食中毒細菌の穀類加工品での生残とカテキン類の効果

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Survival of Foodborne Pathogens in Grain Products and the Effect of Catechins

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We investigated the survival of bacterial pathogens in grain products. *Salmonella* Enteritidis and *Staphylococcus aureus* survived for more than 3 weeks in grain flakes. Although *S.* Enteritidis in grain flakes reached a level of less than 10^2 CFU/g, the surviving organisms grew rapidly in milk and reached 10^9 CFU/g after 25-hr incubation. When catechins were present on the grain flakes, the numbers of *S.* Enteritidis and *S. aureus* were significantly decreased. In addition, catechins reduced the survival and growth of *S.* Enteritidis, *S. aureus* and *Bacillus cereus* when added to cooked grain products. This study indicates that *S.* Enteritidis, *S. aureus* and *Bacillus* can survive for a long time in grain products and that catechins impair their survival and growth.

Key words: foodborne pathogens; grain products; catechins

Introduction

Grain powders, such as corn flour and soy powder, may be contaminated with *Salmonella*[1] from animals[2),3] and soil bacilli[4]. However, bacteria can not grow in grain products, such as grain flakes and cooked grain, because the manufacturing process includes heating and drying[5]. Nevertheless, the spores of *Bacillus cereus* may survive after pasteurization during food processing. Therefore, during the storage of cooked foods under optimal growth conditions, the spores may germinate and then the vegetative cells grow to cause foodborne infections[6]. Grain products associated with foodborne infections have also been reported; examples include grain flakes contaminated with *Salmonella*[7),8], dumpling contaminated with *Staphylococcus aureus*[9] and adzuki bean paste contaminated with *B. cereus*[10]. In Japan, 141 cases of foodborne infections (123 cases of *Salmonella* Enteritidis and 18 cases of *S. aureus*) associated with adzuki bean paste, rice cake or dumpling have occurred[11]. To find a way to control microorganisms in food, we evaluated catechins commonly found in tea extracts in this study. Catechins have antibacterial activity against pathogens such as pathogenic *Vibrio* species[12)-14)], *S. aureus*[15], *Escherichia coli* O157 : H7[16], *S.* Enteritidis[17], and vegetative cells and spores of *Clostridium botulinum*[18]. The antibacterial activity is attributed to the galloylester and pyrogallol moieties of the catechins, and the activity attributed to catechol and pyrogallol appears to be equal to that of the galloylester[19]. Despite many reports on the antibacterial activity of catechins, there has been no report on the use of catechins as antibacterial food additives.

In the present study, we investigated the survival of bacterial pathogens in grain flakes. In addition, the antibacterial activities of catechins against the bacterial pathogens in the grain flakes, and the inhibition of bacterial growth were examined during storage of cooked grain products and grain flakes.

Materials and Methods

Preparation of food samples.

Three kinds of grain flakes: corn flakes, brown rice flakes and wheat bran flakes were purchased in retail shops in Tokyo. We dipped the corn flakes in solutions of catechins from green tea (Sanfenone, Taiyo Chemical Co., Ltd., Yokkaichi, Japan) at concentration of 1.0, 5.0 and 50 mg/mL and catechins were bound to the surface of the flakes at the final concentrations of 0, 0.1, 0.5 and 5.0 mg/g, respectively. We found that 1 g of grain flakes absorbed 0.1 mL of catechin solution. The food treated with catechins was stored at 25℃ until used for experiments. The following three kinds of cooked grain products prepared in the laboratory were treated with catechins at the concentration of 1 mg/g or not treated. A portion (1 mL) of catechin solution at the concentration of 100 mg/mL was added to 100 g of cooked food

sample, *i.e.*, adzuki bean paste (adzuki bean powder 180 g, sugar 300 g and water 500 mL), shiratama dumpling (glutinous rice powder 50 g, rice powder 50 g and water 100 mL) and warabimochi: a jelly-like confection made from starch (sweet potato starch 85 g, kudzu vine starch 10 g, bracken starch 5 g, sugar 50 g and water 500 mL).

