Fate of *Escherichia coli* O157 Cells Inoculated into Lightly Pickled Chinese Cabbage during Processing, Storage and Incubation in Artificial Gastric Juice

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Received 13 April, 2015/Accepted 3 September, 2015

Fate of *Escherichia coli* O157 cells was evaluated when inoculated into each step after production of lightly pickled Chinese cabbage. The efficacy of surface sterilization by 100 mg/L of chlorine water for 10 min on raw leaves (6.0 log CFU/g) was 2.2 log CFU/g reduction. No meaningful change of the population of *E. coli* O157 (3.5 log CFU/g to 1.5 log MPN/g) contaminated into 19 kinds of products was observed. These results indicated the difficulty of estimating the viable count of the cells between contaminated on farms and further processing and storage steps. The population of *E. coli* O157 (3 log CFU/g to 1 log MPN/g) inoculated into the Chinese cabbage products was reduced less than 0.6 log CFU/g after 2 h-incubation at 37°C in artificial gastric juice. Prevention from initial contamination of *E. coli* O157 on the ingredients of Chinese cabbage products is important to reduce the risk of food poisoning because the reduction of the bacterial counts after processing and consumption are limited.

**Key words**: Lightly fermented vegetable / Food poisoning / *Escherichia coli* O157 / Viable cell counts.

**INTRODUCTION**

Lightly pickled vegetable products are prepared by putting washed (and cut) raw vegetables into 2-3% brine (with some ingredients) and kept overnight to several days at low or room temperature. This kind of food is produced commonly in broad areas of East to Southeast Asian countries such as Japan, Korea, Vietnam, Lao PDR, Cambodia, Thailand and Myanmar (Inatsu et al., 2005a). The production of lightly picked vegetables in Japan was 0.15 million t/y in 2000s to 2010s (MAFF, 2012) and these values were estimated to be equivalent to 15 g per person living in Japan a day (calculated by simple arithmetic).

The production of lightly pickled vegetables decreased to 0.10 million t/y in 2012 due to the occurrence of an outbreak of Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 (169 patients, 8 dead) that was suspected to be caused by lightly pickled (fermented) Chinese cabbage produced in Hokkaido prefecture in Japan. In that case, the route of contamination could not be identified even though the same *Escherichia coli* (*E. coli*) strain was isolated from patients and the suspected product (Kataoka et al., 2013; Higashi, 2014). Five other outbreaks of pathogenic *E. coli* in Japan had been reported relating to the consumption of lightly pickled vegetables. They are caused by (1) *E. coli* O157:H7 in lightly pickled turnip (June 2000, Saitama prefecture, 7 patients, 3 dead) (Uehara et al., 2000), (2) EHEC O157:H7 in Japanese styled Kimuchi (August, Kanto area, 29 patients) (Ozeki.Y. et al., 2003), (3) EHEC O157:H7 in lightly pickled cucumber (June 2002, Fukuoka prefecture, 100 patients) (Ozeki.N. et al., 2003), (4) Enterotoxigenic *E. coli* O6:H16 in lightly pickled (fermented) Chinese cabbage (August 2005, Chiba prefecture, 401 patients) (Kimura et al., 2006) and (5) EHEC O157:H7 in lightly pickled egg plant and shiso (August 2011, Tochigi prefecture, 15 patients) (Naito et al., 2012). Recently, an outbreak

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that may be caused by the consumption of lightly pickled cucumber was occurred in Shizuoka prefecture of Japan in July to August 2014 (Shizuoka public health center, 2014). In that case, 510 peoples got ill and 114 of them were hospitalized by the infection of \(E.\) \(coli\) O157. An outbreak of Enterotoxigenic \(E.\) \(coli\) O169 in Kimuchi (1642 patients) was occurred in Korea in 2012 (Cho et al., 2013).

The pathogenic bacteria may contaminate into the products from several sources such as ingredients, workers, or the manufacturing environment. It is important to know what portion of contaminated pathogenic bacteria will be killed (1) by the sterilization process of raw materials, (2) during storage after manufacturing or (3) in the stomach after consumption to evaluate the whole microbiological risk of this kind of food. To clarify the three points above, we examined the fate of inoculated \(E.\) \(coli\) O157 (1) in the raw material of lightly pickled Chinese cabbage after sterilization, (2) in purchased Chinese cabbage products and (3) in artificial gastric juice.

