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Isolation and Identification of Pathogenic Microorganisms from Soybean Sprouts

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Abstract

Raw soybean sprouts were tested for contamination with the following bacteria which have potential for pathogenesis or food spoilage: *Salmonella* spp., *Escherichia coli* O157:H7, *Yersinia enterocolitica*, *Vibrio parahaemolyticus*, *Aeromonas hydrophila*, *Plesidomonas shigeloides*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus*, *Clostridium perfringens*, *Campylobacter jejuni*, *Erwinia* spp., and *Fusarium* spp. Three of the above strains were isolated from the sprouts, and identified by morphological and biochemical methods including an API kit and ATB automated identification system. The isolate cultured in Cereus selective agar, a selective medium, was a Gram-positive, rod shaped, anaerobic spore former. The biochemical and culture tests revealed the following characteristics: catalase-positive, no growth on Simmon's citrate, NO_2 production and requirement of arginine for growth; the ATB automated identification system gave 99.8% agreement for the identification of *Bacillus cereus* to the species level. The isolate cultured in MacConkey agar selective medium was Gram-negative, rod shaped and a gas former; the ATB-system gave 99.9% agreement for the identification of *Aeromonas hydrophila* to the species level. The isolate found in Pseudomonas isolation agar was Gram-negative, rod shaped, cytochrome oxidase-positive, a reducer of nitrates to nitrogen, and pyocyanin producer; the ATB-system gave 99.9% agreement for the identification of *Pseudomonas aeruginosa* to the species level. These results indicate that the three bacteria species present in the soybean sprouts were *Bacillus cereus*, *Aeromonas hydrophila*, and *Pseudomonas aeruginosa*. *Salmonella* spp., *Escherichia coli* O157:H7, and *Yersinia enterocolitica*; which are associated with serious disease in humans, were not isolated from soybean sprouts examined in this study.

Key words: soybean sprouts, pathogenic microorganisms, isolation, identification

INTRODUCTION

Recently, the use of cook-chilled food has increased in hospital and institutional foodservices. This tendency is a result of the need for organizations to become more efficient by minimizing the costs associated with labor, equipment and energy by introducing new systems for efficient large scale food production. The production of cook-chilled food involves: pre-cooking preparation (washing, formulation, rehydration etc.), preparation of a partially cooked product, and heating in a hermetically sealed container until the food is completely cooked. The packaged product is then rapidly chilled and refrigerated until used. Foods prepared by these processes have been proven to be more palatable and nutritious than traditionally sterilized products (1).

One of the main problems and limitations associated with cook-chilled food is their relatively short shelf-life. However, shelf-life can be prolonged by improvements in hygiene and other methods to reduce microbial contamination that result in spoilage and pathogenesis. Microbial risk assessment is becoming a crucial procedure in the safety of foods. The presence of certain microorganisms can be used as an indicator of food safety and be useful for detection of incorrect processing. Detection and quantification of harmful microorganisms provide information on initial contamination, but further information will be necessary to determine which bacteria will remain at the point of consumption.

Soybean sprouts are a year around fresh vegetable and a popular food eaten at least once a week in Korea. Furthermore, soybean sprouts are a highly nutritive and economical food that helps prevent protein deficiency resulting from a rice-based diet (2). Therefore, cook-chilled products, combining other popular foods with soybean sprouts, might provide nutritious but economical diets with a minimum of cooking time and labor.

Although cook-chilled foods have become increasingly popular for reasons of their high sensory quality and convenience, little data is available on microbiological contam-

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ination of Korean cook-chilled foods. Therefore, raw soybean sprouts were investigated as a model of cook-chilled foodstuff and examined for the presence of pathogenic bacteria. Three strains, among fourteen pathogenic microorganisms tested, were isolated and identified by morphological and biochemical methods including an API kit and ATB automated identification system.

MATERIALS AND METHODS

Sampling

Fresh soybean sprouts were purchased from a foodservice company in Korea. Soybean sprouts were transported to the laboratory under low temperature (<7°C), stored at 4°C, and analyzed within 24 h.

Isolation of pathogenic microorganisms

Soybean sprouts were screened for the presence of 14 bacteria by culturing in differential media (Table 1), as a way of monitoring microbial contamination with pathogenic microorganisms. Each sample (10 g) was weighed and aseptically transferred into a sterile homogenizer jar containing 90 mL of 0.1% sterile peptone water and homogenized for 2 min at 11,000×g. Homogenates were plated onto the isolation medium and incubated as described in Table 1. Serial dilutions were performed if required.

