Contamination patterns and molecular typing of *Bacillus cereus* in fresh-cut vegetable salad processing

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Abstract  Microbiological contamination of fresh vegetables is a recent food safety concern. *Bacillus cereus* has been recognized as the most frequently detected foodborne pathogen in fresh-cut salads in Korea. Prevalence and level of contamination of *B. cereus* was determined in samples from processing lines of packaged fresh-cut vegetable salad manufacturing companies (A, B, C, and D). *B. cereus* was detected in the 27.3–30.8 % of food samples (raw material, salads in washing steps, and final products) and the contamination levels were up to $9.5 \times 10^2$ CFU/g, while detection rates in environmental samples were very low. Molecular subtyping of *B. cereus* strains detected in the processing environments (company A) and retail products (companies A and E) was conducted to reveal the contamination sources during processing. High genetic similarity was found in the bacterial strains obtained from the processing lines and retail products of company A. This result suggested that *B. cereus* strains of same clone may have circulated in the products prepared in the same company. Genetic similarities were also observed among part of the *B. cereus* isolates obtained from the processing line of company A and from the retail products of company E, suggesting that some of the *B. cereus* clones may have originated from the raw materials. The identified information can be used to develop the intervention technology for fresh-cut vegetable processing.

Keywords  *B. cereus* · Fresh-cut vegetable salad · Molecular typing · Processing · Repetitive-sequence-based PCR method

Introduction

Foodborne diseases caused by pathogenic bacteria and viruses are major health concerns worldwide. Although, traditionally, food of animal origin has been primarily implicated in outbreaks, incidences of contaminated fruits and vegetables have been increasing, reflecting rising consumer’s demand for fresh and minimally processed fruits and vegetables (Hackl et al. 2013). Changes in agricultural methods and in food processing and distribution, such as washed and pre-packaged leafy greens, may also have increased the risk of outbreaks due to increased consumption of fresh produce (Berger et al. 2010). In a previous study on the microbial quality of retail fresh vegetable products, the most frequent foodborne pathogen to be found in fresh produce was *Bacillus cereus*, with a contamination rate of 37.5 % (Jo et al. 2011). *B. cereus* is a gram-positive spore forming bacterium that can cause two types of foodborne illness. Emetic syndrome is related to cereulide, a cyclic dodecadepsipeptide produced by a nonribosomal peptide synthetase (Toh et al. 2004). Diarrheal syndrome is caused by enterotoxins such as nonhemolytic, hemolysin BL, and cytK (Ankolekar et al. 2009; Ceuppens et al. 2011).

Because fresh vegetable products are usually consumed without cooking, when contaminated with foodborne pathogens, these vegetable products can be a major route of
human exposure to pathogens (EFSA 2011; Hackl et al. 2013). Therefore, the fresh produce industry should adopt various risk management practices designed to prevent pathogenic contamination in raw materials and cross-contamination during processing. Conventional surface sanitation methods can reduce the microbial load, but cannot eliminate pathogens if present (Olaimat and Holley 2012). Therefore, identifying the contamination sources of *B. cereus* in fresh-cut salad processing is important to develop the intervention technology. Information on the contamination and molecular typing of *B. cereus* will help understanding the microbial contamination routes during processing. A limited study on the contamination of *B. cereus* in the processing steps of vegetable salads has been provided.

In this study, contamination patterns of *B. cereus* were identified for the processing steps of fresh-cut salad production. To identify the contamination routes and to develop proper intervention technologies for salad processing steps, molecular typing of *B. cereus* strains obtained during the processing and retail stages of fresh-cut salad products was conducted using rep-PCR method.

**Materials and methods**

**Sample collection**

Fresh-cut vegetables and environmental samples were collected from various steps of four different fresh-cut vegetable salad processing lines (companies A, B, C, and D), located in Gyeonggi and Chungbuk in Korea. Food and environmental samples were collected from processing steps using sterile plastic bag and cotton swabs in the Ringer solution (Oxoid, Milan, Italy). Machinery swab samples were collected from the environmental surfaces of processing lines. The collected samples were transported to the laboratory within 2 h and then were stored at 4 °C until analysis. In order to compare the genetic similarities between *B. cereus* isolated from processing lines and from retail samples of fresh-cut vegetable salad products, *B. cereus* isolates obtained from companies A and E in our previous study were used (Jo et al. 2011).

