), respectively.

Data Analysis

The IonXpress barcodes and low quality reads were trimmed from the read sequences (6,971,712 sequence reads, 1,864,023,980 bp) using CLC Genomic Workbench software ver. 7.0 (CLC Bio, Aarhus, Denmark) under the criteria of (i) lead length ranged between 280 and 340 bp and (ii) quality score <0.003. The trimmed sequences were then blasted against NCBI 16S rRNA database ver. 197. METAGENOME@KIN program (World Fusion, Tokyo, Japan) was used accordingly to draw bar charts.

Experimental Salt-Soaking of Napa Cabbage

Fresh napa cabbages were purchased at a market in Tokyo. After washing with tap water twice, the fresh produce were aseptically cut with sterile scissors to obtain a 25 g portion.

The cut samples (25 g each, $n = 3$) were then soaked with 225 mL of salt solution at a final concentration of 0, 2 or 10%, followed by incubation at 15C. After a 3-day incubation, the samples were homogenized with 225 mL of BPW and then subjected to indicator bacterial counts and the pyrosequencing analysis as described earlier. Pre-soaked samples were also tested in parallel. The pyrosequencing data were deposited to the DDBJ under accession number DRA002513.

RESULTS AND DISCUSSION

Kinetics of Indicator Bacteria and Incidence of STEC, *Salmonella* spp. in Lightly Pickled Napa Cabbages Through the Manufacturing Process

Chemical sanitizers, such as chlorine and chlorine dioxide, have been widely utilized to minimize contamination with pathogenic microorganisms in fresh vegetables during the manufacturing process (Gil *et al.* 2009; Keskinen and Annous 2011). After the prerequisite program for pickle production was revised to use NaClO to disinfect of fresh vegetables as of 1 December 2013 in Japan (MHLW 2013), we examined the microbiological quality of the lightly pickled vegetables at manufacture to evaluate the effectiveness of the revision. In collaboration with a manufacturer, materials (fresh vegetables), intermediate products after salt-soaking or disinfection, and the final products were collected as well as swab samples from the facilities/equipment used for cutting the produce (env1-3) or used for cutting prior to packaging (env4-6) (Fig. 1). TVCs exhibited reduced trends following the processing, although no statistically significant difference existed between the processes (Table 1). The TVCs in the materials were 4.68 ± 4.64 log cfu/g, which exhibited a one-tenth reduction after the salt-soaking (10% NaCl for 3 days). Following disinfection with NaClO (0.012% for 10 min), the TVCs were further decreased to 3.95 ± 3.67 log cfu/g and the means in the final products were 3.76 ± 2.92 log cfu/g (Table 1). By contrast, coliform counts underwent a one-sixtieth reduction from 3.48 ± 3.58 log cfu/g in the materials to 1.70 ± 2.00 log cfu/g after the salt soaking and 1.94 ± 2.24 log cfu/g after disinfection (Table 1). No coliforms were detected from the final products or the cutting boards used for cutting the disinfected intermediates (Table 1), indicating the effective decontamination of coliforms throughout the processing under hygienic environments.

The TVCs or coliform counts in the swab samples ranged from 0.69 to 2.84 log cfu/100 cm^2 and from 0 to 1.00 log cfu/100 cm^2, respectively (Table 1). No STEC and *Salmonella* spp. were detected in any of the samples tested herein (data not shown). Thus, we could demonstrate that the manufacturing process of the lightly pickled napa cabbages at the collaborative factory, which was in accordance with the revised prerequisite program (mainly composed of the addition of disinfection procedure for materials with NaClO and HACCP-based control), effectively reduced the numbers of coliforms with no detection of the two major pathogens.

