Journal of Food Protection, Vol. 48, No. 5, Pages 438-441 (May 1985)

Evaluation of the Microbiological Safety of Tempeh Made from Unacidified Soybeans

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(Received for publication October 3, 1984)

ABSTRACT

Studies were done to evaluate the safety of tempeh made from unacidifed soybeans and inoculated with different bacterial pathogens. Pathogens were added to either the soybeans before fermentation by *Rhizopus oligosporus* or the tempeh after fermentation and steaming. In the latter method, the inoculated products were incubated at several different temperatures (5, 10, 15 and 25°C). *Clostridium botulinum* (types A and/or B) toxin was produced in 2 d during the fermentation and within 5 d at 25°C or 4 wk at 15°C in tempeh inoculated and incubated in vacuum packages after fermentation and steaming. *Staphylococcus aureus* grew very well (>6-$\log_{10}$ CFU/g increase) in 2 d during the fermentation, and grew from ca. 10^3 CFU/g to 10^8 CFU/g in 7 d at 25°C and 21 d at 15°C in tempeh inoculated after fermentation and steaming. Staphylococcal enterotoxins were detected in some of these samples. *Salmonella typhimurium* also grew well during the fermentation (>6-$\log_{10}$ CFU/g increase in 1 d), but grew relatively slowly at 25 and 15°C in tempeh inoculated after fermentation and steaming. *Yersinia enterocolitica* grew very well (>6-$\log_{10}$ CFU/g increase) in 1 d during the fermentation, and also grew well in tempeh inoculated after fermentation and steaming, with a >6 $\log_{10}$ CFU/g increase in 2 d at 25 or 15°C and 5 d at 10°C. Results of these studies indicate the need for maintaining: (a) a high level of sanitary practices during production and (b) good refrigeration (≤5°C) of the product following fermentation until it is used.

Tempeh is usually made of soybeans fermented with *Rhizopus oligosporus (9)*, although it could be made of other grains (*6*). The beans are cooked, mixed with spores of *R. oligosporus,* and incubated at about 30°C until dense, white mycelia of the mold grow and bind the beans together to make a cake- or patty-like product (*6,9*). Tempeh is one of the most important soybean foods in Indonesia (*6*) and is becoming increasingly popular in the United States. Tempeh is so easy to make that it is commonly made at home. Commercially, in the United States, the fermented tempeh is fried or steamed, and packaged in plastic film.

Because tempeh is made of cooked whole soybeans, which is a very nutritious medium, it is quite susceptible to microbial growth. In the United States, tempeh has an expected shelf life of several weeks if handled properly. However, the product is often displayed on a produce counter with little refrigeration or sometimes on a display table without refrigeration. Little is known about the microbiological safety of tempeh during production or at the retail and consumer level. The purpose of this study was to determine the ability of foodborne bacterial pathogens to grow or produce toxin in tempeh during production and when the heat-treated, packaged product is held at different storage temperatures.

MATERIALS AND METHODS

Bacterial cultures and methods of enumeration

Four foodborne bacterial pathogens were studied. These included: *Clostridium botulinum, Staphylococcus aureus, Salmonella typhimurium* and *Yersinia enterocolitica.*

A ten-strain spore mixture of *C. botulinum,* consisting of five strains each of types A and B (*8*) in water, was used. Immediately before inoculating tempeh, the spores were heat-shocked at 80°C for 15 min. Immediately after inoculation, *C. botulinum* (three samples per batch) was enumerated using a 5-tube most probable number (MPN) method with Trypticase-peptone-glucose-yeast extract broth as the growth medium (*4*).

