Original Article

Detection of *Bacillus cereus* and Gram-negative Bacteria Communities in Commercial Sesame in Japan

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Abstract

Sesame seeds are used in many traditional Japanese foods for their flavor and taste but commercial sesame can be highly contaminated with bacteria. We therefore examined the bacterial populations, including *Bacillus* spp., Enterobacteriaceae bacteria and standard plate count bacteria in sesame samples purchased from markets in Japan. A total of 8 sesame samples were tested, of which 4 (50.0%) harbored 4.3–5.6 log cfu/g standard plate count and 2.7–4.3 log cfu/g Enterobacteriaceae bacilli. *Pantoea dispersa*, *P. septica* and *P. agglomerans* were identified by a MALDI-TOF MS-based test. One (12.5%) sample harbored 3.6 log cfu/g *Bacillus cereus,* but this strain lacked the gene encoding of the enzyme responsible for cereulide synthesis and did not produce enterotoxin. *B. cereus* was also isolated from a heated sample (98 °C for 20 mins). Metagenome analysis showed that 4 samples were contaminated with bacteria belonging to the 5 genera *Pantoea*, *Serratia*, *Pseudomonas*, *Xanthomonas* and *Rosenbergiella*. *Pantoea* and *Pseudomonas* DNA was detected in all positive samples but the bacterial load varied. Our study revealed that sesame can become contaminated with Enterobacteriaceae bacteria and *B. cereus*. Dishes containing contaminated sesame could potentially cause *B. cereus* food poisoning, although the *B. cereus* isolates obtained in this study did not contain the gene encoding the enzyme responsible for cereulide synthesis and did not produce enterotoxin. To prevent food poisoning caused by bacterial contamination, it is important to roast sesame seeds at a sufficiently high temperature, do not leave the cooked food with sesame at room temperature for a long time and avoiding cross-contamination from sesame to ready-to-eat food.

Keywords: *Bacillus cereus*, Contamination, Gram-negative Bacterial Communities, Sesame, Japan

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Introduction

In Japan, national food poisoning data is collected by the Ministry of Health, Labor and Welfare. *B. cereus* was detected in 34 outbreaks of food poisoning and 568 affected patients between 2015 and 2019. The foods causing the 34 cases were mainly made in rice dishes and the dishes had been kept at room temperature for many hours. We started to research about the elucidation of risk factors in rice dishes such as "Chahan: fried rice with other ingredients" , "Maze-gohan : rice cooked with other ingredients" and "Takikomi-gohan: boiled rice with fish and vegetables mixed in advance". Our previous report (Kato et al., 2018; Kato et al., 2020) about rice and sticky rice contamination suggested that some uncooked rice had *Bacillus* species and enterobacteriaceae bacteria contaminations. Another previous report (Kato et al., 2019) showed that some commercial *sekihan* (8 of 34 samples) had also *Bacillus* species and enterobacteriaceae bacteria contaminations. Japanese taste, *sekihan,* literally means "red rice" and it is steamed sticky rice with boiled red beans. *Sekihan* applies sesame before eating. In the report, we thought that the major contamination source of the bacilli in *sekihan* is sesame.

Sesame (*Sesamum indicum*) seeds are a high-volume food crop. Whole seeds are used in baking and cooking oil is extracted from the seeds. Sesame is primarily grown on small farms in developing countries and in Japan. Sesame is sprinkled with salt on the surface of *sekihan*, rice balls or a traditional Japanese rice dish. In addition, sesame is used to flavor dishes such as *goma-ae*, which consists of vegetables in a sesame sauce with sesame tofu and pickles.

Sesame can become highly contaminated with bacteria such as *Salmonella* spp. and *Bacillus* spp. (Brockmann *et al*., 2004; Kato *et al*., 2019; Meinen *et al*., 2019). We previously reported (Kato *et al*., 2019) that 3 of 12 sesame samples contained 2.00–4.84 log cfu/g *Bacillus* spp. and 4 of 12 sesame samples contained 2.00–4.85 log cfu/g standard plate count bacilli.

The matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) test for identification of bacteria species and metagenomic analysis of samples was developed and is ubiquitous these days. Here, we examined 8 sesame samples from markets in Tokyo, Japan for *Bacillus* spp., enterobacteriaceae bacteria and standard plate count bacteria. *Bacillus cereus* isolates were subjected to PCR and reverse passive latex agglutination to detect the genes encoding the enzymes responsible for the synthesis of cereulide (CRS) and enterotoxin, respectively. The enterobacteriaceae isolates were subsequently identified at the species level by biochemical test or MALDI-TOF MS test. In addition, we used metagenomics to analyze the gram-negative bacteria in the sesame samples using a prokaryotic 16S ribosomal RNA gene (Kõiv *et al.,* 2019).

Materials and Methods

***Sesame samples and laboratory handling*:** A total of 8 sesame samples (S-1 to S-8) were examined in this study. Sample numbers S-1 to S-3 were purchased at a large grocery store, S-4 at a farmer's market in a rural area and S-5 to S-8 at convenience stores. The labels on the packages of samples S-6 to S-8 stated "roasted sesame". The sesame samples were held at room temperature in the stores, kept in refrigerators at 3–5 °C in our laboratory, and analyzed for bacterial populations within one week of collection.

Standard plate counts, Enterobacteriaceae bacteria counts and identification of Enterobacteriaceae bacteria: The sesame samples (10 g) were homogenized gently by hand in 90 mL of phosphate buffered saline (PBS; pH 7.0) for approximately 30 secs. We collected supernatant liquid from the sample, then, diluted 10^0- to 10^3-fold in PBS. A portion (50 µL) of each PBS-diluted sample was inoculated onto two plates each of SPC agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and VRBG agar (Oxoid Ltd., Basingstoke, UK) using a spiral plating method (EDDY JET2W, IUL Instruments, Barcelona, Spain). The SPC agar plates were incubated at 35 °C for 48 h and the VRBG agar plates were incubated at 35 °C for 24 h, after which the number of bacterial colonies was counted. The 1–3 enterobacteriaceae isolates grown on VRBG agar were identified using a commercial identification kit (ID Test EB-20, Nissui Pharmaceutical Co. Ltd.). Bacteria that could not be identified using this kit were sent to the ICLAS Monitoring Center in Kawasaki, Japan (https://www.iclasmonic.jp/en/microbiology/mquick.html) for identification using a MALDI-TOF MS test. In addition, 1 mL of sample homogenate was transferred to a microcentrifuge tube (Safe-Lock, 1.5 mL; Eppendorf, Hamburg, Germany) and heated at 98 °C for 20 mins on a heat block (THB-2, Dry block bath, AS ONE, Osaka, Japan), after which a 50-µL aliquot was inoculated onto SPC agar using the spiral plating method. If a colony grew on either the SPC agar or the VRVG agar plate, but not both, we considered this as 100 spc or cfu/g.

***Metagenomic analysis of gram-negative bacteria in the sesame samples*:** The sample homogenate (1 mL) was transferred to a Safe-Lock microcentrifuge tube and the DNA was extracted from 140 µL of supernatant using the gram-negative protocol provided with the DNeasy Blood & Tissue kit (Qiagen GmbH, Hilden, Germany) and dissolved in 200 µL of Buffer AE (Nippon Gene Co., Ltd., Tokyo, Japan). Metagenomic analysis of the 16S V3 and V4 region followed the instructions provided on the Illumina website (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf) for 16S Metagenomic Sequencing Library Preparation: preparing 16S Ribosomal RNA Gene Amplicons for the Illumina Miseq System (Part # 15044223 Rev.B), except that we used Tks Gflex™ DNA Polymerase Low DNA (Takara Bio Inc., Kusatsu, Japan) and 2x KAPA HiFi HotStart Ready Mix (Roche Diagnostics, Rotkreuz, Switzerland). Metagenomic DNA sequencing was carried out using the Illumina MiSeq platform (MiSeq Reagent Nano Kit V2; Illumina, San Diego, CA). For read clustering and analysis, we used the CD-HIT-OTU workflow for Illumina data (Li *et al*., 2012). Representative operational taxonomic unit (OTU)

