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Note

Cronobacter spp. in Commercially Available Dried Food in Japan

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A total of 140 samples of dried food sold in Japan were surveyed and tested for the presence of viable bacteria, distribution of coliform bacteria, and contamination with *Cronobacter* spp. The samples were purchased from retail stores in Tokyo and Kanagawa Prefecture. Out of the 140 samples tested, viable bacteria were found in 135 samples and coliform bacteria were found in 23 samples. Qualitative and quantitative testing revealed the presence of *Cronobacter* spp. in 35 (25.0%) and 11 samples (7.9%), respectively. The most commonly found *Cronobacter* species were *C. sakazakii*, with the next most common, in order, being *C. muytjensii* and *C. turicensis*. The actual numbers of *Cronobacter* species in the tested dried foods were low, but the widespread contamination particularly in dried herbs and vegetables was confirmed.

Key words：*Cronobacter* spp. / Dried food / Dried herbs / Dried spices / Dried vegetables.

The genus *Cronobacter* had been used for a single species *Enterobacter sakazakii* (Farmer et al., 1980); however, in 2007, it was renamed and reclassified as the *Cronobacter* species consisting of *C. sakazakii, C. malonaticus, C. turicensis, C muytjensii, C. dublinensis, C. dublinensis* subsp. *dublinensis, C. dublinensis* subsp. *lausannensis, C. dublinensis* subsp. *lactaridi*, and *C.* genomospecies (Iversen et al., 2007; Iversen et al., 2008). *C. condimenti* and *C. universalis* were also added as new species in 2012 (Joseph et al., 2012). *Cronobacter* spp. are gram negative, nonspore-forming, facultative-anaerobic, motile bacteria belonging to the family Enterobacteriaceae. They are known to cause meningitis, enteritis, and sepsis, particularly in infants through contaminated formula milk; furthermore, they are known to cause food borne illnesses (Muytjens et al., 1983; Simmons et al., 1989; Van Acker et al. 2001). Outbreaks caused by *Cronobacter* most typically originated in infant milk formula, but they can spread to many other kinds of food. They are contaminants in food factories, and their presence has been confirmed not only in factories producing powdered milk but in cereal and potato powder processing plants (Kandhai et al., 2004). In particular, in powdered milk factories, dust from the powder in the filling process was highly contaminated by *Cronobacter* (Reich et al., 2010). Contamination has also been found in common food products such as brown rice (Jung and Park, 2006), herbs and spices (Iversen and Forsythe, 2004), ready-to-eat food (Baumgartner et al., 2009), dry cereals and raw ground meat (Kandhai et al., 2010). However, the source of the contamination is not always known. There are few studies on *Cronobacter* species in common types of Japanese food, and not enough surveys have been conducted on the contamination of food sold in the market. This survey therefore set out to investigate the extent of contamination of bacteria, especially *Cronobacter* spp. in dried food sold in Japan.

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A hundred and forty samples of dried food were purchased from retailers in the Tokyo metropolitan area and in Kanagawa Prefecture from 2008 to 2010. The survey was conducted on 42 kinds of dried spices, 34 varieties of dried herbs, 17 types of dried vegetables, 16 kinds of dried seafood, 6 kinds of dried fruits, 6 types of dried tea leaves, and 19 other kinds of dried foods (Table 1).

The dried food samples were prepared by being ground in a blender or by mortar and pestle. Briefly, 5 g or 10 g sample of food material was suspended in ten times the volume of buffered peptone water (BPW; Oxoid, UK), homogenized in a stomacher (Stomacher 400 Lab blender, Seward Medical London, UK) for 1 min.

The number of viable bacteria was estimated by plating appropriate dilutions onto plate count agar (PCA; Difco, Detroit, USA), incubation the plates at 37 ±1°C for 48 h and counting the colonies. Coliforms and *Escherichia coli* were counted by plating appropriate dilutions onto Chromocult Coliform Agar (CCA; Merck, Darmstadt, Germany), and incubation at 37±1℃ for 24 h. The red coliform colonies and blue *E. coli* colonies were counted.