Tracing the Alteration of Bacterial Community Composition in the Lightly Pickled Napa Cabbages at Manufacture

Leafy vegetables are known to harbor large bacterial populations, with the composition of these populations varying strongly across plant species (Redford *et al.* 2010) due to variations in metabolites, physical characteristics and symbiotic interactions with the host plant and other microbial inhabitants (Lindow and Brandl 2003; Hunter *et al.* 2010). Based on the differential kinetics of the TVCs and coliform counts in the lightly pickled napa cabbages throughout processing, we hypothesized whether the bacterial community composition might be altered at each processing step, thereby affecting those bacterial counts. To address this issue, two representative intermediate samples were randomly selected, which were subjected to 16S rRNA pyrosequencing analysis. Finally, 18361–104969 trimmed sequence reads were blasted, resulting in the identification of 77 families/194 genera in the eight samples. Representative bacterial genera exhibiting dominance or drastic changes throughout the processing were described.

(i) *Pseudomonas* spp., dominant genera in the material samples. The relatively high population genera in the materials were *Pseudomonas* (28.2%), *Aureimonas* (16.3%) and *Sphingomonas* (15.5%) (Fig. 2 and Supporting Information Table S2). Among these genera, the *Pseudomonas* spp. markedly decreased in yields after salt-soaking (average 1.4%) and then increased to 11.0% after disinfection (Fig. 2 and Supporting Information Table S2). The final products contained *Pseudomonas* spp. at approximately 5.7% (Fig. 2 and Supporting Information Table S2).

(ii) *Leuconostoc* and *Rhizobium* spp. exhibit increased yields after salt-soaking. After the salt-soaking, 33.5 or 26.2% of the bacterial communities were *Leuconostoc* and *Rhizobium* spp., respectively, which exhibited yields in the samples at other stages of less than 5% except for sample D2 (Fig. 2 and Supporting Information Table S2). It is likely that these genera commensally colonize leafy vegetables, such as lettuce, spinach and napa cabbage (Jun *et al.* 2011; Leff and Fierer 2013). Considering the predominant distribution of *Leuconostoc* spp. in traditional Korean seafood, such as "Ojingeo Jeotgal," which contains high concentrations of NaCl (Jung *et al.* 2013) or fermented vegetables, such as