**MATERIALS AND METHODS**

All media used in this experiment were purchased from Nissui Pharmaceutical Co. Ltd. The experimental methods were the same as previously published (Inatsu et al., 2004; 2005b; 2011) except for small modifications. The spontaneous mutants of \(E.\) \(coli\) O157 (CR-3, MN-28, MY-29, DT-66 and CR-273) used for the experiments below were isolated from calf feces. The mixture of the strains was used for inoculation study to minimize the diversity of the survivability among \(E.\) \(coli\) O157 strains. The concentration of each of the cultures used for the preparation of inoculums was adjusted to similar value (8.2 to 8.4 log CFU/mL). The combination of rifampicin resistant strains and rifampicin containing non-selective media (such as tripticase soy agar: TSA) were commonly used to measure survivability of bacteria in foods because of its high selectivity and high efficacy of injured bacteria detection (Moyne et al., 2011).

**Bactericidal effectiveness of sodium hypochlorite water on Chinese cabbage leaves**

Chinese cabbage (\(Brassica\) \(rapa\) spp. \(Chinensis\)) was purchased from supermarkets in Tsukuba City, Japan. Leaves of vegetables (650 g) were cut into 3 cm X 3 cm square pieces and mixed well in a plastic bag. Each of the vegetables was dipped into a mixture of five spontaneous rifampicin resistant \(E.\) \(coli\) O157:H7 strains (CR-3, MN-28, MY-29, DT-66 and CR-273) and dried for 1 h. The inoculated vegetables (150 g) were submersed into 1.5 L of 100 mg/L of sodium hypochlorite (NaClO) solution or sterile distilled water (DW) (in a 3 L plastic beaker) for 10 min with gentle mixing by a glass rod. The remaining washing water on the surface of the leaves was removed by using a salad spinner for 1 min. The numbers of viable cells on these leaves were enumerated by pour plate method after 1 min treatment by a stomacher machine (Günze industry, MC-D type 400D). A 50 mg/L rifampicin containing tripticase soy agar (TSA) and sorbitol MacConkey agar (SMAC) were used for the measurement of recovered \(E.\) \(coli\) O157 in the sample (detective limit: 200 CFU/g). These experiments were repeated 8 times on different days by using lots of different vegetables purchased from different shops. The obtained experimental results were subjected to statistical analysis by Turkey-Kramer’s multiple comparison tests.

**Change of the viable cell counts of inoculated \(E.\) \(coli\) O157 in lightly pickled Chinese cabbage during cold storage**

Lightly pickled Chinese cabbages commercially produced by 19 different manufacturers in 12 prefectures were used for experiments. Three portions (10 g each) from each of the collected samples were mixed with 90 g of distilled water and their pH value and salt concentration was analyzed by using Horiba D-54 (Kyoto) and Atago PAL ES-1 (Tokyo), respectively. The pH and salt concentration of each of the samples was calculated from the average values of 3 measurements.

A mixture of early stationary phase cultures of five rifampicin resistant strains of \(E.\) \(coli\) O157 shown above was mixed with each of the collected samples (3.5, 2.5, 2.0 and 1.0 log CFU or MPN (Most Probable Numbers) /g and stocked at 10°C for 7 d. Viable cells of \(E.\) \(coli\) O157 recovered from contaminated samples were enumerated by pour plate method same as written above. The most probable numbers (MPN) of viable cells in 25 g of sample were estimated by using 225 mL of rifampicin (50 mg/L) containing tripticase soy broth and “EC-blue MPN plate” (Nissui, Tokyo). The existence of \(E.\) \(coli\) in the compartments of a plate was confirmed by the development of typical colony on Eosin-Methylene Blue (EMB) agar plate from a loop of culture. The MPN numbers of \(E.\) \(coli\) in the tested samples was estimated by 5 tube method (each for 10 mL, 1 mL and 0.1 mL). The viable cell numbers or MPN of contaminated \(E.\) \(coli\) O157 into each of the samples were analyzed at least twice. Comparison of the obtained results from 19 kinds of samples was subjected to statistical analysis by Student’s t-tests or Turkey-Kramer’s multiple comparison tests.