Strains and culture

S. Enteritidis (from liquid egg: strain no. SEC 315), *S. aureus* (American Type Culture Collection: ATCC 25923) and *Bacillus cereus* (Japan Culture Microorganisms: JCM2152) were used. *S.* Enteritidis, *S. aureus* and *B. cereus* were cultured in 10 mL of tryptic soy broth (TSB; Becton Dickinson, Sparks, MD) for 24 hr at 37℃. The cultures were centrifuged at 3,000×*g* for 15 min at 4℃. The cell pellet of each strain was suspended in phosphate-buffered saline (PBS; Nissui Co., Tokyo, Japan). The procedure was repeated twice to wash the cells. Finally the cells of each strain were suspended in 10 mL of PBS. These bacterial suspensions were used for inoculation of grain flakes. A 10^4-fold dilution was used as the bacterial solution for inoculation of cooked grain products. To confirm the inoculation level of the bacterial strains, the cultures were diluted with PBS to 10^{-6}. A 0.1 mL aliquot was plated onto tryptic soy agar (TSA; Becton Dickinson). After incubation at 37℃ for 24 hr, the number of colonies was counted.

Microbiological quality of the food sample

Aerobic bacteria in the food samples were counted and it was confirmed that the samples were not contaminated with *Salmonella*, *S. aureus* and *B. cereus* as follows. Samples (10 g) were homogenized with 90 mL of PBS with a stomacher (Seward Co., Ltd., London, UK) for 1 min or a pulsifier (Microgen Bioproducts Ltd., UK) as a vibratory mixer for 15 sec. To count aerobic bacteria, 0.1 mL of the homogenate and each of serial 10-fold dilutions in PBS were plated onto nutrient agar (NA; Nissui) and incubated at 37℃ for 24 hr. The food samples were confirmed not to be contaminated with *Salmonella*, *S. aureus* and *B. cereus* using homogenate in BPW for *S.* Enteritidis, TSB with 7.5% NaCl for *S. aureus* and TSB with polymixin (50 unit/mL) for *B. cereus*. After incubation at 37℃ for 18 hr, the enrichment cultures of *S.* Enteritidis, *S. aureus* and *B. cereus* were streaked onto CHROMagar *Salmonella* (CHROMagar, Paris, France), Mannitol salt agar (Eiken Chemical, Tokyo, Japan) and NGKG (Niussi), respectively. Colonies were observed after incubation at 37℃ for 24 hr.

Survival of S. Enteritidis and S. aureus in grain flakes

Corn flakes, brown rice flakes and wheat bran flakes were transferred to a stomacher bag in 10 g amounts and inoculated with *S.* Enteritidis or *S. aureus* solution (0.1 mL) at the inoculation level of approximately 10^6 CFU/g. These samples were sealed with a heat-sealer and incubated at room temperature for 56 days, then homogenized with 90 mL of PBS in a pulsifier for 15 sec. To determine the survival of pathogens, aliquots of 0.1 mL of serial 10-fold dilutions was plated onto TSA as a non-selective agar medium and incubated at 37℃ for 24 hr. Then the TSA-plated colonies of *S.* Enteritidis were transferred to CHROMagar *Salmonella* and the colonies of *S. aureus* were transferred to mannitol salt agar, from which they were picked up with needles for identification.

Growth of *S.* Enteritidis and *S. aureus* in grain flakes with milk. Samples (each 10 g) of corn flakes, brown rice flakes and wheat bran flakes, inoculated with *S.* Enteritidis and *S. aureus* as described above, were kept at room temperature for 35 days to reach an undetectoble level by plating method described above. Then 90 mL of pasteurized milk purchased in a retail shop in Tokyo was poured onto the samples, and incubated at 25℃ (room temperature) for 24 hr. To determine the growth of *S.* Enteritidis and *S. aureus* in grain flakes with milk, the cultures with milk were diluted 10^{-1} to 10^{-6}-fold in PBS. The dilutions (0.1 mL) were plated onto CHROMagar *Salmonella* and mannitol salt agar medium (Oxoid) with 30% egg yolk (EYMSA). Colonies were observed after incubation at 37℃ for 24 hr. The experiments were performed in quadruplicate.

