Survival of Inoculated *Escherichia coli* O157:H7 in Japanese Sweet Dumplings during Storage

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An outbreak of *Escherichia coli* O157:H7 occurred due to the consumption of sweet dumplings in Japan. We examined the survival of *E. coli* O157:H7 inoculated into several types of sweet dumplings to evaluate the progress of the residual contaminating pathogens after the production or packing processes. For all 4 types of tested typical sweet dumplings, no significant reduction in the viable cell counts of inoculated *E. coli* O157:H7 (3 log CFU/g) was observed during storage at -20°C and 5°C for 5 weeks. Approximately 1 log CFU/g of reduction was observed after storage for 5 weeks at 10°C and 15°C, which corresponded to the growth of the naturally contaminating fungi. Similar results were obtained when we used several types of commercially distributed sweet dumplings. It is of vital importance to prevent the cross contamination of sweet dumplings after the heating process in order to reduce the risk of foodborne illness.

Key words: Enterohaemorrhagic *E. coli* / Confection / Food poisoning.

Sticky steamed rice cakes such as *Khanom Chan* in Thailand, *Banh da lon* in Vietnam, *Num Kom* in Cambodia, *Nian Gao* in China and *Tteok* in Korea are popular, widely consumed confections throughout East and Southeast Asia. Japanese raw confections such as *Dango* (steamed rice dumpling) or *Mochi* (steamed rice cake) are included in this food category. These confections are basically a mixture of sticky or ordinary rice flour and sugar that is steamed to make a sticky starch gel. The possibility of developing food poisoning from these foods has been recognized because many such confections are made in small household enterprises and are often sold on the streets in different countries (Henson, 2003; Notermans and Batt, 1998).

Contamination of sticky steamed rice cakes by toxin(s)–producing *Staphylococcus aureus* (Houjyo et al., 2001; Ishii et al., 1988; Kaneko et al., 1982; Saito et al., 2001; Tsuji et al., 2003; Uematsu and Kaneko, 1989) and *Bacillus cereus* (Huruse et al., 2004; Matsuoka et al., 2003) has caused many outbreaks of toxic foodborne diseases. The accumulated, heat-resistant enterotoxin(s) that are produced by *S. aureus* or the spores of *B. cereus* can contaminate the raw ingredients of sticky steamed rice cakes and do not lose their toxicity or viability, even after the heating process. Therefore, they are a risk factor in food poisoning (Okahisa et al., 2008).

Compared to cases caused by these foodborne pathogens, it is difficult to find reports on the outbreaks of illnesses involving other infectious foodborne pathogens (such as *Salmonella enteritica* or enterohaemorrhagic *E. coli*) caused by sticky steamed rice cakes in recent years. However, we experienced *E. coli* O157:H7 outbreaks that were caused by the consumption of *Dango* or *Kashiwa Mochi* in May 2011 (Anonymous, 2011; Kabae, 2013; Yuuki et al., 2011). In brief, 287 people who ate *Kashiwa mochi* and 4 types of *Dangos* became ill, and 1 elderly man died. The contamination of the raw ingredients of these foods after the heating
process was estimated to continue for 5 days, but the exact routes of contamination could not be evaluated clearly by speaking to workers or using methods such as swab tests. In addition, an outbreak caused by dumplings with sweet bean paste (Tsukimi-Manjyuu) that were contaminated with Salmonella Enteritidis occurred in August 2001 (Tsukada et al., 2004). If contaminating pathogenic bacteria could easily die, a simple short-time storage would reduce the risk of food poisoning. In contrast, if the bacteria can survive for a long time, we need to invest much effort to insure the safety of the food. Because we could not find any evidence about this kind of confection to consider these points, we evaluated the survival of E. coli O157:H7 inoculated into different types of Dango and Kashiwa Mochi and kept at different temperatures in the period from production to consumption.

Five types of major Japanese sticky steamed rice cake, Mitarashi-Dango (dumplings covered with syrup made of sugar and soy sauce), An-Dango (dumplings covered with mashed and cloth-filtered sweet bean paste), Goma-Dango (dumplings covered with mashed sesame mixed with sugar), Maccha-Dango (dumplings covered with white bean paste mixed with Japanese green tea powder) and Kashiwa-Mochi (steamed rice cake with sweet bean paste inside and wrapped in an oak leaf), were used for the experiments. The Kashiwa Mochi and 4 types of Dango were manufactured from May to August 2011 by 4 different factories and were sold in several local markets around Tsukuba City, Japan. All of the used samples did not contain food preservatives such as benzoic acid or sorbic acid.