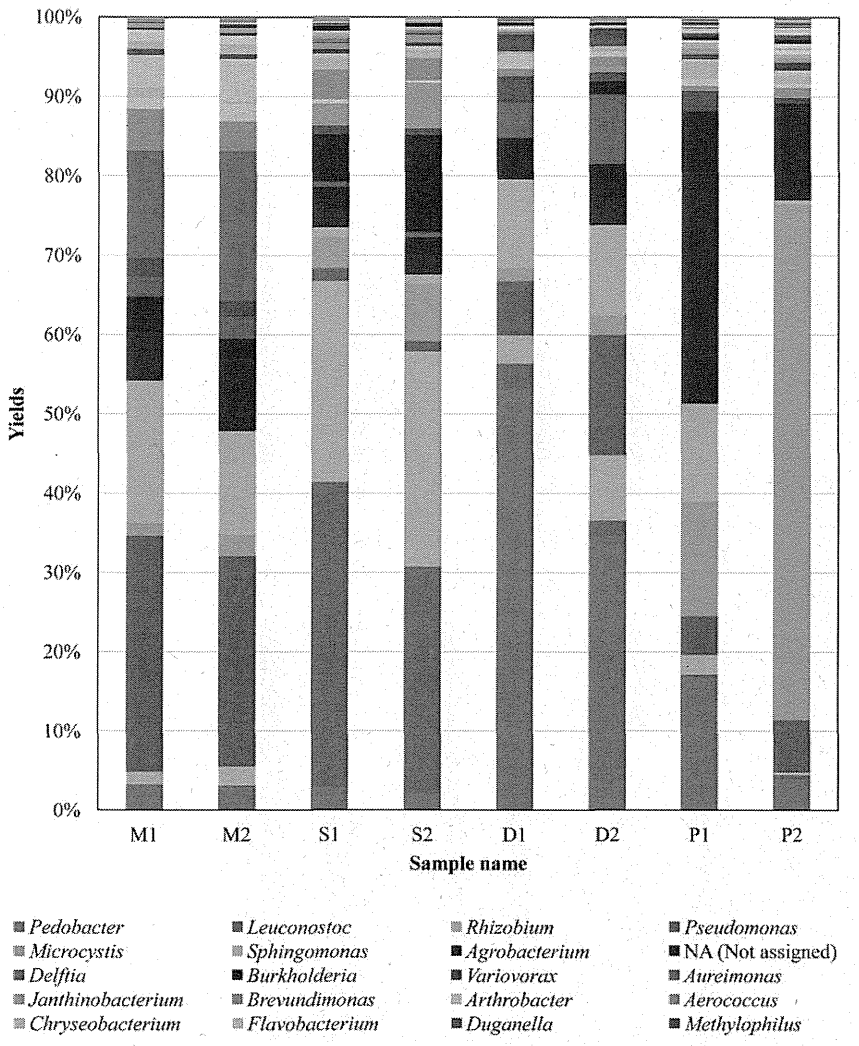

FIG. 2. PROCESS-BY-PROCESS DYNAMICS OF BACTERIAL COMMUNITY COMPOSITION ASSOCIATED WITH LIGHTLY PICKLED NAPA CABBAGES DURING THE MANUFACTURING PROCESS

Relative abundance of bacteria genus based on 16S rRNA pyrosequencing analysis is shown. Twenty dominant genera are shown with the explanatory note. Sample names represent material (M), salt-soaking (S), disinfection (D) and the final products (P), respectively. The entire data set is listed in Supporting Information Table S2.

"kimchi," which is also processed with soaking in more than 10% salt (Kim *et al.* 2000), our data suggested that the salt-soaking process expanded the dominance of salt-tolerant bacteria, hence hindering the yields of others.

(iii) *Pedobacter* spp. increases dominance after disinfection. The disinfection process increased the relative yields of *Pedobacter* (43.9%) in the intermediate samples (Fig. 2 and Supporting Information Table S2). Although these bacteria are mainly distributed in the rhizosphere, they were also detected from the phyllosphere of lettuce during NaClO washing of the vegetables surface (Kwon *et al.* 2007; Jackson *et al.* 2013). Considering these reports, our data explaining the increased yields of *Pedobacter* spp. thus suggest that the disinfection process might target the surface-localized bacteria on the vegetable to decontaminate them, while rhizosphere-originated *Pedobacter* residing internally could expand its yields during and after the process.

(iv) *Microcystis* spp. The yields of *Microcystis* were relatively increased (39.8%) in the final products (Fig. 2 and Supporting Information Table S2). These bacteria abound in eutrophic and hypertrophic freshwater bodies worldwide (Kardinaal *et al.* 2007; Vareli *et al.* 2009) and exhibit tolerance to low temperature and darkness (Wu *et al.* 2008). Considering the lower yields (<5% except for the sample S2) before this process (Fig. 2 and Supporting Information Table S2), it could be considered that the increased yields of this bacterium might originate from the surrounding environments during the packaging process. The variation of the two final product samples (F1, 14.4%; F2, 65.1%) (Supporting Information Table S2) as well as the greater yields of *Flavobacterium* spp. that inhabit water environments (Shewan and McMeekin 1983) in the F1 and F2 (0.40 or 0.79%, respectively) than the samples post-disinfection (D1, 0.019%; D2, 0.08%) (Supporting Information Table S2) might support the above hypothesis.

