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Effect of environmental conditions during soya-bean fermentation on the growth of *Staphylococcus aureus* and production and thermal stability of enterotoxins A and B

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During tempe manufacture, *Staphylococcus aureus* was able to survive in newly started soya-bean soaks (final pH 4.7), whereas it died during soaks with accelerated souring (final pH 4.0). No *S. aureus* were detected after subsequent heat treatment of soaked beans. *S. aureus* strain S6 could produce enterotoxins A and B if inoculated on cooked soya-beans. Its growth and enterotoxin production were not influenced by inoculation rate nor by the presence of actively growing *Rhizopus oligosporus*, the fungus responsible for tempe formation. Enterotoxin levels were highest after 48 h fermentation. Presence of lactobacilli (*L. brevis* or *L. plantarum*) during the tempe fermentation did not influence staphylococcal growth, but enterotoxin levels were reduced. Reduced oxygen pressure did not affect staphylococcal growth, but resulted in lower enterotoxin levels after 48 h incubation. The thermal stability of enterotoxin A in tempe was significantly lower than in Brain Heart Infusion broth of comparable pH. Heating of enterotoxin-containing tempe mash reduced enterotoxin A by 99.7% as measured with ELISA and animal feeding methods.

Key words: *Staphylococcus aureus* growth; Staphylococcal enterotoxins; Thermal stability; Tempe; Soya-bean; Fermented products

Introduction

Tempe is a legume- or cereal-based food obtained by fungal solid-state fermentation. Soya-bean tempe originated in Indonesia and presently meets an increasing interest in other regions as well. The manufacturing process has been reviewed extensively (Ko and Hesseltine, 1979). It includes soaking dehulled soya-beans in water overnight, followed by boiling or steaming, discarding the boiled water,

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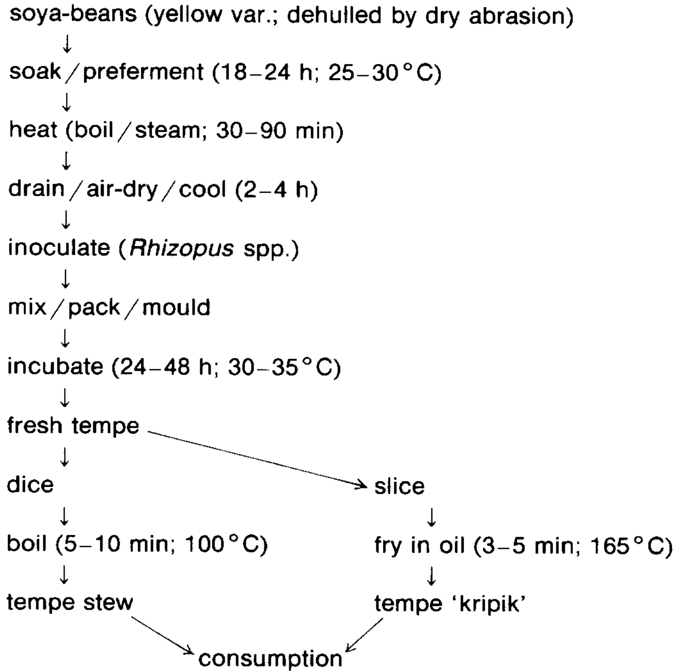

Fig. 1. Tempe manufacture and preparation for consumption.

allowing the beans to air-dry and cool, inoculating them with spore preparations of *Rhizopus* spp., usually *R. oligosporus*, and fermenting the inoculated beans in perforated containers (Fig. 1). The resulting fresh tempe is a firm mass of beans and fungal mycelium which has not sporulated due to the limited oxygen supply. This product has a limited shelf-life and should be prepared for consumption the same day or be kept refrigerated. Preparation prior to consumption consists of heating, e.g. boiling in stew or frying in oil.

The nutrient composition, high water activity and neutral pH of fresh soya-bean tempe provide a suitable environment for large numbers of bacteria and yeasts accompanying the fungal mycelium. A survey of commercial tempe (Samson et al., 1987) revealed the presence of *Bacillus cereus* and *Staphylococcus aureus* (> 4 log CFU/g in 12% and 16% of all samples, respectively).

