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Detection of Foodborne Pathogens and Analysis of Aflatoxin Levels in Home-made *Doenjang* Samples

− Research Note −

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Abstract

***Doenjang* is a traditional Korean fermented soybean product that provides a major source of protein. In this study, a total of 18 different home-made *doenjang* samples were examined for the presence of foodborne pathogens and the total aflatoxin levels. Using an enzyme-linked immunosorbent assay to assess microbial quality and potential public health risk, we showed that total coliform levels in the *doenjang* samples ranged from 0 to $4.43 \pm 2.32 \times 10^6$ CFU/g, and the maximum limit of *Bacillus cereus* was $4.67 \pm 2.0 \times 10^5$ CFU/g. However, other foodborne pathogens, such as *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *Salmonella* spp., were not detected among the tested samples. One of the samples (S3) showed a maximum limit of 42.2 ± 9.1 μg/kg for aflatoxin levels, which was above the safety limit allowed by the Codex Alimentarius Commission (CAC) regulatory agency. Further research is necessary to determine whether and how *doenjang* safety can be improved via elimination/reduction of microbial contamination during fermentation and storage or using microbial starter cultures for its fermentation.**

Key words: *doenjang*, aflatoxin, microbial quality, food safety

INTRODUCTION

Contamination of foods and feeds that may be hazardous to human and animal health is a world-wide problem. Specifically, bacterial contamination by foodborne pathogens is an important food safety issue. Rapid and accurate identification of bacterial pathogens isolated from food samples is important both for food quality assurance and for tracing the outbreaks of bacterial pathogens within the food supply.

Aflatoxins are a group of extremely toxic fungal metabolites produced by some species of *Aspergillus* during the growth of these fungi on foods and feeds. These toxins are considered significant threats to both human and animal health because they are potent carcinogens, teratogens and mutagens. Previously, Park et al. reported the presence of aflatoxin in barley and corn-based foods as well as in dairy products and traditional Korean soybean products such as *meju* (1). *Doenjang* is traditionally prepared by mixing and fermenting *meju*. The quality of the *doenjang* is affected by the microflora involved, the fermentation process and the basic ingredients used, which could be soybean or a combination of soybean and grains. In our previous study conducted on the safety issue of *doenjang* samples, we revealed that some of the *doenjang* samples contained biogenic amines (2), although at relatively lower levels than those for other kinds of soybean products, as discussed in previous reports (3).

Reddy et al. investigated the amount of aflatoxin in Indian soybean food samples, and these samples were found to be free from aflatoxin (4). Also it was reported that aflatoxin level in Egyptian soybean was found to be 5 to 35 ppb (5). In addition, Crane et al. hypothesized that the high incidences of stomach cancer in the Korean peninsula were probably due to the consumption of aflatoxin-contaminated *doenjang* products (6). There is an enormous use of fermented soybean foods, such as soy sauce and soybean paste, as part of everyday meals in Korea. Although soybeans themselves are not good substrates for the production of aflatoxins, some scientists have suspected that soy sauce and soybean paste may contain the toxins due to the microbial contamination of raw material used for the preparation of various soybean products (7). On the other hand, it has been also claimed that aflatoxins may be destroyed during the natural process of ripening, if they were present to begin with (8). Hence, in our continuous efforts to confirm the food safety of fermented food in Korea, we analyzed the level of aflatoxin in fermented soybean samples of *deonjang*.

The main objective of this study was to further identi-

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fy the presence of foodborne pathogens and to detect total aflatoxin levels in 18 fermented soybean samples of *doenjang*.

MATERIALS AND METHODS

Samples

A total of 18 Korean traditional fermented soybean paste samples (*doenjang*) were collected in March-April 2009 from various households, located in areas of Daegu-si and Gyeongsangbuk-do districts, Republic of Korea. A couple of samples were sold to the public. Collected samples were stored at 4°C until analysis. The fermentation period of all *doenjang* samples was approximately 1 year.