The pH, brix, salt concentration and water activity (aw) were measured as the chemical characteristics of each sample of Dango or Mochi by using a pH meter (D-54, Horiba, Ltd. Kyoto, Japan), refractometer (PAL-1, ATAGO Co., Ltd. Tokyo, Japan), salt meter (PAL-ES1, ATAGO Co., Ltd. Tokyo, Japan) and aw measuring apparatus (Novasina aw CENTER, Novasina Co., Ltd. Lachen, Switzerland), respectively. Each sample was diluted 10-fold with pure water for measurements, except when performing the Aw measurement. The mean and standard deviation (SD) values of 4 different lots of samples were calculated (Table 1).

A cocktail of phosphate buffered saline (PBS)-washed brain heart infusion broth cultures of rifampicin-resistant, spontaneous mutants of four E. coli O157:H7 strains (CR-3, MN-28, MY-29 and DT-66) that were isolated from calf feces was used for inoculation (Inatsu et al., 2004). All of these strains are eaeA-positive and stx-negative. The mixture of the strains was used for the inoculation study to minimize the diversity of the survivability among E. coli O157 strains. The combination of rifampicin resistant strains and rifampicin containing non-selective media (such as tripticase soy agar : TSA) has been commonly used to measure the survivability of bacteria in foods because of its high selectivity and high efficacy in detecting injured bacteria (Moyne et al., 2011).

A 0.025-mL inoculum (4 log CFU/mL) was spotted onto 10 points on the surface of each of the 16 divided samples (11 to 15 g for each block). These inoculated samples were packed in a sterile plastic box and stored at each of 4 temperatures (-20, 5, 10 and 15°C). Each block of stored (or just-inoculated) samples was taken randomly and homogenized for 1 min in a stomacher after being transferred into a sterile stomacher bag and mixed with 9 times the weight of phosphate buffered saline (PBS). Additionally, ten-fold serial dilutions with PBS were performed, and each 1 mL of the diluted samples was pour-plated with trypticase soy agar (TSA) or sorbitol-MacConkey (SMAC) agar (Nissui Co. Ltd. Tokyo) containing 0.05 mg/mL rifampicin (Wako pure chemical Co. Ltd. Tokyo) to enumerate the inoculated E. coli. The most probable number (MPN) of E. coli in the sample was also measured using mEC-broth with novobiocin (Nissui Co. Ltd. Tokyo) by incubating the inoculated tubes for 24 hours at 42°C. The existence of E. coli in the MPN tubes (5 tubes for each 3 levels) was confirmed by typical colony formation on EM agar (Nissui Co. Ltd. Tokyo) with (or without: for control study) 0.05 mg/mL rifampicin.

All experiments were repeated three times with dupli-

### Table 1. pH, brix, salt concentration and water activity (Aw) of the sample surfaces of several types of Dango and Mochi varieties

| Sample       | pH    | Brix  | Salt conc. | Aw     |
|--------------|-------|-------|------------|--------|
| Mitarashi-Dango | 4.42±0.14 | 42.8±3.3 | 1.29±0.22 | 0.92±0.01 |
| An-Dango     | 5.21±0.04 | 40.7±1.2 | 0.00±0.00 | 0.93±0.00 |
| Goma-Dango   | 5.04±0.16 | 28.2±5.5 | 0.93±0.08 | 0.93±0.00 |
| Macha-Dango  | 8.49±0.43 | 28.0±1.7 | 0.00±0.00 | 0.92±0.00 |
| Kashiwa-Mochi | 4.57±0.12 | 31.3±5.6 | 0.15±0.17 | 0.93±0.01 |

The mean and standard deviation (SD) values of 4 different lots of samples are shown.
SURVIVAL OF E. coli O157 IN SWEET DUMPLINGS

Station, Mitarashi-Dango and Goma-Dango showed values of approximately 1%, which were believed to have originated from the use of soy sauce as a cooking material. Meanwhile, the Aw did not show any difference among the Dango varieties.