sequences were identified using the SILVA database (https://www.arb-silva.de/) (Quast *et al*., 2013), taking the hit with the highest bit-score as the representative organism for this OTU. The figure of relative frequency of bacterial phyla found in positive sesame samples was drawn using an online application (https://view.qiime2.org/).

B. cereus ***counts, bacterial identification and toxin tests***: Sesame samples (10 g) were homogenized gently by hand in 90 mL PBS (pH 7.0) for approximately 30 secs. Two parallel 1-mL homogenate samples were collected in Safe-Lock microcentrifuge tubes; one sample was heated at 98 °C for 20 mins in a heat block; the other, which was not heated, was used as a control. A portion (50 µL) of each heated sample and corresponding control was inoculated onto Brilliance Bacillus Cereus Agar (Oxoid Ltd.) using the spiral plating method. The inoculated agar plates were incubated at 35 °C for 18 h, after which the typical colonies were counted. *B. cereus* colonies are blue/green on Brilliance Bacillus Cereus Agar. The colonies were identified using a commercial identification kit (API® 50 CH; bioMérieux, Marcy-l'Étoile, France) and the API_50_CHB database of APIweb™ (https://apiweb.biomerieux.com) and a MALDI-TOF MS-based test. *B. cereus* colonies identified using APIweb™ were examined using a *B. cereus* (CRS gene) PCR Detection kit (Takara Bio Inc.) to detect the gene encoding the enzyme responsible for CRS synthesis and the gene encoding lecithinase. Assays were also performed to detect enterotoxin, using a Reverse Passive Latex Agglutination kit (CRET-RPLA, Denka Seiken Co. Ltd., Tokyo, Japan).

Results

Standard plate counts and enterobacteriaceae counts of 8 sesame samples and identification of enterobacteriaceae (Table 1): Four of 8 (50.0%) sesame samples (samples S-1 to S-4) harbored 4.3–5.6 log cfu/g standard plate count and 2.7–4.3 log cfu/g enterobacteriaceae bacilli. Three of these samples (S-1 to S-3) were obtained from a large grocery store and S-4 was from a farmer's market. No bacilli were isolated from the 4 samples (S-5 to S-8) purchased at convenience stores. The packaging for samples S-6 to S-8 was labeled "roasted sesame". The enterobacteriaceae isolates could not be identified using ID test EB-20, but the enterobacteriaceae species *Pantoea dispersa*, *P. septica* and *P. agglomerans* were identified by a MALDI-TOF MS-based test.

Table 1 Bacterial counts and species of isolates from samples

Sample ID	Standard plate counts (Log spc/g)	*Enterobacteriaceae* counts (Log cfu/g)	Identification method of *Enterobacteriaceae* isolates		*Bacillus* spp. counts (Log cfu/g)	Identification method of *Bacillus* isolates	
			ID Test EB-20	MALDI-TOF MS-based test		API 50 CH	MALDI-TOF MS based test
S-1	4.73	3.6	N.I. a)	*P. dispersa*	-		
S-2	5.63	4.3	N.I.	*P. septica*	-		
S-3	4.26	3.3	N.I.	*P. agglomerans*	3.6 b)	*B. cereus*	*B. cereus*
S-4	4.34	2.7	N.I.	*P. dispersa*	-		
S-5	-	-			-		
S-6	-	-			-		
S-7	-	-			-		
S-8	-	-			-		

Sample number S-1, S-2 and S-3 were obtained from a big grocery store, S-4 was from farmer's market in local area, and S-5, S-6, S-7 and S-8 were from convenience stores in Japan. Sample number S-6, S-7, and S-8 labeled "roasted sesame" on packaging.
a) No Identification
b) Heat treatment (98°C, 20min) sample showed 2.6 log cfu/g of *B.cereus* .