Detection and enumeration of coliforms

For the detection of coliforms, each *doenjang* sample (10 g) was homogenized with 90 mL of sterile water and diluted with peptone water to $10^1 \sim 10^6$ using a serial dilution method. One mL of each diluted sample was poured onto a desoxycholate lactose agar media (Difco, Sparks, MD, USA) using pour plate technique. The plates were incubated at 35°C for 24 hr. The numbers of colonies formed on the plates were counted as colony forming units (CFU) of sample. A single observed colony was streaked onto the eosin methylene blue (EMB) agar (Difco) and incubated at 35°C for 24 hr (9). Confirmatory identification tests were performed by streaking the observed colonies on nutrient agar (NA, Difco) plates and then the grown colonies were further confirmed by using Gram's staining techniques.

Detection and enumeration of *Bacillus cereus*

To analyze *B. cereus*, 10 g of each fermented soybean sample of *doenjang* was homogenized with a blender in 90 mL of sterile water and serially diluted to $10^1 \sim 10^6$ with KH_2PO_4 buffer solution (pH 7.2). Enumeration of *B. cereus* was performed by spreading 0.1 mL of each diluted sample ($10^1 \sim 10^6$) onto the surface of mannitol-egg yolk-polymyxin agar (MYP) media (Oxoid, Hampshire, England), and plates were incubated at 30°C for 24 hr (9). Confirmatory tests were performed by streaking the observed colonies on NA media and then the grown colonies were further confirmed by using an API 50 CHB kit (bioMerieux, Marcy l'Etoile, France).

Detection of *Salmonella* spp.

To identify *Salmonella* spp., each *doenjang* sample (10 g) was aseptically transferred in 90 mL of peptone water and incubated at 35°C for 18 hr. A 0.1 mL of aliquot culture was transferred into 10 mL of Rappaport Vassiliadis broth (Merck, Darmstadt, Germany) for the enrichment of bacterial growth, and then incubated at 42°C for 24 hr. Finally, 0.1 mL of exponentially grown culture was spread onto MacConkey agar plates (Becton, Dickinson and Company, Sparks, MD, USA) and incubated at 35°C for 24 hr (9). Typical colorless lactose non-fermenting colonies were taken as presumptive *Salmonella* spp. Confirmatory tests were done by streaking the observed colonies on NA media and then the grown colonies were further confirmed by using an API 20E kit (bioMerieux).

Detection of *Staphylococcus aureus*

For the identification of *S. aureus*, each *doenjang* sample (10 g) was aseptically transferred in 90 mL of tryptic soy broth (Becton, Dickinson and Company) with 10% NaCl and incubated at 35°C for 16 hr. Further, 0.1 mL proportion of each sample was spread onto the surface of Baird Parker (BP) agar (Oxoid) supplemented with egg yolk-tellurite emulsion (Oxoid) and incubated at 37°C for 24 hr. Colonies with typical *S. aureus* morphology (*i.e.*, black, convex with or without light halo on BP agar) were subjected to microscopic examination and confirmed by streaking the colonies on NA plates and then identified by using API Staph kit (bioMerieux).

Detection of *Escherichia coli* O157:H7

For *E. coli* O157:H7 detection, 10 g portions of each *doenjang* sample were transferred in 90 mL of modified *E. coli* broth with novobiocin (Merck) and incubated at 35°C for 24 hr. After 24 hr, 0.1 mL of each sample was spread onto Sorbitol MacConkey (SMAC) agar (Becton, Dickinson and Company) and incubated at 35°C for 24 hr. Typical colorless colonies formed on the SMAC agar plates were streaked onto EMB agar and incubated at 35°C for 24 hr. Blue-black colonies with a green metallic shine were taken as presumptive *E. coli* O157:H7 (9). Confirmatory tests were done by streaking the observed colonies on NA plates and then the grown colonies were further identified using API 20E kit (bioMerieux).

Determination of total aflatoxin

Extraction of aflatoxin from *doenjang* samples was performed according to the instruction of the producer (Neogen Corp., Lansing, MI, USA) supplied with the quantitative aflatoxin test kits (Veratox for total aflatoxin). A 10 g of each fermented soybean paste sample of *doenjang* was homogenized with 50 mL of 70% methanol for 2~3 min at room temperature, and filtrated with Whatman filter paper No. 2. The filtrates were stored in the refrigerator until they were assayed for the level of total aflatoxin in the tested samples of *doenjang* by Neogen Veratox kit. (Neogen Corp.) Each filtrate was applied to ELISA analysis without further purification. Optical densities of the developed colors were measured

in comparison to known concentrations of four reference standards by using Infinite M 200 ELISA reader (TECAN, Mannedorf, Switzerland) at 650 nm. Results were calculated using Neogen's Veratox data reduction software that converts the absorbance values into concentration values. All steps of experiment were repeated three times.