S. aureus strains 196 E (enterotoxin A and D producer) and 361 (enterotoxin C_2 producer) were kindly provided by M. S. Bergdoll, University of Wisconsin-Madison. Cultures were stored in brain heart infusion (BHI; Difco) agar slants at 4°C. For each experiment, cultures were grown overnight in BHI broth at 37°C, harvested by centrifugation, washed and resuspended in 0.01 M phosphate-buffered saline, pH 7.5 (PBS). The cell suspensions were diluted appropriately with PBS and mixed to give an equal number mixture of the two strains. *S. aureus* was enumerated by plate count on Baird-Parker agar (Difco) incubated at 37°C for 48 h. Typical *S. aureus* colonies (black, shiny and convex surrounded by clear zone) were counted and randomly chosen isolates were confirmed for coagulase activity.

S. typhimurium strains 40252 and 40467 were obtained from the Wisconsin State Laboratory of Hygiene, Madison, WI. Each strain was stored on Trypticase soy agar (TSA; BBL Microbiology Systems) at 4°C. For each experiment, cultures were grown overnight in Trypticase soy broth (TSB;BBL Microbiology Systems) at 37°C. Cells of each strain were harvested by centrifugation, washed and resuspended in PBS, then diluted and mixed

to give an equal number mixture of the two strains. Hektoen enteric agar (Difco) was used for enumeration of *S. typhimurium (24 h at 37°C)*. Randomly selected colonies were confirmed as *S. typhimurium* by agglutination with the appropriate antisera (Difco).

Y. enterocolitica strains 34/1B and FRI-YE16 were used. *Y. enterocolitica* 34/1B (serotype 0:8), kindly provided by C. C. G. Aulisio, Food and Drug Administration, Washington, DC, was originally isolated from unchlorinated water used in the manufacture and packaging of tofu which caused food-poisoning incidents (*1*). *Y. enterocolitica* FRI-YE16 (serotype 0:3) is a virulent strain originally isolated from a porcine tongue (*2*). Cultures were stored on TSA slants at 4°C, and for each experiment, overnight cultures were grown in TSB at 25°C. Cells of each strain were harvested by centrifugation, washed and resuspended in PBS, then an equal number mixture of the two strains was made. Cells were enumerated on yersinia selective agar base containing yersinia selective supplement (Oxoid) incubated at 25°C for 48 h. Randomly selected colonies were confirmed as virulent *Y. enterocolitica* by agglutination with antiserum (WA-SAA; *3*). Biochemical characteristics of these isolates were determined by the API-20E diagnostic system (Analytab Products, Plainview, NY).

Preparation of tempeh

Tempeh was prepared according to a procedure described by H. L. Wang, Northern Regional Research Center, U.S. Department of Agriculture, Peoria, IL (personal communication), which is as follows. Dry, cracked soybeans were soaked in cold tap water for ca. 30 min, then washed and floating hulls were removed. The washed soybeans were boiled on an electric stove in about three volumes of water for 30 min. During this time, hulls that floated to the surface were removed with a ladle. The cooked soybeans were then drained, spread as a thin layer (1 to 2 cm thick) over a double layer of sterile cheesecloth, covered with another double layer of sterile cheesecloth, and excess moisture was removed by blotting with paper toweling. The cooked beans were allowed to cool to ca. 37°C. A freeze-dried spore preparation of *R. oligosporus* NRRL 2710 (obtained from C. W. Hesseltine, Northern Regional Research Center, U.S. Department of Agriculture, Peoria, IL; *10*) was mixed with the soybeans to obtain an inoculum level of ca. 2.2×10^7 spores/kg of dry beans (1 kg of dry beans produced ca. 2 kg of cooked and drained beans). The inoculated beans (ca. 60 g of cooked, drained soybeans per petri dish) were then packed tightly in plastic petri dishes (100×15 mm, Falcon Labware, Becton Dickinson Labware, Oxnard, CA). The inoculated soybeans were then incubated at 30°C until white mycelia grew to cover the entire surface of the soybean patty, but not enough growth to allow the mold to produce black spores. It generally took about 24 to 28 h for the mycelia to cover the entire surface. The petri dishes were not stacked during fermentation because during mycelial growth the temperature of tempeh rises and will not dissipate if petri dishes are stacked. An excessive increase in temperature results in slow growth or even death of mycelia.