For *Bacillus cereus*, the homogenate was prepared by heating to 80°C for 10 min, immediately cooling in cold water, inoculation onto Cereus selective agar (Merck Laboratories, Germany), and incubating for 24 hr at 30°C. The pink colonies on the Cereus selective agar were isolated and inoculated into tryptic soy agar (TSA) for 24 hr at 30°C. To detect *Aeromonas hydrophila*, the samples (25 g) were diluted with 225 mL alkaline peptone water (APW, pH 8.6) and homogenized for 2 min at 11,000×g. After the homogenate (10 mL) was cultured on 90 mL of APW (pH 8.6) at 30°C for 24 hr, the culture was plated onto MacConkey agar (Difco Laboratories, USA) at 30°C for 24 hr. For *Pseudomonas aeruginosa*, the homogenate (10 mL) was cultured on asparagine medium (in 90 mL; DL-asparagine 3.0 g, potassium phosphate dibasic 1.0 g, magnesium sulfate 0.5 g) at 35°C for 24 hr, and the positive cultures streaked onto Pseudomonas isolation agar with CFC selective agar supplement (Difco Laboratories, USA) for the detection of fluorescent pseudomonads. Finally, the isolated fluorescent colonies were cultured on a TSA plate and incubated at 35°C for 24 hr.

Identification of microbial isolates

Representatives of predominant colony types from each of the media were isolated and identified to the species level using conventional morphological and biochemical criteria, including Gram staining, cell shape, motility, catalase, oxidase, and oxidative or fermentative utilization of glucose (3).

The presence of *B. cereus* was further identified using the API 50 CHB, API 20 E kit, and ATB automated identification system (bioMerieux Co., Ltd., France). Additional tests for growth factor (arginine, lysine, ornithine, and tryptophane) requirements, growth on Simmon's citrate medium, and utilization of 2-keto gluconate were conducted according to Bergey's Manual of Determinative Bacteriology (3). For *A. hydrophila*, oxidase-positive colonies were incubated on KIA slant agar and MIL medium for 24 hr at 30°C, and then the colonies obtained were subcultured in TSA under the same conditions. The API 20 E kit and ATB identification system were used for further identification. Identification of *P. aeruginosa* was performed using the API 20 NE kit and ATB identification system. The isolated colonies which were pyocyanin producers and oxidase positive were inoculated onto KIA slant agar, SIM medium, and O-F medium at 35°C for 24 hr; hydrolysis of gelatin was observed after culturing it at 35°C for 2 weeks.

Table 1. Media and culturing conditions for isolation of pathogenic microorganisms in raw soybean sprouts

Microorganisms	Isolation conditions	Growth conditions
Salmonella spp.	Hektoen enteric agar, 35°C, 24 hr	Selenite F broth, 35°C, 24 hr
Escherichia coli O157:H7	Sorbitol MacConkey agar, 37°C, 24 hr	Modifed EC medium, 35°C, 24 hr
Yersinia enterocolitica	Yersinia selective agar, 35°C, 24 hr	Peptone sorbitol bile broth, 10°C, 10 days
Vibrio parahaemolyticus	TCBS agar, 35°C, 24 hr	APW (pH 8.6) with 2% sodium chloride, 35°C, 24 hr
Aeromonas hydrophila	MacConkey agar, 35°C, 24 hr	APW (pH 8.6), 30°C, 24 hr
Plesidomonas shigeloides	MacConkey agar, 35°C, 24 hr	APW (pH 8.6), 30°C, 24 hr
Pseudomonas aeruginosa	Pseudomonas isolation agar, 35°C, 48 hr	Asparagine broth, 35°C, 24 hr
Staphylococcus aureus	Mannitol salt agar with egg yolk, 35°C, 24 hr	Tryptic soy broth with 10% sodium chloride, 35°C, 24 hr
Listeria monocytogenes	Oxford agar, 30°C, 48 hr	Listeria enrichment broth, 30°C, 24 hr
Bacillus cereus	Cereus selective agar, 30°C, 24 hr	Tryptic soy broth, 30°C, 24 hr
Clostridium perfringens	Clostridium perfringens agar, 35°C, 24 hr	Cook meat medium, 35°C, 24 hr
Campylobacter jejuni	Abeyta-hunt agar, 35°C, 48 hr	HUNT medium, 35°C, 24 hr
Erwinia spp.	MAT, 30°C, 48 hr	Nutrient broth, 30°C, 48 hr
Fusarium spp.	Potato dextrose agar (pH 3.5), 25°C, 10 days	Potato dextrose broth, 25°C, 7 days

RESULTS AND DISCUSSION

Of the fourteen strains that can cause spoilage or be used as indicators of food safety, three strains were isolated from soybean sprouts and identified by morphological and biochemical methods as well as API identification kit and ATB automated identification system.