**Reduction of the viable cell counts of \(E.\) \(coli\) O157 in artificial gastric juice with lightly pickled Chinese
cabbage

Mixture of rifampicin resistant *E. coli* O157 was inoculated to 7 kinds of lightly pickled Chinese cabbage products same as written above. The used samples (No. 2, 4, 6, 7, 9, 11 and 13 on Table 2) were chosen based on the different use of food additives. Each of the inoculated samples (25 g) was mixed with 250 mL of artificial gastric juice (sodium chloride 2.0 g/L, Hydrogen chloride 7.0 g/L, pH 1.2) (Takemura et al., 2011) or 2.0 g/L sodium chloride and incubated for 3 h at 37°C after 1 min treatment by a stomacher machine. Viable cell counts or MPN of recovered *E. coli* O157 were measured after adjusting their pH to 6.5 – 7.5 by adding 1 mol/L of sodium hydroxide. Three tests were performed for each of the 7 samples and the results were subjected to statistical analysis by Student's t-tests.

### RESULTS

A mixture of Five *E. coli* O157 strains was inoculated on the surface of Chinese cabbage by dipping method and washed with 100 mg/L of NaClO solution or DW for 10 min (Table 1). The differences of the numbers of recovered *E. coli* O157 between two kinds of enumeration agars (TSA-Rif and SMAC-Rif) were 0.6 (before washing), 0.7 (DW washing) and 0.4 (NaClO washing) log CFU/g, respectively. A 1.3 or 2.2 log CFU/g reduction of TSA-Rif enumerated *E. coli* O157 on the leaves was achieved by DW washing or NaClO washes, respectively. Similar results were obtained by the enumeration with SMAC-Rif.

The values of pH and salt concentration of 19 cabbage

### TABLE 1. Bactericidal effectiveness of Chlorine water for *E. coli* O157 on the surface of Chinese cabbage leaves.

|                          | Viable cell counts (log CFU/g) |
|--------------------------|--------------------------------|
|                          | Before Washing | DW Washing | NaClO Washing |
| TSA-Rif                  | 6.0±0.2<sup>a</sup> | 4.7±0.3<sup>b</sup> | 3.8±0.6<sup>c</sup> |
| SMAC-Rif                 | 5.4±0.2<sup>a</sup> | 4.0±0.5<sup>b</sup> | 3.4±0.5<sup>c</sup> |

Each of washing treatment was performed 10 min at room temperature. The different characters in the same row mean the statistical difference of the values (P<0.05).

### TABLE 2. Viable cell counts of *E. coli* O157 contaminated into purchased lightly fermented Chinese cabbage before and after 7 d- storage.

| No. | Manufactured prefecture | pH | NaCl (%) | Viable cells of inoculated *E. coli* O157 (log CFU/g) before and after 10°C storage | Used food additives for bacterial growth control |
|-----|-------------------------|----|----------|----------------------------------------------------------------------------------|-----------------------------------------------|
| 1   | Fukushima               | 6.0| 2.2      | 2.6 Day 0; 2.5 Day 7                                                              | acidifer                                     |
| 2   |                        | 5.7| 1.9      | 2.4 Day 0; 2.5 Day 7                                                              | acidifer                                     |
| 3   |                        | 4.8| 1.9      | 2.5 Day 0; 2.6 Day 7                                                              | acidifer                                     |
| 4   |                        | 5.5| 1.9      | 2.5 Day 0; 2.5 Day 7                                                              | acidifer                                     |
| 5   |                        | 5.5| 1.6      | 2.5 Day 0; 2.3 Day 7                                                              | acidifer                                     |
| 6   |                        | 4.2| 2.4      | 2.4 Day 0; 2.3 Day 7                                                              | acidifer, chitosan                           |
| 7   |                        | 4.5| 1.8      | 2.7 Day 0; 2.2 Day 7                                                              | acidifer, ethanol                            |
| 8   |                        | 5.4| 1.9      | 2.4 Day 0; 2.1 Day 7                                                              | acidifer, chitosan, ethanol                  |
| 9   |                        | 4.7| 1.7      | 2.6 Day 0; 2.3 Day 7                                                              | acidifer, chitosan, Yucca extract            |
| 10  |                        | 4.5| 1.8      | 2.5 Day 0; 2.2 Day 7                                                              | acidifer                                     |
| 11  |                        | 4.9| 1.5      | 2.4 Day 0; 2.3 Day 7                                                              | sodium acetate, glycine, fatty acid glyceride|
| 12  |                        | 4.7| 1.9      | 2.3 Day 0; 2.4 Day 7                                                              | acidifer                                     |
| 13  |                        | 4.0| 1.8      | 2.4 Day 0; 2.3 Day 7                                                              | acidifer                                     |
| 14  |                        | 4.5| 1.4      | 2.5 Day 0; 2.3 Day 7                                                              | acidifer, ethanol                            |
| 15  |                        | 4.7| 1.7      | 2.5 Day 0; 2.2 Day 7                                                              | acidifer                                     |
| 16  |                        | 4.4| 2.5      | 2.6 Day 0; 2.3 Day 7                                                              | acidifer                                     |
| 17  |                        | 5.5| 2.3      | 2.5 Day 0; 2.5 Day 7                                                              | acidifer, chitosan, ethanol                  |
| 18  |                        | 5.7| 2.1      | 2.1 Day 0; 2.2 Day 7                                                              | acidifer                                     |
| 19  |                        | 5.3| 2.1      | 2.5 Day 0; 2.4 Day 7                                                              | acidifer                                     |
collected lightly pickled Chinese cabbages were 4.2 to 6.2 and 1.4% to 2.5%, respectively (Table 2). Food additives were used for all of them except for 4 samples. No relationship between the use of acidifier and the value of pH was found (P > 0.05).