Prevalence of* B. cereus *and toxin assays of the isolated* B. cereus *colonies (Table 1): One of the 8 (12.5%) sesame samples harbored 3.6 log cfu/g *B. cereus* and 2.6 log cfu/g *B. cereus* was isolated after heating the sample homogenate at 98 °C for 20 mins. The isolate from sample number S-3 was identified as *B. cereus* by both the API 50 CH and MALDI-TOFMS-based tests. This strain did not harbor the gene encoding the enzyme responsible for cereulide synthesis, and did not produce enterotoxin.

Microbial taxonomic composition: The relative frequencies of the bacterial phyla found in the 4 positive sesame samples are shown in Figure 1. We detected sequences from the 5 genera *Pantoea*, *Serratia*, *Pseudomonas*, *Xanthomonas* and *Rosenbergiella* in these sesame samples. *Pantoea*, *Serratia* and *Rosenbergiella* belong to enterobacteriaceae, *Pseudomonas* belongs to *Pseudomonadaceae*, and *Xanthomonas* belongs to *Xanthomonadaceae*. The genera *Pantoea and Pseudomonas* were isolated from all positive sesame samples but their distribution and proportion in the sesame

samples were different. The predominant genus was *Serratia* in Sample No. S-1, *Pseudomonas* in Sample No. S-2 and *Pantoea* in Samples No. S-3 and S-4. The populations of bacteria in these 4 sesame samples varied.

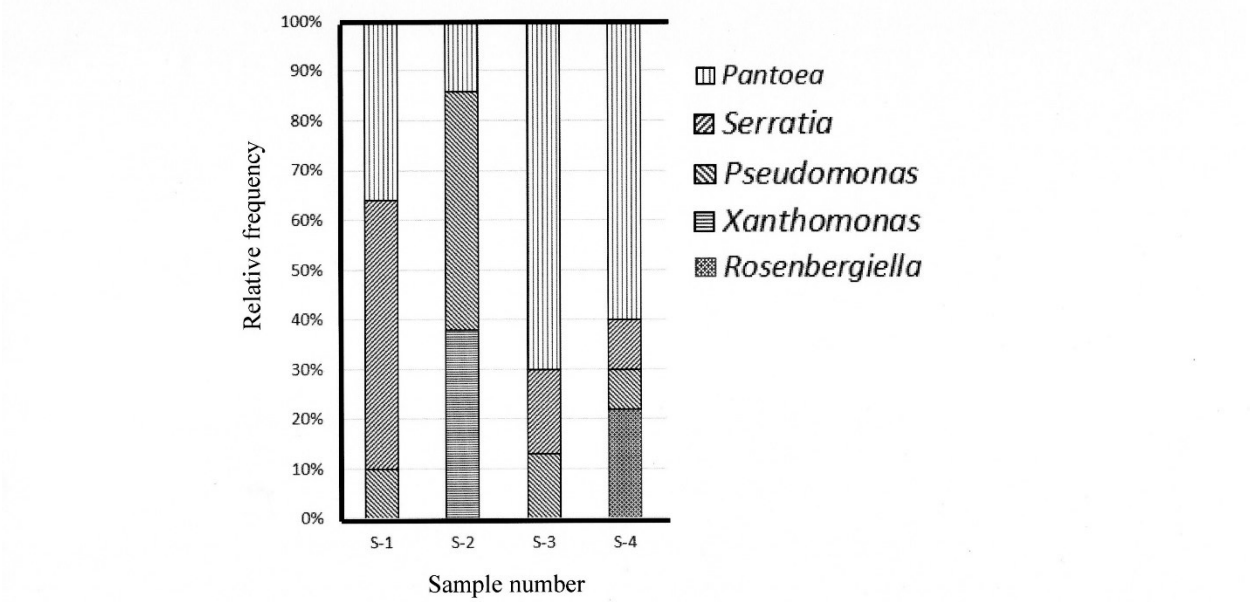

Figure 1 Relative frequencies of bacterial phyla found in 4 positive sesame samples