The isolate obtained from Cereus selective agar was a Gram positive, rod shaped, anaerobe and spore-former. The biochemical and cultural tests exhibited catalase-positive, no growth on Simmon's citrate, NO_2 production, and the requirement of arginine for growth; the ATB automated identification system gave 99.8% agreement for the identification of *B. cereus* to the species level (Table 2). The isolate from MacConkey agar selective medium was Gram negative, rod shaped, and a gas former; the ATB system gave 99.9% agreement for the identification of *A. hydrophila* to the species level (Table 3). The isolate from Pseudomonas Isolation agar was Gram-negative, rod shaped, cytochrome oxidase-positive, a reducer of nitrates to nitrogen, and a pyocyanin producer; the ATB system gave 99.9% agreement for the identification of *P. aeruginosa* to the species level. The colonies in Pseudomonas Isolation agar expressed a distinctive bluish green color (Table 4). On the basis of these results, the three bacterial contaminants were identified as *B. cereus*, *A. hydrophila* and *P. aeruginosa*. Despite the presence of these microorganisms, visible spoilage (such as maceration, soft-rot and discoloration) was absent in the raw soybean sprouts until stor-

Table 2. Identification of the isolate from raw soybean sprouts using Cereus selective agar

Characteristics	Results	Characteristics	Results
Shape	rod	Cellobiose	+
Gram stain	+[1)]	Maltose	+
Spore formation	+	Lactose	-
Cell diameter > 1.0 μm	+	Melibiose	-
Sporangium swollen	-	Saccharose	-
Spore shape	ellipsoidal	Trehalose	-
Spore position	central	Inuline	+
Catalase	+	Melezitose	-
Anaerobic growth	+	Raffinose	-
Egg-yolk lechthinase	+	Starch	+
Glycerol	+	Glycogen	+
Erythritol	-	Xylitol	-
D-Arabinose	-	Gentiobiose	-
L-Arabinose	-	D-Turanose	-
Ribose	+	D-Lyxose	-
D-Xylose	-	D-Tagatose	-
L-Xylose	-	D-Fucose	-
Adonitol	-	L-Fucose	-
β-Methyl-D-xyloside	-	D-Arabitol	-
Galactose	-	L-Arabitol	-
D-Glucose	+	Gluconate	-
D-Fructose	+	2-Keto gluconate	-
D-Mannose	+	5-Keto gluconate	-
L-Sorbose	-	Ortho-nitro-phenyl-galactoside	-
Rhamnose	-	Arginine	+
Dulcitol	-	Lysine	-
Inositol	-	Ornithine	-
Mannitol	-	Simmon's citrate	-
Sorbitol	-	Hydrogen sulfate	-
α-Methyl-D-mannoside	-	Urease	-
α-Methyl-D-glucoside	-	Tryptophane	-
N-Acetyl glucosamine	+	Indole	-
Amygdalin	+	Voges-Proskauer	-
Arbutin	+	Kohn's gelatin	+
Esculin	+	NO_2 production	+
Salicin	+		

[1)]+: Positive, -: Negative.

Table 3. Identification of the isolate from raw soybean sprouts using MacConkey agar

Characteristics	Results	Characteristics	Results
Shape	rod	β-Glucuronidase	-
Gram stain	-[1)]	Malonate	-
Gas from glucose	+	Indole	-
Motility	+	N-Acetyl-β-glucosaminidase	-
Hydrogen sulfide	-	β-Galactosidase	-
Ornithine decarboxylase	-	Glucose	+
Arginine dihydrolase	-	Saccharose	+
Lysin decarboxylase	-	L-Arabinose	+
Urease	-	D-Arabitol	-
L-Arabitol	-	α-Glucosidase	-
Galacuronate	-	α-Galactosidase	-
5-Ketogluconate	-	Trehalose	+
Lipase	+	Rhamnose	-
Phenol red	+	Inositol	-
β-Glucosidase	+	Celiobiose	+
Mannitol	+	Sorbitol	-
Maltose	+	α-Maltosidase	+
Adonitol	-	L-Aspartic acid arylamidase	-
Palatinose	-		

[1)]+: Positive, -: Negative.