**B. cereus analysis**

*B. cereus* was analyzed according to the Korea Food code (MFDS 2014). In brief, 25 g of a sample was added to 225 mL of phosphate-buffered dilution water and homogenized for 2 min using a BagMixer Blender (Interscience, Saint-Nom la Bretèche Arpents, France). The homogenate was serially diluted and inoculated onto mannitol-egg yolk-polymyxin B agar (Merck, Darmstadt, Germany). Typical pink colonies surrounded by a zone of precipitation were selected for presumptive *B. cereus* colonies after 24-h incubation at 30 °C. The presumptive colonies were confirmed biochemically using the VITEK® 2 compact (Biomerieux, Marcy l’Etoile, France) and further analyzed for identification of *B. cereus* according to the Korea Food code (MFDS 2014). All the identified strains were stored at −70 °C until genotyping using rep-PCR.

**Genotyping of B. cereus isolates**

An automated repetitive-sequence-based PCR (rep-PCR) system (DiversiLab, Biomerieux, Marcy l’Etoile, France) was used to identify the genetic similarities of the *B. cereus* strains. For the confirmed *B. cereus* strains, DNA was extracted using the UltraClean microbial DNA isolation kit (Mo Bio Laboratories, Solona Beach, CA, USA) and Rep-PCR amplification was performed using the DiversiLab Bacillus Kit (Bacterial Barcodes, Inc., Athens, GA, USA). The PCR conditions included an initial denaturation at 94 °C for 2 min, 35 cycles at 94 °C for 30 s, 60 °C for 30 s, 70 °C for 90 s, and a final extension at 70 °C for 3 min. The specific controls included in the kit were used for validation of amplifications. The obtained amplicons were separated using microfluidic chips and analyzed using the DiversiLab system. Dendrograms were created based on the rep-PCR results using the DiversiLab software (version 3.3), which adopts the Kullback–Leibler method for the calculation of percent similarities.

**Results and discussion**

**Contamination of B. cereus**

The occurrence of *B. cereus* in the food and environmental samples from processing lines of four different companies (A, B, C, and D) was determined to identify the possible contamination routes of fresh vegetable products. As can be seen in Fig. 1, fresh-cut salad processing lines have various steps including cutting and trimming of raw materials, disinfecting, washing, draining of water, and packaging steps, in general. A total of 31 vegetable samples, 19 swab samples of the processing surfaces, and water samples of the disinfecting and washing steps were collected from four processing lines. *B. cereus* was detected in 29.0 % of the vegetable samples of processing lines, with the highest contamination rate for vegetables collected from the disinfection and washing steps (i.e., 30.8 %). The contamination levels were up to $9.5 \times 10^2$ CFU/g, which was below the MFDS (2014) guideline for *B. cereus* in food consumed without further cooking. The detection rates of *B. cereus* in food samples at different steps were...
The contamination level of the final products was found to decrease by approximately 1 log CFU/g. On the microbiological hazard analysis in fresh-cut vegetable processing plants, Kim et al. (2011) reported that no pathogenic microorganisms such as S. aureus were detected in the processing plant; however, total aerobic bacteria and coliform groups of the samples were found to increase after the second washing and spin-drying steps, due to cross-contamination from the spin-dryer. Little information on the fate of B. cereus during the processing of fresh-cut vegetable salad products has been reported.

In the 16.7% of the environmental samples including swabs and water samples of the processing lines, B. cereus was contaminated with the concentration of up to $2.2 \times 10^3$ CFU/g. The lack of detection of B. cereus in the machinery surfaces indicated that there is a low probability of cross-contamination due to surface contamination during processing steps of pre-treatment, transport, and packaging of vegetable salad products.

**Genotyping of B. cereus isolates**

From the four different salad production companies (company A, B, C, and D) that were subjected to analysis for B. cereus contamination, company A was selected for further analysis to identify the route of B. cereus contamination in salad products available in the retail market. A total of 13 B. cereus isolates comprised 10 isolates of retail salmon products of company A and 3 isolates obtained from the processing line of company A were analyzed for genetic similarity using the rep-PCR. The rep-PCR can be applied for molecular subtyping of bacteria and identification of contamination sources. The sources of infections by Listeria monocytogenes had been identified using the rep-PCR method (Blatter et al., 2010; Grisold et al., 2010).

As can be seen in Fig. 2, the rep-PCR results assigned 11 out of the 13 B. cereus isolates to 3 clusters consisting of two or more isolates with a similarity >95%. The biggest cluster had five isolates (No. 4–8), and consisted of one isolate obtained from food samples of the processing line and four isolates from the retail salad products of company A. In the clusters of B. cereus isolates (No. 11–3) as well as in B. cereus isolates (No. 1–2), high genetic similarity was found among the B. cereus samples isolated from food samples of the processing lines and retail products of the same company. Among the entire set of 13 isolates, based on the rep-PCR results, only two isolates showed similarity levels of less than 95%. These results suggest that some clones (No. 1, 8, 11) observed in the food samples of the processing lines might be circulated in the salad products of company A.