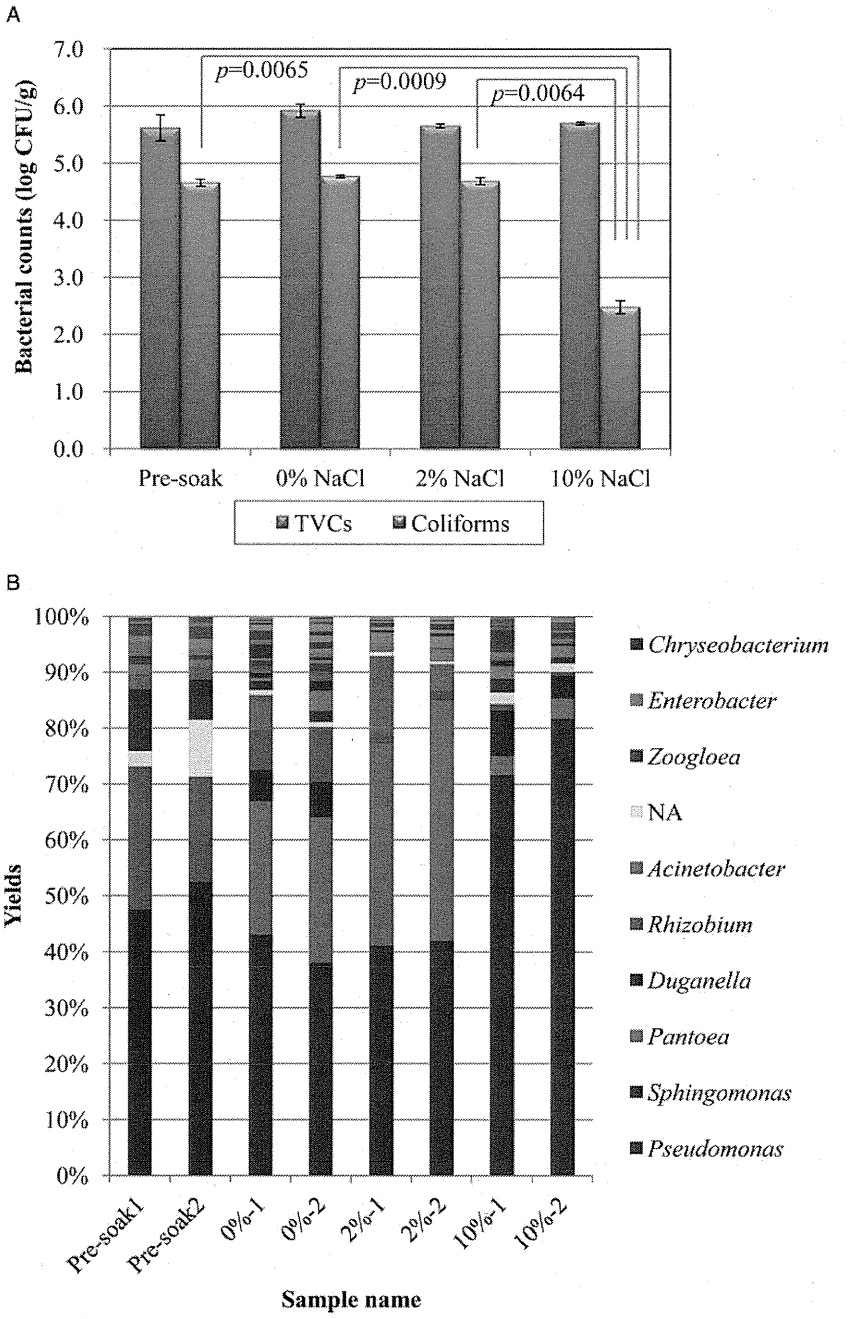

FIG. 3. BACTERIAL KINETICS IN NAPA CABBAGE THROUGHOUT SOAKING IN DIFFERENT CONCENTRATION OF SALTS
(A) Napa cabbages ($n = 3$ each) were experimentally soaked with different concentrations of NaCl (0, 2 and 10%) for 3 days at 15C. Indicator bacterial counts (total viable counts [TVCs] as red bars, coliform counts as blue bars) are shown. *P* values represent comparisons between the coliform counts in 10% salt samples and the others. (B) Comparison of bacterial community composition in napa cabbage samples before and after soaking in different concentrations of salt ($n = 2$ each). The genus-level taxonomic bar charts are shown. Ten dominant genera are shown in the explanatory note. The entire data set is listed in Supporting Information Table S3.