Table 4. Identification of the isolate from raw soybean sprouts using Pseudomonas isolation agar

Characteristics	Results	Characteristics	Results
Shape	rod	Assimilation of arabinose	-
Gram stain	-[1)]	Assimilation of mannose	-
Pyocyanin production	+	Assimilation of mannitol	+
Reduction of nitrates to nitrogen	+	Assimilation of N-acetyl-glucosamine	+
Indole production	-	Assimilation of maltose	-
Acidification of glucose	-	Assimilation of gluconate	+
		Assimilation of caprate	+
Arginine dihydrolase	-	Assimilation of adipate	+
Urease	-	Assimilation of malate	+
Hydrolysis of esculin	-	Assimilation of citrate	+
Hydrolysis of gelatine	+	Assimilation of phenyl-acetate	-
β-Galactosidase	-	Cytochrome oxidase	+
Assimilation of glucose	+		

[1)]+: Positive, -: Negative.

age time was longer.

Salmonella spp., *Escherichia coli* O157:H7, and *Yersinia enterocolitica*, which are associated with human pathogenesis, were not isolated from the sample; an important observation for evaluating the survival and growth of pathogens that occasionally occur in raw and cook-chill products.

Most vegetables, including soybean sprouts, have a high moisture content which provides an environment conducive to microbial growth. Many bacteria capable of causing food-borne illness have been isolated from a wide range of salad vegetables (4). Dufrenne et al. reported that an average of 5~10% of reported food-borne diseases with known etiology are caused by *B. cereus* (5). *B. cereus* emerged as an important food-poisoning organism because of its cosmopolitan distribution. *Bacillus* spp. was reported to be the dominant aerobic bacteria in cooked, pasteurized and chilled vegetable foods (6,7). *Aeromonas* spp. is also responsible for spoilage and has been cultured from green salad, coleslaw (8), pre-made salad samples (9), fresh asparagus, broccoli and cauliflower (10), and commercial mixed vegetable salads (11). Furthermore, *Pseudomonas* species and epiphytic strains of Enterobacteriaceae are primarily found in the microflora of fresh vegetables under ambient conditions (12).

The presence of *Listeria monocytogenes* on raw vegetables has been documented by several investigators (13-15). In contrast to this study, several microorganisms capable of causing human illness such as *Escherichia coli*, *Salmonella* spp. and *Staphylococcus aureus* have been isolated from vegetables (16,17). Also, the large number of total microorganisms, contamination indicators (*E. coli* and coliforms) and pathogens (*S. aureus* and *B. cereus*) detected in vegetarian food samples revealed that their presence presented a potential health hazard to consumers (18).

The microorganisms mentioned above are widely distributed in soil (19,20) and are therefore potential contaminants of vegetables. In the same sense, Kaneko et al. (21) proposed that intact vegetables may be contaminated with non-faecal coliforms of soil origin and suggested that *E. coli* detected in fresh vegetables might be derived from the environment of food factories and from contact with interior surfaces of equipment that are difficult to clean (22). Vegetables are exposed to various sources of contaminations, in addition to those encountered during their processing. The incidence of *S. aureus*, *B. cereus*, *E. coli* and coliforms were found to be the highest in unpackaged foods, and lowest in vacuum-packed vegetarian foods (18).

Pathogens, when present on raw vegetables, may not be fully eliminated by commercial washing procedures, and heating can have the unintended effect of reducing or removing naturally competitive organisms, allowing pathogens to grow more rapidly. For instance, *Bacillus* and *Clostridium* spp. produce heat-resistant spores that can survive exposure to 100°C for more than 5 min (23).

Although *B. cereus*, *A. hydrophila*, and *P. aeruginosa* were isolated as the major species in raw soybean sprouts, we previously determined that they were readily killed by a blanching treatment (100°C for 8 min) used in the cook-chill process (data not shown). However, the cook-chill process does not always prevent their growth, because they are able to grow at refrigeration temperatures (10,24,25). Therefore, cook-chill products must be protected by a combination of preservation techniques and stored under controlled chill temperatures to prevent growth of pathogenic and spoilage organisms (26). Also, it is very important to prepare sanitation procedures and standards for vegetable preparation using microbiological monitoring with careful attention to determining appropriate indicator organisms according to the storage temperature and period. Accordingly, further study is required to confirm the safety of vegetables, including soybean sprouts, for use in cook-chill foods.

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