Statistical analysis

Data are presented as mean±standard deviation of three independent experiments.

RESULTS AND DISCUSSION

Detection and enumeration of coliforms

The detection of coliforms was confirmed using pour plate technique onto a desoxycholate lactose agar media. Various kinds of microorganisms present in foods or food products could be used to examine the microbiological quality and safety of foods. As shown in Table 1, among the analyzed samples of *doenjang*, coliform bacteria was found in the range of 0 to $(4.43\pm2.32)\times10^6$ CFU/g. Previous analysis of foodborne pathogens has also confirmed the presence of coliform bacteria in some food and food products (10). According to Gilbert et al., the detection limit range of >2, $2\sim3$ and <3 log CFU/g for coliform bacteria was found to be satisfactory, acceptable and unsatisfactory, respectively (11). Coliforms can influence food safety and preservation because these organisms are the indicators of fecal contamination. The detection of coliforms is widely used as a means of measuring the effectiveness of sanitation programs, indicating their presence as a substantially increased risk of the presence of pathogens (12). However, the presence of coliforms in 50% of the *doenjang* samples indicates the possibility of poor personnel hygiene, insufficient production conditions or cross-contamination in manufacturing process.

Detection and enumeration of *B. cereus*

Detection of *B. cereus* is important due to its ability to produce emetic toxins on food or food products stored at room temperature, which may cause gastrointestinal illness in human beings. In the present study, *B. cereus* colonies were observed on MYP agar plates in 6 tested *doenjang* samples namely S7, S9, S11, S13, S17 and S18 (Table 1). The limit of *B. cereus* in the tested *doenjang* samples was found in the range of 0 to $4.67\pm2.0\times10^5$ CFU/g (Table 1). As shown in Table 1, among the tested samples of *doenjang*, all the samples showed either no or little growth of *B. cereus* except 4 samples, S9, S13, S17 and S18, in which the detection limit of *B. cereus* was not in agreement with the threshold limit ($>10^4$ CFU/g) defined by the Korea Food and Drug Administration (KFDA) (9). Results of percentage confirmatory identification for the presence of *B. cereus* in 6 *doenjang* samples have been summarized in Table 2.

Detection of *E. coli* O157:H7, *Salmonella* spp. and *S. aureus*

E. coli O157:H7 is considered an important causative agent of diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (13). *S. aureus* is considered the third most important cause of disease in the world amongst

Table 1. Determination of foodborne pathogenic bacteria and aflatoxin in 18 Korean traditional (home-made) *doenjang* samples

Sample	Coliform (CFU/g)	*Bacillus cereus* (CFU/g)	*Staphylococcus aureus* (CFU/g)		*Escherichia coli* O157:H7 (CFU/g)		*Salmonella* spp. (CFU/g)		Total aflatoxin (μg/kg)
		MYP[2]	BP[3]	API test	SMAC[4]	API test	MA[5]	API test	
S1	$(1.33\pm0.25)\times10^4$	−	−	−	−	−	−	−	1 ± 0.03
S2	−[1]	−	−	−	−	−	−	−	8.7 ± 3.9
S3	−	−	−	−	−	−	−	−	42.2 ± 9.1
S4	−	−	−	−	−	−	−	−	2.1 ± 1.2
S5	−	−	−	−	−	−	−	−	1.3 ± 0.6
S6	−	−	−	−	−	−	−	−	−
S7	$(4.63\pm2.73)\times10^4$	$(2.0\pm0.57)\times10^3$	−	−	−	−	−	−	2.6 ± 1.1
S8	$(1.9\pm0.95)\times10^3$	−	−	−	−	−	−	−	−
S9	$(9.47\pm2.73)\times10^3$	$(1.27\pm0.57)\times10^4$	−	−	−	−	−	−	1.3 ± 0.5
S10	$(3.47\pm0.08)\times10^3$	−	−	−	−	−	−	−	−
S11	$(1.23\pm0.53)\times10^4$	$(6.67\pm0.62)\times10^2$	−	−	−	−	−	−	0.7 ± 0.02
S12	$(3.27\pm1.10)\times10^3$	−	−	−	−	−	−	−	−
S13	−	$(4.67\pm2.0)\times10^5$	−	−	−	−	−	−	0.6 ± 0.04
S14	$(4.43\pm2.32)\times10^6$	−	−	−	−	−	−	−	0.7 ± 0.01
S15	$(3.9\pm1.17)\times10^6$	−	−	−	−	−	−	−	1.6 ± 0.5
S16	−	−	−	−	−	−	−	−	1.5 ± 0.6
S17	−	$(2.67\pm1.10)\times10^5$	−	−	−	−	−	−	−
S18	−	$(1.67\pm0.57)\times10^5$	−	−	−	−	−	−	−