Growth of *S.* Enteritidis, *S. aureus* and *B. cereus* in cooked grain products and the inhibitory effect of catechin. The cooked grain products (adzuki bean paste, shiratama dumpling and warabimochi) treated with/without catechins were transferred to a stomacher bag (10 g) and inoculated with *S.* Enteritidis, *S. aureus* and *B. cereus* at the level of 10^2 CFU/g, and then incubated at 4 or 25℃ for 3 days. To determine the growth of the pathogens in the cooked grain products and the inhibitory effect of catechins, samples were homogenized with 90 mL of PBS in a pulsifier for 15 sec or a stomacher for 1 min. Each serial 10-fold dilution (0.1 mL) was plated onto XLD, EYMSA and NGKG. Colonies were observed after incubation at 37℃ for 24 hr. The experiments were performed in quadruplicate.

Enrichment of food homogenate and plating of enrichment culture

To confirm that the population of each pathogen was below the detection limit, the homogenates (100 mL) of grain flakes and cooked grain food were added to 100 mL of enrichment broth prepared at double strength: buffered peptone water (BPW; Oxoid, Basingstoke, Hampshire, UK) for *S.* Enteritidis; TSB with 7.5% NaCl for *S. aureus*; TSB with polymixin (50 unit/mL) for *B. cereus*. Incubation was carried out at 37℃ for 18 hr. The enrichment cultures for *S.* Enteritidis, *S. aureus* and *B. cereus* were streaked onto XLD (Oxoid), EYMSA and NGKG, respectively. Colonies were observed after incubation at 37℃ for 24 hr.

Confirmation of *S.* Enteritidis, *S. aureus* and *B. cereus*. A portion of the colonies suspected to be *Salmonella* on CHROMagar *Salmonella* and XLD was tested for agglutination with a *Salmonella* antibody kit (the *Salmonella* latex test, Unipath, Oxoid). A portion of the suspected *S.*

aureus colonies was tested for coagulase and confirmed to be Gram-positive cocci with the Gram stain and microscopy. A portion of the suspected *B. cereus* colonies was tested for catalase, VP reaction, growth under anaerobic conditions and growth at pH 5.7. The enterotoxin of *B. cereus* in culture was detected using a reverse passive latex agglutination kit (CRET-RPLA, Denka Seiken, Tokyo, Japan). The experiments were performed in quadruplicate.

Statistical analysis.

Significant differences between bacterial populations incubated with catechins and without catechins were analyzed by using Student's *t*-test. A p value < 0.05 was taken to be significant.

Results and Discussion

Aerobic bacteria counts in corn flakes, adzuki bean paste, siratama dumpling and warabimochi were less than 1.9 log, cfu/g though those in brown rice flakes and wheat bran flakes were 3.9 and 5.2 log, cfu/g, respectively. Food samples used in this study were confirmed not to be contaminated with *Salmonella*, *S. aureus* or *B. cereus*.

In the three kinds of grain flakes, the population of *S.* Enteritidis quickly decreased and the organism was undetectable by a plating method at day 14, but remained detectable by enrichment at day 28 (Fig. 1). The population of *S. aureus* decreased and became undetectable by enrichment at day 28. In corn flakes and brown rice flakes, the decrease of the *S. aureus* population was slower than that of the *S.* Enteritidis population. It was demonstrated that foodborne pathogens can survive in grain flakes.