(v) *Escherichia* and *Enterobacter* spp. The proportion of *Enterobacter* and *Escherichia* spp. in the final products were 0.04 and 0.02%, respectively (Supporting Information Table S2). Although these means were slightly higher than those in the other processes (Fig. 2 and Supporting Information Table S2), plate counts indicated that the numbers of viable coliforms were under detection limit (<100 cfu/g) in these samples and in the swab samples from the final cutting process (env 4–6) (Table 1). As the pyrosequencing data track nucleotide composition not only from viable but also from killed bacteria, the genetic proportion of the two bacterial genera suggests their tight associations with the surface of the vegetable, which led to the increased ratio after the disinfection process, despite their efficient killing. Indeed, it is likely that some *E. coli* strains exhibit increased ability to adhere to leafy vegetables through the expression of curli and/or cellulose (Macarisin *et al.* 2012). Perhaps, additional application such as ultrasound and organic acids

during the washing processes (São José *et al.* 2014) might effectively reduce the tight adhesion of these bacteria on the plant surface.

Effects of Salt-Soaking on the Alteration of Bacterial Community Composition Associated with Napa Cabbage

With the possible link between salt-soaking and the dynamics of *Pseudomonas* spp. in napa cabbages, fresh napa cabbages were experimentally soaked in different concentrations of salt (0, 2 and 10%) for 3 days, and the kinetics of the indicator bacterial counts (TVCs and coliform counts) and bacterial community compositions were examined. Soaking with 10% salt did not affect the TVCs in the produce, while exhibiting greater reduction of the coliforms than soaking in lower concentrations of salt or without soaking ($P < 0.05$) (Fig. 3A). The pyrosequencing analysis further revealed the decreased proportion of *Pantoea* spp. in the samples soaked in 10% NaCl compared with the others ($P < 0.05$) (Fig. 3B and Supporting Information Table S3), which suggested its correlations with the reduced numbers of coliforms throughout soaking. The decreased proportion of *Duganella* spp. in the samples after soaking in the 10% salt condition was not in agreement with the data obtained in the pilot study at a factory. One explanation for the contradiction might be the differential bacterial community between the fresh produce used in each study. As an additional support, this genus exhibits species-dependent variation in salt tolerance (Abdel Wahab and Zahran 1979).

In contrast, the metagenomic data also showed increased yields of *Pseudomonas* spp. in association with elevated concentrations of NaCl (Fig. 3B and Supporting Information Table S3), further supporting our data in the pilot study (Fig. 2 and Supporting Information Table S2). Thus, these data demonstrated that the salt concentration was one of the major determinants for the altered proportion of the *Pseudomonas* spp. as well as dominant bacteria that were categorized as coliforms in napa cabbage at the soaking step during manufacture of light pickles.

CONCLUSION

We demonstrated that the revised prerequisite program improved the microbiological quality of the lightly pickled vegetables. The metagenomic analysis further revealed the process-to-process dynamics of the bacterial community composition, providing evidence for the increased efficacy of the high salt-soaking, in combination with disinfection procedures, to decontaminate enteric pathogens from the produces. Further study would be required to evaluate the impact of the revised program on the improved microbial safety in the commercially distributed products through epidemiological surveillance. Nevertheless, our data suggest that a metagenomic approach in combination with plate counts assays can better our understanding of microbial safety, and link to improvement of hygienic practices in a series of fresh or minimally processed produce at manufacture.

ACKNOWLEDGMENTS

We thank the manufacturers for their kind cooperation of the pilot study and sample collection. This work was supported financially in part by a grant from the Ministry of Health, Labour, and Welfare, Japan (H25-shokuhin-ippan-010) and a Grant-in-Aid of Scientific Research from the Japan Society for Promotion of Science (JSPS) (26870869).

REFERENCES

ABDEL WAHAB, A.M. and ZAHRAN, H.H. 1979. Salt tolerance of *Rhizobium* species in broth cultures. Z. Allg. Mikrobiol. *19*, 681–685.