All of the purchased products (total of 61 packs) that were used for the inoculation studies did not harbor E. coli O157:H7 (limit of detection: 0.2 MPN/g). In all of the inoculation experiments, the numbers of developed colonies that were enumerated in SMAC agar were similar to or lower (maximum 0.7 log CFU/g after 2 weeks of storage at 10 or 15°C; data not shown) than those on TSA, which contradicted the existence of partially injured bacteria that could not grow in medium with selective chemicals (Lekkas et al., 2006). Only the averages and standard deviations of the logarithmic values of the viable cells in the samples that were counted on TSA with rifampicin (TSA-Rif) are shown in the time course: Fig.1 A to D) or triplicate (difference among samples: Table 2) samples. The logarithmic values of the viable cell counts or MPN were subjected to statistical analysis. The possibility of natural contamination of E. coli in samples that were not inoculated was also tested using the MPN method. The evaluated data were subjected to Tukey’s honestly significant difference test for multiple comparisons. Significant differences in the logarithmic values of the viable cell counts were established as less than the 5% level of significance.

The pH, brix, salt concentration and Aw of the surfaces of the five tested types of Dango (Mitarashi-Dango, An-Dango, Goma-Dango and Macha-Dango) and Kashiwa-Mochi are shown in Table 1. Machadango showed weak alkalinity, and the others showed weak acidity. Mitarashi-Dango and An-Dango showed higher brix values than the others. As for salt concentration, Mitarashi-Dango and Goma-Dango showed values of approximately 1%, which were believed to have originated from the use of soy sauce as a cooking material. Meanwhile, the Aw did not show any difference among the Dango varieties.

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FIG. 1. A mixture of 4 E. coli O157 strains was inoculated on the surfaces of Dango (A to C) or Mochi (D) samples and stored at 4 different temperatures. The average values and standard deviations of the obtained logarithmic viable cell counts are shown (n=6).
the figures and table below because no practical or meaningful differences in their magnitude or tendency were counted by SMAC-Rif.

The survival of inoculated \textit{E. coli} O157:H7 (3 log CFU/g) on each of the 3 types of \textit{Dango} (\textit{Mitarashi-Dango}, \textit{An-Dango} and \textit{Goma-Dango}) and \textit{Kashiwa-Mochi} that were stored at 4 temperatures (-20, 5, 10 and 15°C) was evaluated (Fig.1 A to D). No significant change (P>0.05) in the viable cell counts of the inoculated \textit{E. coli} was observed in any of the tested samples under -20 or 5°C storage for 5 weeks. A slight (0.3 to 1.0 log CFU/g) to much larger (0.8 to 1.6 log CFU/g) reduction in the number of \textit{E. coli} was observed after 5 weeks of storage at 10 and 15°C, respectively. Once the quick reduction in viable counts after 2 or 3 weeks of storage occurred, the immediate growth of naturally contaminating fungi was observed (data not shown). In the cases of the four types of tested confections, increasing the temperature of storage (in -20 to 15°C) enhanced the reduction of \textit{E. coli} in the samples. No clear difference in the tendency for survival of the inoculated \textit{E. coli} among these tested samples was found.

The differences in the viabilities of \textit{E. coli} O157:H7 after storage of the four types of \textit{Dango} that were produced by four manufacturers are shown in Table 2. Similar to the results shown in Fig.1 A to 1 C, a significant (P<0.05) but slight reduction in the viable cell counts (0.4 to 1.5 log CFU/g) of the inoculated \textit{E. coli} (3 log CFU/g initial) in six of the eight tested samples that were stored for 2 weeks at 15°C was observed. The viable cell counts of the \textit{E. coli} inoculated into each of the tested samples did not change significantly (P>0.05) for 4 weeks at -20 or 5°C. The \textit{E. coli} inoculated on the \textit{Maccha-An} samples tended to reduce their viability slightly more than on the other types of samples, despite there being no clear differences in their basic features (Table 1). \textit{Maccha} with Japanese green tea contains several types of epigallocatechin or epicatechin compounds that suppress the growth of bacteria by inhibiting DNA gyrase activity via interaction with their ATP binding site or other mechanisms (Gradisar et al., 2007). However, this antimicrobial activity was reported to not be high against \textit{E. coli} (Kim et al., 2004).

As shown in Fig.1 A to D and Table 1, the \textit{E. coli} inoculated on the tested sticky steamed rice cakes survived at least 4 weeks under storage conditions, and the cakes maintained their commercial quality. In addition, increasing the storage temperature tended to enhance the loss of viability in the inoculated \textit{E. coli}. A similar phenomenon was reported with \textit{E. coli} in artificially inoculated chocolate and biscuit cream (Baylis et al., 2004). In the case of a similar Japanese confection (\textit{Nerikiri}), the decreased rate of viable cell counts of naturally contaminating coliforms tended to be lower and corresponded with the reduction of the storage temperature within a range of 10 to 30°C (Fujikawa et al., 1996).