Grain flakes are usually consumed very soon after milk is poured on them. But children and old people may allow the grain flakes to soften in milk, and may leave them for a long time before eating them. In this study, milk was poured into the three kinds of grain

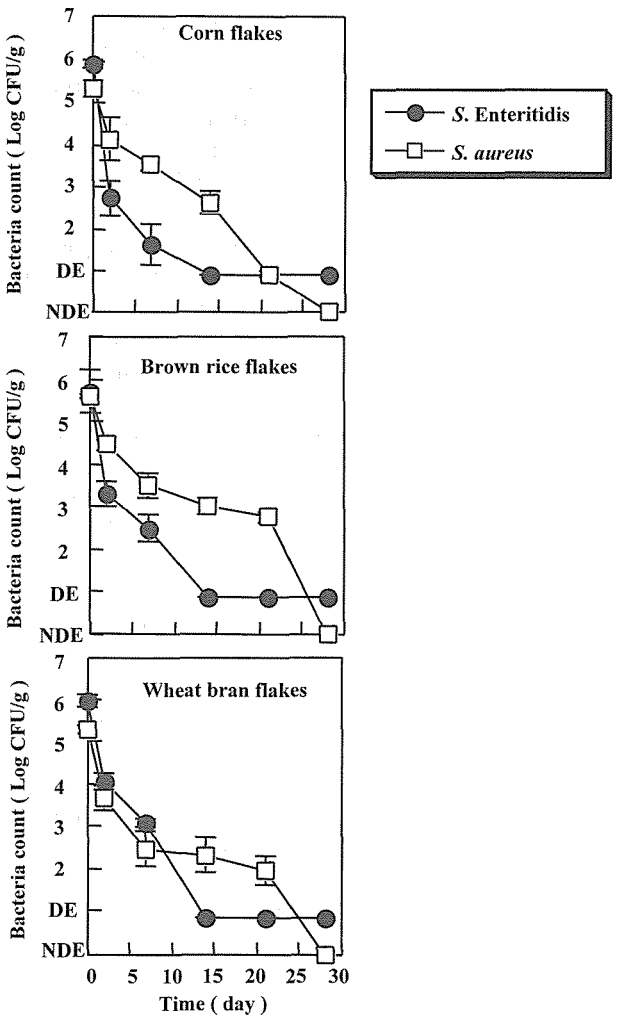

Fig. 1. Survival of *S.* Enteritidis and *S. aureus* in grain flakes.

Bars represent standard errors. DE: detected in enrichment culture but not detected by the plating method, NDE: not detected in enrichment culture.

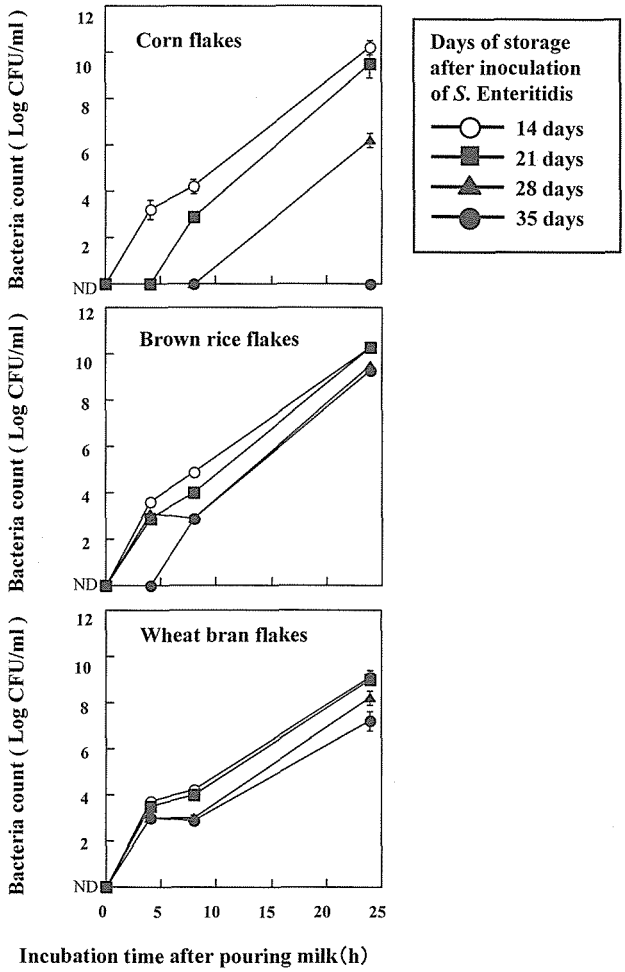

Fig. 2. Growth of *S.* Enteritidis in grain flakes with milk.