A mixture of five E. coli O157 strains was inoculated into each of the collected samples and stored for 7 d at 10°C. The viable cell counts of each of samples before and after 7 d- storage was shown on Table 2. No relationship between the pH of used samples and log reduction of inoculated E. coli O157 was found (R² = 0.20). No relationship between the salt concentration of used samples and log reduction of inoculated E. coli O157 was found (R² = 0.10). No relationship between the use of food additives and log reduction of inoculated E. coli O157 was found (P > 0.05).

The different initial load (3.5 and 2.5 log CFU/g or 2.0 and 1.0 log MPN/g) of the mixture of E. coli O157 strains were inoculated into each of 19 collected samples and stored for 7 d at 10°C (Figure 1). Despite the levels of the initial load, less than 0.5 log CFU (or MPN)/g reduction of the viable cells in the samples was observed.

Five strains of E. coli O157 were inoculated into each of 7 collected samples and mixed with artificial gastric juice or DW (Table 3). Despite the level of the initial load (3 to 1 log CFU or MPN/mL), no significant reduction of the numbers of E. coli O157 in the mixture of the sample and DW was observed after 3 h incubation at 37°C. In the cases of 3 or 2 log CFU/mL initial load, only a little (less than 0.6 CFU/mL) reduction of the numbers of recovered E. coli O157 from the mixture of sample and artificial gastric juice was observed after 3 h- incubation at 37°C.

**DISCUSSION**

Several types of lightly pickled vegetables were related to outbreaks of pathogenic E. coli in Japan and other countries. Not only preventing the contamination of pathogenic bacteria into the final product or on the raw material of them essential to reduce the risk of food poisoning, but the implementation of effective prerequisites programs or good practices at farm or facilities will enhance the safety of food as well.

The “critical control point (CCP)” in hazard analysis and critical control point (HACCP) system is defined as “A step at which control can be applied and is essential to prevent or eliminate a food safety hazard or reduce it to an acceptable level” (Codex Alimentarius Commission, 2003). Washing (surface sterilization) of the raw material of lightly pickled Chinese cabbage is thought to be a CCP to reduce the risk of foodborne illnesses caused by lightly pickled vegetable. “A guide line for lightly pickled vegetable” published by Ministry of Health Welfare and Labor, Japan, recommends using 100 mg/L or 200 mg/L of effective chlorine containing water for 10 or 5 min washing, respectively (MHWL, 2013). As shown on Table 2, washing by 100 mg/L chlorine water for 10 min (the recommended condition by MHWL) reduced the viable cell counts of surface attached E. coli O157 on Chinese cabbage leaves 2.2 log CFU/g (TSA-Rif enumerated). Acidic electrolyzed water or other sanitizers have been reported to exhibit similar or lower level of bactericidal activity as chlorine water (Dai et al., 2012; Forghani et al., 2013). In contrast, washing leaves by water could reduce only 1.3 log CFU/g of surface attached E. coli O157 in the same condition. In general, the use of chlorine based sanitizers will exhibit 10 times higher effectiveness for surface washing (and disinfection) of vegetable leaves than water. In addition, the use of sanitizer instead of