Inoculation and incubation of tempeh with pathogens

Two methods were used to inoculate the test organisms and incubate the inoculated tempeh. *Method A* involved inoculating cooked soybeans with both washed cells of the test bacteria (each type of pathogen was tested individually) and spores of *R. oligosporus,* and incubating soybeans in petri dishes at 30°C for a maximum of 3 d. During this time *R. oligosporus* grew, and after ca. 36 h parts of the tempeh became black indicating the development of some *Rhizopus* spores. *Method B* consisted of spraying test bacteria on fermented, steamed tempeh patties, vacuum packaging the tempeh in an oxygen impermeable film (Curwood, Inc., New London, WI; oxygen transfer rate less than 0.5 $cm^3/1$ atm/100 cm^2), and incubating at several different temperatures. Details of the procedure are as follows. Tempeh patties (covered by *R. oligosporus* mycelia following ca. 24 to 28 h of incubation at 30°C) were removed from petri dishes, wrapped in sterile aluminum foil, and steamed in a chamber with flowing steam for 30 min. The steamed patties were cooled to room temperature and inoculated with the test bacteria (each type of pathogen was tested individually) by an aerosol delivered through a plant sprayer. The sprayer was adjusted to discharge an average of 0.2 ml of aerosol per application. For safety considerations, tempeh was inoculated by the aerosol procedure in a disposable glove box. The inoculated patties were individually vacuum packaged with a laboratory vacuum packaging unit (Model 24 V, Packaging Aid Corp., San Francisco, CA), and incubated at 5, 10, 15 or 25°C.

Analysis of samples

An entire patty of tempeh was blended for 2 min with two volumes of double distilled water using a Stomacher Lab Blender (Model 400, Seward UAG, London). For samples inoculated with *C. botulinum,* the blended tempeh was centrifuged ($5{,}000 \times g$, 5 min) and the supernatant fluid was used for the mouse toxicity assay (*4*). Samples inoculated with other organisms were blended, diluted appropriately in PBS, and plated. Triplicate samples were analyzed for each treatment at each sampling interval, and an average of these results was reported.

Some samples containing greater than 10^6 cells of *S. aureus*/g were tested for the presence of staphylococcal enterotoxins (A, C_2 and D) by an enzyme-linked immunosorbent assay (*5*). These samples were stored frozen (-20°C) until enterotoxin assays were performed.

RESULTS

C. botulinum toxin was produced in tempeh within 2 d during the fungal fermentation (Method A; Table 1). When tempeh was inoculated after steaming and incubated in vacuum pouches (Method B), botulinal toxin was produced within 5 d at 25°C, 4 wk at 15°C, and not at all by 6 wk at 5 or 10°C (Table 2).

S. aureus grew in tempeh during the fungal fermentation, with about a 5- and 6-$\log_{10}$ CFU/g increase in 1 and 2 d, respectively (Table 1). Furthermore, staphylococcal enterotoxins were detected in both the 2- and 3-d samples (Table 1). When *S. aureus* was applied to steamed tempeh after fermentation, the organism grew well at 25°C (ca. 3-$\log_{10}$ CFU/g increase in 2 d), slowly at 15°C (ca. 3-$\log_{10}$ CFU/g increase in 2 wk), and poorly at 5 and 10°C (ca. 1-$\log_{10}$ CFU/g increase throughout 6 wk of incubation). Small amounts (<1.8 ng/g) of enterotoxins were detected in samples incubated at 15°C for 2 wk and at 25°C for 1 wk (Table 3).

S. typhimurium grew extremely well during the fermentation of tempeh, with a 6-$\log_{10}$ CFU/g increase in 1 d (Table 1). The organism did not grow as well when applied to tempeh after fermentation and held at 5 to

25°C (Table 4). Greatest growth (ca. 4.5-$\log_{10}$ CFU/g increase) occurred in 1 wk at 25°C and little growth (<1-$\log_{10}$ CFU/g increase) occurred by 6 wk at 5°C.