Qualitative detection of *Cronobacter* spp. was performed according to the method of ISO/TS 22964:2006, (ISO/TS 22964 and IDF/RM 210-first edition 2006-02-01: Milk and milk products-Detection of *Enterobacter sakazakii*, 2006). Briefly, a 5 g or 10 g sample of food material was suspended in ten times the volume of buffered peptone water, homogenized in a stomacher for 1 min in a stomacher, and incubated at 37±1℃ for 18 h. After incubation, 0.1 mL of the BPW suspension was inoculated in 10 mL of LST-vancomycin medium (LVM; Oxoid, UK), and incubated at 44±1℃ for 24 h. Next, a loopful of the LVM suspension was streaked onto Chromocult Enterobacter Sakazakii Agar (ESA; Merck, Darmstadt, Germany), and incubated at 44±1℃ for 24 h. The blue-green colonies were streaked onto Trypticase Soy Agar (TSA; Difco, Detroit, USA) to make a pure sample. After incubation at 25±1℃ for 48 h, the purity of the sample could be checked by confirming that all colonies were yellow. Quantitative results were calculated by stepwise dilutions of the sample liquid in BPW, plating diluted suspensions onto ESA plates, incubation at 44±1℃ for 24 h, and counting the blue-green colonies.

Bacterial identification was performed by first blue-green colonies on ESA were transferred to pure cultures on TSA. These were then identified using common

TABLE 1. Dried Food Samples.

Sample Type	Number of Samples	Dried food
Spices	42	Elder, Orange Peel, Laurel, Black Pepper, White Pepper, Pink Pepper, Garlic, Poppy Seed, Star Anise, Caraway Seed, Cinnamon Stick, Japanese Pepper, Salada mix, Pepperoncino, Rosehip, Fennel Seed, Anise, Dill, Ajwain, Citrus Peel, Fenugreek, Citrus Chamomile, Nutmeg, Ginger, Turmeric, Paprika, Clove, Juniper Berry, Cannabis, Mustard, Pepper, Gardenia, Horseradish, Cumin, Blue Poppy Seed, Mace, Allspice, Garammasala, Cardamom, Licorice, Cut Red Pepper, Red Pepper.
Herbs	34	Saint Johns Wort, Cresson, Tarragon, Oregano, Parsley, Basil, Thyme, Sage, Rosemary, Peppermint, Coriander, Lemongrass, Lavender, Lemon balm, Chamomile, Jasmine Flower, Nettle Leaf, St. johns wort, Citrus & Apple, Hibiscus, Mint, Marigold Petal, Savory, Mugwort, Camomilemint, Citrus & Chamomile, Flower Blend, Jasmine, Lemon Ginger, Celery, Kaffirlime Leaf, Laurel, Marjoram, Hop.
Vegetables	17	Tomatoes, Daylily, Mulukhiyya, Jews Ear Fungus, Spinach, Stem Lettuce, Green Onion, Taro Stem, Japanese Radish Julienne, Gourd Shavings, Black Fungus, Chopped Onion, Onion, Snow Fungus, Komatsuna, Cut Japanese Radish, Japanese Radish Leaf.
Seafood	16	Mekabu (Sea Weed), Konbu (SeaTangle), Wakame (Sea Weed), Tenngusa (Sea Grass), Surume (Dried Squids), Katsuoko (Dried Fermented Bonito Powder), Saketoba (Dried Salted Salmon), Hijiki (Sea Weed), Funori (Seaweed), Dried Shrimps (2), Tororokonnbu (Shredded Tangle), Niboshi (Dried Boiled Sardines), Chirimenjako (Dried Boiled Young Sardines), Aonori (Sea Green Laver), Katsuobushi (Dried Fermented Bonito).
Fruits	6	Dried Olive, Dried Figs, Dried Apricots, Dried Mangoes, Dried Pineapple, Dried Grapes.
Tea Leaves	6	Jasmine Tea, Houjicha, Green Tea, Oolong Tea, Pu-erh Tea, Tie Guan Yin Tea.
Other Food	19	Buckwheat Tea, Wolf Berry, Lotus Seed, Yerba Mate, Pine Nut, Green Lentils, Roasted BarleyTea, Toasted Soybean Flour, Toasted Barley Flour, Tian Tea, Dried grounded Soybean Curd, Almond Dice, Buckwheat Seed, Yellow Beans Hit, Mashed Potato Flakes, Mashed Sweet Potato Flakes, Green Beans Hit, Udon (Dried Wheat Noodles), Harusame (Gelatin Noodles).