ASAKURA, H., SAITO, E., MOMOSE, Y., EKAWA, T., SAWADA, M., YAMAMOTO, A., HASEGAWA, A., IWAHORI, J., TSUTSUI, T., OSAKA, K., *ET AL.* 2012. Prevalence and growth kinetics of Shiga toxin-producing *Escherichia coli* (STEC) in bovine offal products in Japan. Epidemiol. Infect. *140*, 655–664.

GIL, M.I., SELMA, M.V., LÓPEZ-GÁLVEZ, F. and ALLENDE, A. 2009. Fresh-cut product sanitation and wash water disinfection: Problems and solutions. Int. J. Food Microbiol. *134*, 37–45.

GOULD, L.H., WALSH, K.A., VIEIRA, A.R., HERMAN, K., WILLIAMS, I.T., HALL, A.J. and COLE, D. 2013. Surveillance for foodborne disease outbreaks – United States, 1998–2008. MMWR Surveill. Summ. *62*, 1–34.

HANIF, R., IQBAL, Z., IQBAL, M., HANIF, S. and RASHEED, M. 2006. Use of vegetables as nutritional food: Role in human health. J. Agric. Biol. Sci. *1*, 18–22.

HUNTER, P.J., HAND, P., PINK, D., WHIPPS, J.M. and BENDING, G.D. 2010. Both leaf properties and microbe-microbe interactions influence within-species variation in bacterial population diversity and structure in the lettuce (*Lactuca* species) phyllosphere. Appl. Environ. Microbiol. *76*, 8117–8125.

JACKSON, C.R., RANDOLPH, K.C., OSBORN, S.L. and TYLER, H.L. 2013. Culture dependent and independent analysis of bacterial communities associated with commercial salad leaf vegetables. BMC Microbiol. *13*, e274.

JUN, J.Y., LEE, S.H., KIM, J.M., PARK, M.S., BAE, J.W., MADSEN, Y. and JEON, C.O. 2011. Metagenomic analysis of kimchi, a traditional Korean fermented fool. Appl. Environ. Microbiol. *77*, 2264–2274.

JUNG, J., CHOI, S., JEON, C.O. and PARK, W. 2013. Pyrosequencing-based analysis of the bacterial community in

Korean traditional seafood, Ojingeo Jeotgal. J. Microbiol. Biotechnol. *23*, 1428–1433.

KARDINAAL, W.E.A., JANSE, I., KAMST-VAN AGTERVELD, M., MEIMA, M. and SNOEK, J. 2007. *Microcystis* genotype succession in relation to microcystin concentrations in freshwater lakes. Aquatic Microb Ecol *48*, 1–12.

KESKINEN, L.A. and ANNOUS, B.A. 2011. Efficacy of adding detergents to sanitizer solutions for inactivation of *Escherichia coli* O157:H7 on Romaine lettuce. Int. J. Food Microbiol. *147*, 157–161.

KIM, J., CHUN, J. and HAN, H.U. 2000. *Leuconostoc kimchii* sp. nov., a new species from kimchi. Int. J. Syst. Evol. Microbiol. *50*, 1915–1919.

KWON, S.W., KIM, B.Y., LEE, K.H., JANG, K.Y., SEOK, S.J., KWON, J.S., KIM, W.G. and WEON, H.Y. 2007. *Pedobacter suwonensis* sp. nov., isolated from the rhizosphere of Chinese cabbage (*Brassica campestris*). Int. J. Syst. Evol. Microbiol. *57*, 480–484.

LAUNDERS, N., BYRNE, L., ADAMS, N., GLEN, K., JENKINS, C., TUBIN-DELIC, D., LOCKING, M., WILLIAMS, C. and MORGAN, D. 2013. Outbreak of Shiga toxin-producing *E. coli* O-157 associated with consumption of watercress, United Kingdom, August to September 2013. Euro Surveill. *18*, 1–5.

LEFF, J.W. and FIERER, M. 2013. Bacterial communities associated with the surfaces of fresh fruits and vegetables. PLoS ONE *8*, e59310.