[1]Not detected. [2]Mannitol-egg yolk-polymyxin agar plate. [3]Baird-Parker agar plate. [4]Sorbitol MacConkey agar plate. [5]MacConkey agar plate.

Table 2. Identification of *Bacillus cereus* in *doenjang* samples using API test

Sample	API test		
	Identification	% ID	T Index
S7	*B. cereus*	94.8	0.74
S9	*B. cereus*	98.9	0.95
S11	*B. cereus*	99.6	0.95
S13	*B. cereus*	99.5	0.82
	B. mycoides	66.7	0.96
S17	*B. cereus*	30.3	0.91
	B. cereus	81.1	0.95
S18	*B. mycoides*	16.1	0.91

the reported foodborne illnesses (14). In Korea, 5.26% of the total cases of bacterial food poisoning caused by *S. aureus* were reported in the year 2009 (10). The United States Food and Drug Administration established that harmful doses of staphylococcal enterotoxin occurs at a population greater than 10^5 organisms per gram of contaminated food (15). *Salmonella* is an important worldwide foodborne pathogen, which is ubiquitously distributed in the environment and commonly colonizes the intestinal tract of animals, especially of poultry and swine, the major components of human food chain (16). In Korea, in the year 2009, the percentage of food poisoning cases caused by *Salmonella* spp. was estimated to be 7.46% (17).

In the present study, none of *doenjang* samples was found to contain *E. coli* O157:H7, *Salmonella* spp. or *S. aureus* (Table 1). The sensitivity of these pathogens to the salt content present in *doenjang* samples might explain their absence. Previously, Kim et al. reported that none of the tested soybean paste samples of various food products showed the presence of *Salmonella* spp. and *E. coli* O157:H7 (18).

Determination of total aflatoxin

Immunoassay tests for aflatoxin refer to the specific interaction between antibodies and aflatoxins, therefore, additional confirmation steps are not as important as with other methods. The advantages of the enzyme immunoassays are their ease of use and the short-time required for the analysis. The results for the quantitative analysis of aflatoxin levels, determined in 18 *doenjang* samples by Veratox total aflatoxin test kit (enzyme immunoassay) are presented in Table 1. In this study, the amount of total aflatoxin levels among the tested *doenjang* samples was found to be in the range of 0 to 42.2 $\pm$9.1 μg/kg. According to KFDA, the samples containing 10 μg/kg aflatoxin may be assumed to be hazardous (17), however, the Codex Alimentarius Commission (CAC), a joint FAO/WHO Food Standards Program, adopted a hazardous limit of 15 μg/kg for total aflatoxin (19). It was confirmed in this study that, with the exception of one of the samples (S3), the limit of total aflatoxin levels in the *doenjang* studied was found below the limit estimated by CAC. The variations in the levels of aflatoxin in food samples might be influenced by various factors, which include climate, region, moisture content, storage conditions and processing of the samples (20).

CONCLUSION

In this study, none of the tested *doenjang* samples showed the presence of foodborne pathogenic bacteria, including *S. aureus*, *E. coli* O157:H7 and *Salmonella* species. However, some of the tested samples showed the presence of coliforms and *B. cereus*. In addition, one of the tested *doenjang* samples (S3) contained aflatoxin with a maximum limit of 42.2$\pm$9.1 μg/kg. In future, a comparative analysis on various home-made *doenjang* samples from various locations in Korea could also be an interesting strategy to compare their quality characteristics and food safety.

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