characteristics such as the Gram staining, cytochrome oxidase tests and oxidation fermentation tests. Based on the results from these tests, identification was established by analysis of the 16s rRNA using PCR, with the following methods (Sasaki et al., 1997;2008): for bacterial DNA extraction, Insta Gene DNA Purification Matrix (Bio-Rad), for PCR reaction, Takara Premix *Taq* (Takara Bio. INC), for primers, 10F (forward) and 800R (reverse), for purification of products, NucleoSpin Extract Ⅱ (Takara Bio. INC), for sequencing of purified products, Big Dye Terminater Cycle Sequencing Kit (Applied Biosystems), and for analysis of base sequences, DNA Sequencer, ABI PRISM Analyzer 3100 (Applied Biosystems). Each sequence was compared with the NCBI (The National Center for Biotechnology Information) database. The homology for genus and species was more than 97%.

The viable bacteria and coliforms found in the 140 samples of dried food are given in Table 2. The number of viable bacteria detected in dried foods ranged from $<10^1$ to 10^7 CFU/g. Counts most frequently ranged from 10^2 to 10^4 CFU/g. The number of viable bacteria were particularly high in dried spices and herbs. Coliforms were found in 23 samples, and most of these were detected in dried spices and herbs at 10^2 CFU/g. *E. coli* was not detected in any of the 140 samples.

Table 3 summarizes the results of the isolation of *Cronobacter* spp. from the samples investigated in this study. The qualitative test detected *Cronobacter* species in 35 (25.0 %) of the 140 samples. The frequency was especially high in dried herbs (47.1%) and dried vegetables (35.3%). On the other hand, there was no contamination of *Cronobacter* spp. detected in dried seafood, dried fruits or dried tea leaves. The quantitative test detected *Cronobacter* contamination in 11 samples (7.9%), with the highest bacterial numbers being found in dried herbs and dried vegetables. Contamination appeared to be particularly high in dried herbs. There were positive correlations between the viable bacteria-coliforms counts and *Cronobacter* counts, and these results roughly corresponded to other studies (Jung and Park, 2006; Iversen and Forsythe, 2004).

A previous study showed that not only some food for

TABLE 2. Incidence and contamination level of viable bacteria counts and coliforms counts in dried food.

Sample type	No. of samples	Level of contamination, No. of samples												
		Viable bacteria counts (CFU/g)*								Coliform counts (CFU/g)				
		$<10^1$	10^1	10^2	10^3	10^4	10^5	10^6	10^7	$<10^1$	10^1	10^2	10^3	10^4
Spices	42	4	7	12	8	7	2	1	1	37	1	3	1	
Herbs	34	1	1	3	7	16	5		1	23	1	5	4	1
Vegetables	17		1	2	5	5	3		1	14		2		1
Seafood	16	1		3	2	7	3			15			1	
Fruits	6			4	2					6				
Tea Leaves	6	2		1	2			1		6				
Other Food	19	7	3	4	2	2	1			16	1		1	1
Total	140	15	12	29	28	37	14	2	3	117	3	10	7	3

*: Colony forming unit/g.

TABLE 3. Incidence and contamination level of *Cronobacter* spp. in dried food.