LINDOW, S.E. and BRANDL, M.T. 2003. Microbiology of the phyllosphere. Appl. Environ. Microbiol. *69*, 1875–1883.

LYNCH, M.F., TAUXE, R.V. and HEDBERG, C.W. 2009. The growing burden of foodborne outbreaks due to contaminated fresh produce: Risks and opportunities. Epidemiol. Infect. *137*, 307–315.

MACARISIN, D., PATEL, J., BAUCHAN, G., GIRON, J.A. and SHARMA, V.K. 2012. Role of curli and cellulose expression in adherence of *Escherichia coli* O157:H7 to spinach leaves. Foodborne Pathog. Dis. *9*, 160–167.

MHLW. 2013. Food Poisoning Statistics. http://www.mhlw.go.jp/stf/seisakunitsuite/bunya/kenkou_iryou/shokuhin/syokuchu/04.html#j4-2 (accessed April 1, 2013).

NIID. 2013. Enterohemorrhagic Escherichia coli infection cases as of April, 2013. IASR. *34*, 126 http://www.nih.go.jp/niid/ja/iasr-sp/2251-related-articles/related-articles-399/3520-dj3992.html (accessed April 1, 2013).

REDFORD, A.J., BOWERS, R.M., KNIGHT, R., LINHART, Y. and FIERER, N. 2010. The ecology of the phyllosphere: Geographic and phylogenetic variability in the distribution of bacteria. Environ. Microbiol. *12*, 2885–2893.

SÃO JOSÉ, J.F., MEDEIROS, H.S., BEMARDES, P.C. and ANDRADE, N.J. 2014. Removal of *Salmonella enterica* Enteritidis and *Escherichia coli* from green peppers and melons by ultrasound and organic acids. Int. J. Food Microbiol. *190C*, 9–13.

SHEWAN, J.M. and MCMEEKIN, T.A. 1983. Taxonomy (and ecology) of *Flavobacterium* and related genera. Annu. Rev. Microbiol. *37*, 233–252.

SIVAPALASINGAM, S., HOEKSTRA, R.H., MCQUISTON, J.R., FIELDS, P.I. and TAUXE, R.V. 2004. *Salmonella bacteriuria*: An increasing entity in elderly women in the United States. Epidemiol. Infect. *132*, 897–902.

SLAYTON, R.B., TURABELIDZE, G., BENNETT, S.D., SCHWENSOHN, C.A., YAFFEE, A.Q., KHAN, F., BUTLER, C., TREES, E., AYERS, T.L., DAVIS, M.L., *ET AL.* 2013. Outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 associated with romaine lettuce consumption, 2011. PLoS ONE *8*, e55300.

VARELI, K., PILIDIS, G., MAVROGIORGOU, M.C., BRIASOULIS, E. and SAINIS, I. 2009. Molecular characterization of cyanobacterial diversity and yearly fluctuations of microcystin loads in a suburban Mediterranean Lake (Lake Pamvotis, Greece). J Environ Monit *11*, 1506–1512.

WENDEL, A.M., JOHNSON, D.H., SHARAPOV, U., GRANT, J., ARCHER, J.R., MONSON, T., KOSCHMANN, C. and DAVIS, J.P. 2009. Multistate outbreak of *Escherichia coli* O157:H7 infection associated with consumption of packaged spinach, August-September 2006: The Wisconsin investigation. Clin. Infect. Dis. *48*, 1079–1086.

WU, Z.X., SONG, L.R. and LI, R.H. 2008. Different tolerances and responses to low temperature and darkness between waterbloom forming cyanobacterium *Microcystis* and a green alga *Scenedesmus*. Hydrobiologia *596*, 47–55.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Oligonucleotide primers used for the 16S rRNA pyrosequencing analysis.

Table S2. Summary of bacterial community composition associated with the lightly pickled napa cabbages at manufacture.

Table S3. Summary of bacterial community composition associated with napa cabbages before and after soaking with different concentrations of NaCl.