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**TABLE 3.** Reduction of the viable cell counts of inoculated E. coli O157 into lightly fermented (pickled) Chinese cabbage dispensed and incubated in artificial gastric juice at 37°C.

| Initial load  | Time | DW (log CFU or MPN/mL) | Artificial gastric juice (log CFU or MPN/mL) |
|--------------|------|------------------------|---------------------------------------------|
|              |      |                        |                                             |
| 3 log CFU/mL | 0 hr | 3.1 ± 0.1              | 3.0 ± 0.3                                   |
|              | 3 h  | 3.3 ± 0.1              | 2.6 ± 0.3                                   |
| 2 log CFU/mL | 0 hr | 2.4 ± 0.5              | 2.3 ± 0.3                                   |
|              | 3 h  | 2.3 ± 0.1              | 1.7 ± 0.3                                   |
| 1 log MPN/mL | 0 hr | 0.9 ± 0.5              | 1.1 ± 0.6                                   |
|              | 3 h  | 1.4 ± 0.4              | 0.8 ± 0.6                                   |

*mean found the statistically significant result of viable cell counts between DW and artificial gastric juice.
water will be helpful to prevent cross contamination during the washing process.

Based on the definition of "deadline of consumption" in food safety law in Japan, commercially purchased food items that show the date are expected to be eaten up by the time limit to ensure the safeness after storage under defined (suitable) condition. We examined the fate of contaminated *E. coli* O157 after 7 d-storage because most of lightly pickled (fermented) vegetable producers set the date less than 7 d after production (Table 2, Figure 1). Over all to see, the viable cell counts of contaminated *E. coli* O157 did not changed in the range of tested initial load (Figure 1). This result showed that *E. coli* O157 that contaminated into lightly pickled (fermented) Chinese cabbage at (or after) the step of its production will be consumed even though the level of contamination was low (10 MPN/g). Similar results were obtained for Japanese style Kimuchi or lightly fermented cucumber (Inatsu et al., 2004: Breidt and Caldwell, 2011). This conclusion is able to adapt to all of tested commercially purchased items including those using food additive(s) to control undesirable overgrowth of microorganisms (Table 2). Chitosan, mustard extract and other food additives have been used commercially and some of them are reported to be effective for control the over growth of yeast and lactic acid bacteria in fermented vegetables to keep good quality longer (Miyao, 1997; Savard et al., 2002). No meaningful effectiveness of food additives to reduce the population of contaminated *E. coli* O157 during storage at 10°C was observed in this study however. Enterohemorrhagic *E. coli* is reported to grow under condition at pH4.4, water activity 0.95 and 7-8°C (FSC, 2010). So it was difficult to reduce the population of contaminated *E. coli* O157 in tested items because the min. pH and max salt concentration of them was 4.2 and 2.5% (Table 2).

Even *E. coli* O157 will be contaminated into lightly pickled (fermented) Chinese cabbage: there is no problem if the bacteria will be killed effectively in the stomach after consumption. However less than 1 log CFU/mL of *E. coli* O157 in artificial gastric fluid with the food was observed at 3 log CFU/mL to 1 log MPN/mL of initial load (Table 3). This result means more than one tenth of contaminated *E. coli* O157 in consumed food will survive in stomach.

In brief, the population of *E. coli* O157 attached on the surface of Chinese cabbage leaves will decrease 1/100 order max by surface disinfection. No meaningful change of population of that *E. coli* O157 will occur during processing and storage of lightly pickled (fermented) Chinese cabbage. The consumed *E. coli* O157 will reduce its population 1/10 max. Simply to say, if producers of lightly pickled (fermented) Chinese cabbage use raw material that contains 1000 CFU/g of *E. coli* O157 and consumers eat 10 g of the product, 10 CFU of *E. coli* O157 is calculated to reach the intestine of the consumer. This number of *E. coli* O157 is thought to be sufficient to cause foodborne illness because the consumption of 11-50 CFU (in sea food salad) or 2-9 CFU (in beef liver) of *E. coli* O157 were reported to cause outbreaks (FSC, 2010).

ACKNOWLEDGEMENT

This work was funded by the "research project for food safety" (Ministry of Agriculture, Forestry and Fishery, JAPAN).

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