Discussion

We investigated the prevalence of bacterial contamination in samples of sesame to assess the risk factor for food poisoning in sesame products sold in Japan. We previously reported (Kato *et al*., 2019) that 3 of 12 sesame samples contained 2.00–4.84 log cfu/g *Bacillus* spp. and 4 of 12 sesame samples contained 2.00–4.85 log cfu/g standard plate count bacilli. In the present study, one of 8 (12.5%) sesame samples contained 3.6 log cfu/g *B. cereus* and 2.6 log cfu/g *B. cereus* after heating the sample homogenate at 98 °C for 20 mins. Dishes containing sesame contaminated with *B. cereus* can cause food poisoning, although the *B. cereus* isolated in this study did not possess the gene encoding the enzyme responsible for cereulide synthesis and did not produce enterotoxin.

Among the sesame samples, 50% (4 of 8 samples) harbored 4.26–5.62 log cfu/g standard plate count and 2.7–4.3 log cfu/g enterobacteriaceae counts and the enterobacteriaceae species *P. dispersa*, *P. septica* and *P. agglomerans* were isolated. No bacteria were isolated from 4 packaged samples (S-5 to S-8) sold by convenience stores and S-6 to S-8 samples were labeled "roasted sesame". Sesame seeds should thus be roasted before use or "roasted sesame" should be bought and used.

Pantoea are gram-negative, non-encapsulated, non-spore-forming, ubiquitous straight rods isolated from geographical and ecological sources such as plant surfaces, buckwheat seeds, human feces and the environment (Asai *et al*., 2019; Gavini *et al*., 1989; Walterson and Stavrinides, 2015). *P. agglomerans* has been isolated from the joint fluids of patients with arthritis, synovitis and osteomyelitis (Cruz *et al*., 2007; Delétoile *et al*., 2009). *P. septica* was first isolated from a human stool sample in New Jersey and has since been frequently isolated from patients with opportunistic infections (Brady *et al*., 2010). *P. dispersa* has been isolated from neonatal sepsis patients (Mehar *et al*., 2013; Panditrao and Panditral, 2018). The *Pantoea* species isolated in this study are abundant in the environment and cause opportunistic infections.

MALDI-TOF MS-based identification tests and metagenomic analysis using a prokaryotic 16S ribosomal RNA gene are widely used in the area of food hygiene (Li *et al*., 2012; Pavlovic *et al*., 2013). We were unable to identify the enterobacteriaceae species in isolates from the sesame samples using the ID Test EB-20 biochemical test but all isolates were identified using the MALDI-TOF MS-based test, indicating that the latter is a useful tool for identifying bacteria, especially in isolates from environmental and food samples (Kato *et al*., 2020).

Metagenomic analysis of gram-negative bacteria in the 4 positive sesame samples showed that *Pantoea* and *Pseudomonas* were common, although the distributed genera and their proportions in the sesame samples were different. This study is the first to report the analysis of bacteria in sesame. We found gram-negative bacteria in sesame sold in Japan and showed that metagenomic analysis is a useful tool for investigating bacterial populations in food samples.

This study revealed that sesame can become contaminated with *B. cereus* and enterobacteriaceae bacteria. Dishes containing contaminated sesame can potentially cause *B. cereus* food poisoning, although the *B. cereus* isolates obtained in this study did not contain the gene encoding the enzyme responsible for cereulide synthesis and did not produce enterotoxin. Isolated *P. dispersa*, *P. septica* and *P. agglomerans* are usually in the environment, and these organisms have been isolated from patients with opportunistic

infections. To prevent food poisoning or infection caused by bacterial contamination, it is important to roast sesame seeds at a sufficiently high temperature, not to leave cooked food with sesame at room temperature for a long time and avoiding cross-contamination from sesame to ready-to-eat food.

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