Y. enterocolitica grew prolifically both during the fermentation of tempeh (>6-$\log_{10}$ CFU/g increase in 1 d; Table 1) and on steamed, fermented tempeh held at 10, 15 or 25°C (>6-$\log_{10}$ CFU/g increase in 5, 2 and 2 d, respectively; Table 5). The organism grew well, but not rapidly, on tempeh at 5°C (>4-$\log_{10}$ CFU/g increase in 1 wk).

In general, all of the inoculated samples held at 15 or 25°C for more than 1 week were putrid.

DISCUSSION

All of the pathogens evaluated grew well during the fungal fermentation of tempeh, indicating that tempeh may be unsafe if any of these pathogens are present when fermentation begins. Fortunately, neither botulinal toxin nor staphylococcal enterotoxin was produced within 24 h of fermentation, the time by which the fermentation of tempeh is often completed. However, both toxins were present in samples at 48 h of fermentation, indicating that such toxins may develop in tempeh produced by a slow, delayed or excessively long fermentation. Hence, it is imperative that tempeh be produced under aseptic conditions, using a high level of sanitary practices.

Of particular concern, botulinal toxin was produced in tempeh within 2 d during the fermentation. Care should be taken to eliminate and avoid contamination of soybeans with *C. botulinum* spores. The risk of the presence of *C. botulinum* can be reduced by thoroughly washing soybeans before use and using sanitary practices at all stages of tempeh preparation.

If soybeans are boiled in water for 30 min, this should be sufficient heat treatment to kill *Salmonella* spp., *S. aureus,* and *Y. enterocolitica*. However, contamination of cooked soybeans with such pathogens by a tempeh-maker can easily occur if good personal hygiene and proper sanitary practices are not used. Care should also be taken to properly sanitize equipment used in the production of tempeh.

Although some tempeh-makers do not acidify soybeans before the fungal fermentation, an acidifer, such as acetic or lactic acid, is often added to soybeans used to produce tempeh in the United States (*6*). The soybeans are soaked and then cooked in acidified water. Perhaps the presence of acid would inhibit the growth of pathogens. However, during fermentation the mold produces ammonia to raise the pH of tempeh to about pH 6.5 to 7.0 (*6,7*), which are ideal conditions for growth of many microbial pathogens. Considering the potential foodborne disease hazard that exists during the fermentation of tempeh produced from unacidified soybeans, a study is needed to evaluate the microbiological safety of tempeh produced from acidified soybeans.

Results of our study also indicate that steamed, vacuum-packaged tempeh is a nutritious medium and an appropriate environment for the growth of microbial patho-

TABLE 1. *Toxin production by* Clostridium botulinum *and growth of* Staphylococcus aureus, Salmonella typhimurium *and* Yersinia enterocolitica *in tempeh during fermentation (Method A).*

Organism	Incubation time (d)			
	0	1	2	3
	No. of toxic samples/No. of samples tested			
C. botulinum[a]	0/3	0/3	1/3	3/3
	Log_{10} (CFU)/g			
S. aureus	1.98	6.85	8.20[b]	8.75[c]
S. typhimurium	1.60	8.32	9.41	9.54
Y. enterocolitica	1.90	8.18	8.45	8.49

[a]Inoculum of 139 *C. botulinum* spores per g of tempeh.
[b]Staphylococcal enterotoxin (SE) A was detected but was less than 1.8 ng/g; SE C_2 was more than 30 ng/g; and SE D was not detected.
[c]SE A was detected but was less than 1.8 ng/g; SE C_2 was more than 30 ng/g; and SE D was detected but was less than 1.8 ng/g.

TABLE 2. *Toxin production by* Clostridium botulinum *in vacuum-packaged tempeh inoculated after fermentation and steaming (Method B)*[a].