The sweet bean paste or boiled-concentrated sweet soy sauce that is used for \textit{(Maccha) An Dango} or \textit{Mitarashi Dango}, respectively, contains approximately 45 to 60 °Brix of sugar, which results in 0.95 to 0.90 of water activity and may partially suppress the growth of \textit{E. coli}. Bacteria produce compatible solutes such as trehalose or glycine betain to cope with hyperosmotic environments (Bremer and Kraemer, 2000). A sigma factor RpoS, which is encoded by the \textit{rpoS} gene, is involved in cellular responses to a diverse number of stresses, including osmotic stress and cold shock.

TABLE 2 Logarithmic values of the viable cell counts (log CFU/g) of \textit{E. coli} O157 on the surfaces of several \textit{Dango} samples before and after storage.

| Items (Manufacturer)       | Before Storage | After 2 weeks | After 4 weeks |
|----------------------------|----------------|---------------|---------------|
|                            | Before Storage | 10°C | 15°C | -20°C | 5°C |
| \textit{Mitarashi-Dango} (FFD) | 3.0±0.0A      | 2.8±0.1\(ABC\) | 2.4±0.1\(C\) | 3.0±0.1\(A\) | 2.6±0.1\(BC\) |
| \textit{Mitarashi-Dango} (MSK) | 2.9±0.0A      | 2.8±0.0\(A\)  | 2.8±0.0\(A\)  | 2.6±0.2\(A\)  | 2.7±0.1\(A\)  |
| \textit{An-Dango} (ASK)     | 2.9±0.1\(A\)  | 2.8±0.1\(A\)  | 2.7±0.1\(A\)  | 2.6±0.1\(A\)  | 2.8±0.0\(A\)  |
| \textit{An-Dango} (FFD)     | 3.0±0.0\(A\)  | 2.8±0.1\(AB\) | 2.6±0.1\(B\)  | 3.0±0.1\(A\)  | 2.8±0.1\(AB\) |
| \textit{An-Dango} (MSK)     | 3.0±0.0\(A\)  | 2.7±0.1\(A\)  | 2.6±0.0\(B\)  | 2.9±0.1\(A\)  | 2.9±0.1\(A\)  |
| \textit{Goma-Dango} (ASK)   | 2.8±0.1\(A\)  | 2.0±0.1\(B\)  | 2.0±0.2\(B\)  | 2.8±0.2\(A\)  | 2.4±0.1\(A\)  |
| \textit{Maccha-Dango} (ASK) | 2.8±0.1\(B\)  | 2.4±0.1\(BC\) | 1.7±0.1\(D\)  | 2.7±0.1\(AC\) | 2.2±0.1\(B\)  |
| \textit{Maccha-Dango} (KPH) | 3.2±0.0\(A\)  | 2.7±0.1\(B\)  | 1.7±0.2\(C\)  | 3.0±0.1\(AB\) | 2.9±0.1\(A\)  |

Items different from those used for the experiments shown in Fig.1 were used for the assay. Three different lots of items were used for each experiment in triplicate (n=9), and the average and standard deviation values are shown. Different superscript characters show statistical differences in the same column.
(Loewen et al., 1998; Munro et al., 1995). The rpoS-dependent accumulation of trehalose occurs in the stationary-phase, which may increase the tolerance to low temperature and high osmolarity (Hegge-Aronis et al., 1991; Kandror et al., 2002). In addition, bacteria left in a long-term stationary-phase genetically adapt to the severe environment (“growth advantage in stationary-phase phenomenon”), which may also increase their tolerance to low temperature and high osmolarity (Bacun-Druzina et al., 2007; Farell and Finkel, 2003). The stationary-phase E. coli under low temperatures were thought to be able to cope with the hard conditions much more easily than slow-growing E. coli at 10 or 15°C due to these mechanisms.

The results shown above suggested that the E. coli O157:H7 that contaminated the sticky steamed rice cakes after the heating process maintained their initial viable cell counts during a commercially meaningful storage period. According to Lee et al. (2006), the viable cell counts of E.coli O157:H7, Salmonella enterica serovar Typhimurium, Listeria monocytogenes, and Staphylococcus aureus inoculated into rice cake flour was reduced over 6 log CFU/g after 30 minutes steaming at 100°C. Because non-spore forming pathogenic bacteria can be killed during the steaming process in manufacturing, it is quite important to prevent cross contamination from water, equipment and the cooking environment or personnel after the heating process.

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