Milk was poured onto grain flakes in which the population of *S.* Enteritidis was undetectable after storage, and incubated at 25℃. Bars represent standard errors. ND: not detected by plating method. :○, 14 days; ■, 21 days; ▲, 28 days and ●, 35 days.

flakes stored for 14, 21, 28 and 35 days after inoculation with *S.* Enteritidis and *S. aureus*. *S.* Enteritidis grew in each kind of grain flakes with milk (Fig. 2), but *S. aureus* did not grow at all (data not shown). In corn flakes and brown rice flakes, the lag phase of *S.* Enteritidis was long (Fig. 2). In wheat bran flakes, *S.* Enteritidis quickly grew in all samples after 4-hr incubation (Fig. 2). The results indicate that small numbers of *S.* Enteritidis in grain flakes could grow in milk and may be a cause of food poisoning.

The number of *S.* Enteritidis was below the detection limit by enrichment at day 7 in corn flakes treated with catechins at the concentrations of 0.5 and 5.0 mg/g ($p<0.01$) (Fig. 3). The population of *S. aureus* was undetectable by enrichment at day 7 at the concentration of more than 0.1 mg/g ($p<0.01$) (Fig. 3). Catechins are known to have antibacterial activity[20]. In this study, catechins impaired the growth of foodborne pathogens in grain flakes and cooked grain products. Catechin treatment might be an effective way to interfere with the survival of foodborne pathogens in corn flakes and other dried grain products.

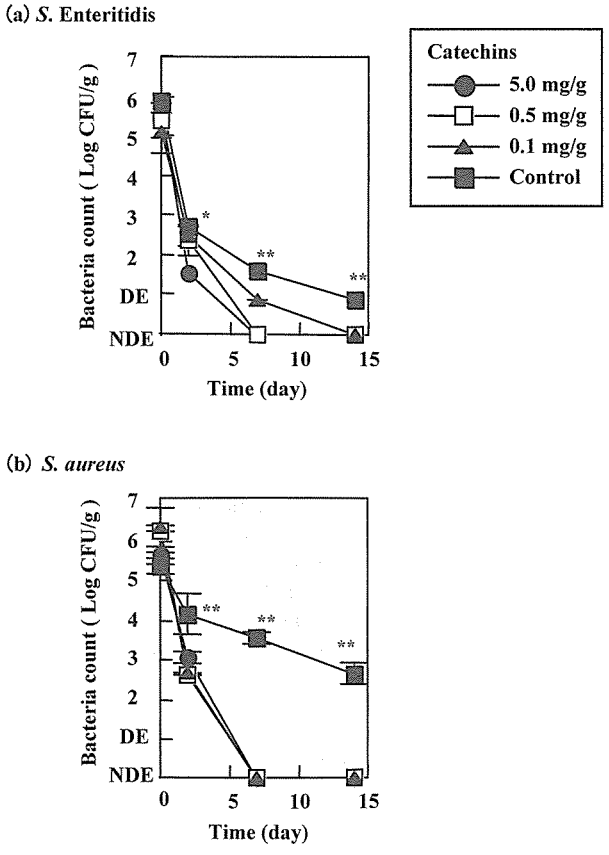

Fig. 3. Inhibitory effect on the growth of *S.* Enteritidis and *S. aureus* in corn flakes treated with catechin.

Bars represent standard errors. *Control *vs.* 0.1 and 0.5 mg/g: $p<0.05$, control *vs.* 5.0 mg/g: $p<0.01$; **Control *vs.* 0.1, 0.5 and 5 mg/g: $p<0.01$. DE: detected in enrichment culture but not detected by the plating method, NDE: not detected in enrichment culture.