Temperature	No. of toxic samples/No. of samples tested at:								
(°C)	0 d	2 d	5 d	1 wk	2 wk	3 wk	4 wk	5 wk	6 wk
5	0/3	-[b]	-	0/3	0/3	0/3	0/3	0/3	0/3
10	-	-	-	0/3	0/3	0/3	0/3	0/3	0/3
15	-	-	-	0/3	0/3	0/3	1/3	3/3	3/3
25	-	0/3	3/3	3/3	3/3	-	-	-	-

[a]Inoculum of 51 *C. botulinum* spores per g of tempeh.
[b]-, not tested.

TABLE 3. *Growth of* Staphylococcus aureus *in vacuum-packaged tempeh inoculated after fermentation and steaming (Method B).*

Temperature	Log_{10} of *S. aureus*/g at:								
(°C)	0 d	1 d	2 d	1 wk	2 wk	3 wk	4 wk	5 wk	6 wk
5	3.34	3.65	3.92	4.79	4.52	4.52	4.68	5.00	3.95
10	3.34	4.34	3.83	5.18	4.32	4.28	4.76	4.26	4.38
15	3.34	3.68	4.90	5.48	6.30[a]	8.00	5.83	7.36	6.18
25	3.34	4.89	6.23	8.04[b]	5.04	7.83	7.00[c]	6.40	6.26

[a]Staphylococcal enterotoxin (SE) A, SE C_2 and SE D were detected but were less than 1.8 ng/g.
[b]SE A was not detected; and SE C_2 and SE D were detected but both were less than 1.8 ng/g.
[c]SE A and SE D were not detected; and SE C_2 was detected but was less than 1.8 ng/g.

TABLE 4. *Growth of* Salmonella typhimurium *in vacuum-packaged tempeh after fermentation and steaming (Method B).*

Temperature	Log_{10} of *S. typhimurium*/g at:									
(°C)	0 d	1 d	2 d	5 d	1 wk	2 wk	3 wk	4 wk	5 wk	6 wk
5	4.23	2.80	3.32	2.48	2.69	3.30	2.63	4.00	4.36	5.00
10	4.23	3.34	3.68	6.54	6.11	4.30	4.89	4.87	4.61	4.91
15	4.23	3.34	4.43	6.26	7.54	6.49	7.08	8.28	6.20	7.40
25	4.23	5.28	5.95	5.77	8.89	8.04	8.26	6.69	7.26	5.74

TABLE 5. *Growth of* Yersinia enterocolitica *in vacuum-packaged tempeh after fermentation and steaming (Method B).*

Temperature	Log_{10} of *Y. enterocolitica*/g at:									
(°C)	0 d	1 d	2 d	5 d	1 wk	2 wk	3 wk	4 wk	5 wk	6 wk
5	1.58	3.76	4.34	5.30	5.86	6.32	7.84	6.08	7.73	7.60
10	1.58	5.46	7.04	8.32	8.80	9.32	9.40	8.60	7.60	8.83
15	1.58	7.20	8.57	9.15	8.88	8.63	9.26	9.23	9.23	9.15
25	1.58	7.72	8.70	8.86	8.90	8.74	8.96	7.41	6.94	4.30

gens. Hence it is imperative to: (a) handle and package heat-treated tempeh in a pathogen-free environment and (b) hold the product under refrigerated conditions (≤5°C). Tempeh producers, retailers and consumers should be aware of this concern and take appropriate measures to prevent microbiological health risks.

ACKNOWLEDGMENTS

We thank Drs. C. W. Hesseltine and H. W. Wang, Northern Regional Research Center, U.S. Department of Agriculture, Peoria, IL, for their helpful assistance and advice. We also thank Dr. M. S. Bergdoll and Ms. Mary Evenson, Food Research Institute, University of Wisconsin-Madison for the cultures of *S. aureus* and staphylococcal enterotoxin assays.

This work was supported by U.S. Department of Agriculture agreement No. 48-519B-2-1173; the College of Agricultural and Life Sciences, University of Wisconsin-Madison; and by contributions to the Food Research Institute.

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