Regarding molecular typing of B. cereus strains isolated from the processing line of company A ($n = 3$) and the retail product of company E ($n = 9$), a relatively small number of B. cereus isolates were found to be clustered with high genetic similarity >95% compared to the case of B. cereus isolates from the processing line and final products of company A. The retail product of company E was selected because the contents of the vegetable salad products were similar to those of retail products of company A. As can be seen in Fig. 3, only 6 out of 12 B. cereus isolates belonged to the clusters consisting of two or more isolates with a similarity >95%. In addition, six isolates remained ungrouped and appeared to represent unique genotypes; this result suggested that no dominant B. cereus clones were circulated in the salad products of company E. However, two B. cereus strains obtained from the processing lines of company A had high genetic similarity of >95% with B. cereus strains obtained from the retail salad products of company E. This result suggests that some of the B. cereus clones had contaminated salad products processed in different companies, i.e., this clone might have originated from the raw materials.

Because B. cereus can be found in variety of natural environments including soil and water (Granum and Lund, 1977), B. cereus contamination of raw materials for salad processing may occur. As shown by the no detection of B. cereus in the machinery surfaces of the processing lines investigated in this study, there was not enough evidence to indicate cross-contamination during processing. The molecular subtyping performed at the strain level for the B. cereus isolates, as well as no contamination of B. cereus in the machinery surfaces of the processing lines (Table 1), indicated that some clones of B. cereus might be the dominant contaminants in the raw materials of salad products. These results suggested that the control of B. cereus contamination in raw materials could be the most important factor in the development of intervention technology to manage the risk of B. cereus in the consumption of fresh vegetable salad products. Analysis of the contamination flow of B. cereus in a zucchini puree processing...
line suggested that efficient cleaning procedures for the equipment surfaces can prevent the growth of *B. cereus* on machinery surfaces (Guinebretiere et al. 2003).

In conclusion, our results on the contamination patterns of *B. cereus* in the processing steps of salad products showed that *B. cereus* was contaminated in 29.0 % of vegetable samples of processing lines, with the highest contamination rate for vegetables collected from the disinfection and washing steps (i.e., 30.8 %) and the contamination levels were up to \(9.5 \times 10^2\) CFU/g. However,
no environmental swap samples of machinery surfaces on the processing line of fresh-cut salad production were found to contain *B. cereus*. Molecular subtyping using rep-PCR showed a high genetic similarity among *B. cereus* strains obtained from company A, which include processing steps and retail products, indicating the possibility that *B. cereus* strains of same clone may have circulated in the products processed in the same company. The observed genetic similarity of part of the *B. cereus* strains isolated from the food samples of the processing line (company A) and from the retail salad products (company E) suggested that some of the *B. cereus* clones had contaminated salad products processed in different companies, which means that this clone may have originated from raw materials. Determination of microbial contamination in the processing steps and molecular subtyping of *B. cereus* strains can help identify the contamination routes of salad processing. These results can be used to develop the intervention technology for fresh-cut vegetable processing.

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Table 1 Detection and quantification of *Bacillus cereus* in the fresh-cut salad and environmental samples obtained from the fresh-cut salad processing steps of four different processing companies

| Samples                  | Description                   | No. of samples, positive/total (%) | Contamination level (CFU/g, min.–max.) |
|--------------------------|-------------------------------|-----------------------------------|---------------------------------------|
| Fresh-cut salad samples  | Raw materials and cutting     | 2/7 (28.6)                        | ND—8.1 × 10^2                        |
|                          | Disinfection and washing      | 4/13 (30.8)                       | ND—9.5 × 10^2                        |
|                          | Dehydration and final products| 3/11 (27.3)                       | ND—2.9 × 10^1                        |
| Sub total                |                               | 9/31 (29.0)                       | ND—9.5 × 10^2                        |
| Environmental samples    | Water (disinfection step)     | 1/6 (16.7)                        | ND—2.2 × 10^1                        |
|                          | Water (washing step)          | 0/9 (0)                           | ND                                    |
|                          | Swabs (equipment surfaces)    | 0/4 (0)                           | ND                                    |
| Subtotal                 |                               | 1/19 (5.3)                        | ND—2.2 × 10^1                        |
| Total                    |                               | 10/50 (20.0)                      | ND—9.5 × 10^2                        |

a ND not detected

b Food and environmental samples were analyzed from four different companies (A, B, C, and D).