Sample type	No. of samples	Qualitative analysis*1		Quantitative analysis*2 Level of contamination, No. of samples			
		−	+	$<10^1$	10^1	10^2	10^3
Spices	42	34	8 (19.0)*3	41	1		
Herbs	34	18	16 (47.1)	27	3	3	1
Vegetables	17	11	6 (35.3)	15		1	1
Seafood	16	16	0 (0.0)	16			
Fruits	6	6	0 (0.0)	6			
Tea Leaves	6	6	0 (0.0)	6			
Other Food	19	14	5 (26.3)	18	1		
Total	140	105	35 (25.0)	129	5 (3.6)	4 (2.9)	2 (1.4)

*1 Qualitative analysis : Determination of *Cronobacter* spp. by enrichment culture.

*2 Quantitative analysis : *Cronobacter* spp. counts (Chromocult Enterobacter sakazakii agar ; Colony forming unit/g).

*3 The number in the parentheses were % of positives to total samples.

early childhood, such as infant milk formulas (2 out of 82 samples) and dried infant food (5 out of 49 samples), but also dried herbs and spices (40 out of 122 samples) were highly contaminated by *Cronobacter* spp. (Iversen, et al. 2004). *Cronobacter* spp. contamination was found in 35 out of 140 samples (25.0%) among common types of dried food in the present study. This frequency is high when compared to overseas studies (Restaino et al., 2006; Friedemann, 2007; Jaradat et al., 2009). This may be related to the origin of the raw materials, and further studies on *Cronobacter* spp. contamination in fresh vegetables, herbs and spices would be useful to clarify the reason.

This study has shown that although the number of contaminating *Cronobacter* may be low, the incidence of contamination is high in commonly sold food, especially in dried herbs and vegetables. Among the Enterobacteriaceae, *Cronobacter* spp. are particularly resistant to desiccation, and so they are likely to survive in dried food. The long -term survival of *Cronobacter* in infant milk formula (Edelson-Mammel et al., 2005) and infant cereals (Lin and Beuchat, 2007) is certainly a concern (Osaili and Forsythe, 2009).

Table 4 shows the species of *Cronobacter* spp. isolated from dried food. Sequencing of 16S rDNA showed common *Cronobacter* species in dried food were *C. sakazakii* (55.2%), *C. muytjensii* (15.2%), and *C. turicensis* (9.0%). The most common species in dried herbs were *C. sakazakii* (46.4%), then *C. muytjensii* (19.1%) and *C. turicensis* (9.1%). *C. sakazakii* was present in a higher proportion of commonly consumed food in Japan. Molloy et al. (2009) conducted tests on *Cronobacter* species in food and the environment, and found that *C. sakazakii* was the most common *Cronobacter* species found in products such as farm animal feed, meat and cereals. Similarly, Hoche et al. (2012) surveyed 399 samples of food and food materials other than dried food, and found 52 instances of *Cronobacter* contamination, of which 54.7% were by *C. sakazakii*, 28.4% were by *C. malonaticus*, and 7.5% were by *C. muytjensii* and *C. dublinensis*. *C. sakazakii* was the most common contaminant, which was coincided with our main finding.

TABLE 4. Contamination of *Cronobacter* spp. in dried food.

Sample type	Number of detected strains					
	C. sakazakii	*C. muytjensii*	*C. turicensis*	*C. dublinensis*	*Cronobacter* spp.	Unidentified
Spices	32 (71.1)*	3 (6.7)	4 (8.9)	5 (11.1)		1 (2.2)
Herbs	51 (46.4)	21 (19.1)	10 (9.1)	12 (10.9)	7 (6.4)	9 (8.2)
Vegetables	18 (60.0)	8 (26.7)			4 (13.3)	
Other Food	15 (60.0)		5 (20.0)			5 (20.0)
Total	116 (55.2)	32 (15.2)	19 (9.0)	17 (8.1)	11 (5.2)	15 (7.1)

*: The number in the parentheses were % of positives to total samples.

If spices and herbs are added after cooking, *Cronobacter* can multiply at room temperature, and should be noted in the use of baby food products containing dried vegetables. Decrease of bacterial contamination would decrease *Cronobacter* contamination; therefore, traditional microbial controlling technology, such as hygienic handling of food, would also contribute to controlling *Cronobacter* contamination.

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