Adzuki bean paste, shiratama dumpling and warabimochi treated with catechins at the concentration of 1 mg/g were inoculated with *S.* Enteritidis, *S. aureus* and *B. cereus*, and incubated at 4 and 25℃. In adzuki bean paste treated with catechins, *S. aureus*, *B. cereus* and *S.* Enteritidis were not detected by enrichment at 4 or 25℃ (Fig. 4a). Although *B. cereus* incubated for 3 days at 4℃ in shiratama dumpling grew in the enrichment broth, catechins significantly impaired the growth ($p<0.01$) (data not shown). The populations of *S. aureus* and *B. cereus* in warabimochi treated with catechins were undetectable by enrichment after having been incubated at 4 and 25℃ for a day ($p<0.01$) (Fig. 4b). Catechins in warabimochi were more effective than in adzuki bean paste and shiratama dumplings, although the reason is not known. The protein content of warabimochi is less than those of adzuki bean paste and shiratama dumplings. Some components of adzuki bean paste and shiratama dumplings might inhibit the activity of the catechins.

The growth of *S. aureus* in warabimochi at 25℃ was inhibited at day 3 (Fig. 4b) although *S. aureus* grew to *ca.* 5 log CFU/g in adzuki bean paste. This might indicate that the high concentration of sugar in adzuki bean paste promoted the growth of *S. aureus*. On the other hand, the population of *B. cereus* in warabimochi increased to *ca.* 5 log CFU/g after incubation for 1 day at

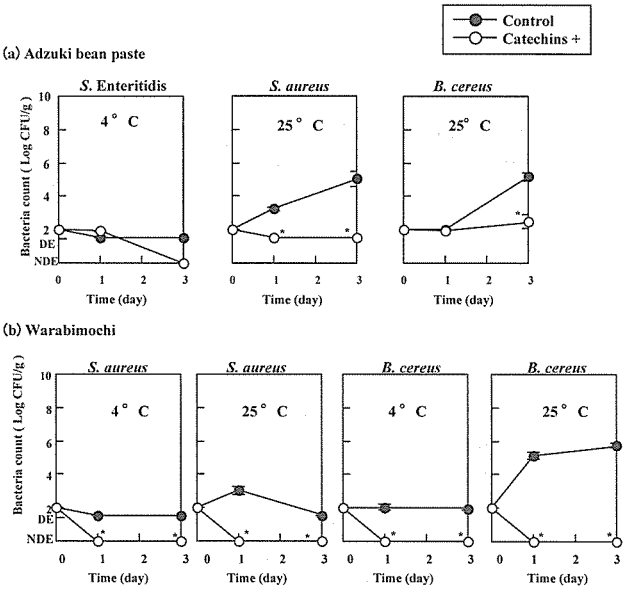

Fig. 4. Inhibitory effect of catechins on the growth of *S.* Enteritidis, *S. aureus* and *B. cereus* in cooked grain products.

(a) *S.* Enteritidis, *S. aureus* and *B. cereus* in adzuki bean paste, (b) *B. cereus* in shiratama dumpling after incubation for 3 days, (c) *S. aureus* and *B. cereus* in warabimochi. The concentration of catechins was 1 mg/g. Temperature was 4 or 25℃. Bars represent standard errors. *Control *vs.* catechins +: $p<0.01$. DE: detected in enrichment culture but not detected by the plating method, NDE: not detected in enrichment culture.

25℃, although that in adzuki bean paste did not change. Again, this might indicate that the high concentration of sugar in adzuki bean paste inhibited the growth of *B. cereus*.

In the present study, the antibacterial activities of catechins were examined in grain products during storage. The results suggest that catechins might be effective as a food additive. While there is no regulatory limit for catechins, the amount of catechins that can be added to food may be limited due to the change in the taste of the food. Because a synergistic effect between catechins and antibiotics was recently reported[21], a combination of catechins with other food additives might allow a decrease in the effective dose of food additives. The addition of catechins to food to control foodborne pathogens should be further investigated. In addition, grain flakes should be tested by enrichment, which is an effective procedure to detect small numbers of foodborne pathogens, because pathogens survive in grain flakes at a low contamination levels for a long time. Grain flakes also should be taken as soon as possible after pouring milk to avoid the possibility of growth of foodborne pathogens.

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