B. cereus was shown (Nout et al., 1987a) to grow rapidly to 8–9 log CFU/g tempe made from non-acidified beans. On the other hand, its growth was inhibited if beans had been acidified to pH $\leqslant 4.85$ by previous lactic acid fermentation.

This paper presents data on growth and production and stability of enterotoxins of *S. aureus* during the various stages of tempe manufacture and preparation. It was observed that growth and production of enterotoxin occurs during processing of *S. aureus* inoculated soya-beans. However, the enterotoxin produced was inactivated during heat treatment.

Materials and Methods

Micro-organisms

Staphylococcus aureus: strain 1 was isolated by us from commercial tempe; 196E (producer of staphylococcal enterotoxin A (SEA), FRI913 (SEA), S6 (SEA + SEB), FRI722 (SEB) and 14458 (SEB) were obtained from the collection of the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands. Strains were maintained on Nutrient Agar slants (Oxoid CM3) at 4° C, after having been incubated 2 days at 37° C. Inoculation of food samples was carried out with young cultures in Brain Heart Infusion broth (BHI) (Gibco, Scotland, No. 152-0680) which had been incubated 2 days at 37° C.

Rhizopus oligosporus: strain NRRL5909 was used. Cultivation, and preparation of spore suspensions were carried out as described previously (Nout et al., 1987a).

Lactobacillus brevis and *L. plantarum* which had been isolated previously (Nout et al., 1987b) from fermenting soya-beans were grown in de Man, Rogosa and Sharpe broth (MRS; Gibco, Scotland, No. 152-3250) for 24 h at 25° C.

Test samples

Soya-bean soaks: freshly started soaks and soaks with accelerated souring were carried out as described previously by Nout et al. (1987b) as soak for spontaneous fermentation and soak with recycling procedure, respectively. Inoculation with a 24 h 37° C BHI culture of *S. aureus* was carried out at the start of the soaking operation.

Cooked soya-beans and tempe: for tempe manufacture, we used the method as published previously (Nout et al., 1987b) employing a soak for spontaneous fermentation. If cooked soya-beans were to be tested, they were transferred aseptically into sterile petri dishes after cooling to ambient temperature, giving a single layer of beans to prevent heat and mass transfer limitations. Inoculation was carried out by adding appropriate dilutions of *R. oligosporus, S. aureus, L. brevis* or *L. plantarum* cultures to the beans, followed by mixing with a sterile spoon. Incubation was at 30° C for 48 h. The influence of reduced oxygen pressure was studied by incubation in jars flushed with a gas mixture of 98% N_2 and 2% O_2 humified by percolation through sterile distilled water at a flow rate of 5 l/h. The O_2 concentration in the exhaust gas was $> 90\%$ of that of the mixture entering the jars. Production of enterotoxin in tempe was carried out by inoculating the cooked soya-beans with *R. oligosporus* and *S. aureus* during the tempe manufacturing process. Fresh, contaminated tempe (40 g) was homogenized for 2 min in a Colworth type 400 Stomacher (Seward & Co., London) with 80 ml PBST (mix 0.07 M NaH_2PO_4 solution in 0.15 M NaCl with 0.07 M Na_2HPO_4 in 0.15 M NaCl to obtain pH 7.2, add 0.05% Tween 20, sterilize 15 min 121° C) prior to experimental heat treatment. Addition of staphylococcal enterotoxin A to non-contaminated fresh tempe was carried out by homogenizing 75 g tempe, 75 ml PBST, and 750 μg enterotoxin A for 2 min in a Colworth type 400 Stomacher to obtain a mash suitable for experimental heat treatment.

Heat treatments

Heat treatments were carried out at 80, 100, and 125°C in a thermostat-controlled glycerol bath in sealed 5 mm inner diameter borosilicate glass tubes (wall thickness 1 mm) containing 3 ml or 3 g mashed sample. Temperatures were monitored using copperconstantane thermocouples placed in the centre of the sample with a centering device.

Analysis

Microbiological analysis

Sample preparation: representative samples of 10.0 g were homogenized for 2 min with 90 ml of sterile peptone saline using a Colworth type 400 Stomacher. Decimal dilution series were prepared using tubes with 9 ml of sterile peptone saline.

S. aureus: surface counts on Baird-Parker agar (BP, Gibco, Scotland, No. 152-0320) sterilized 15 min at 121°C supplemented with 10 ml filter-sterilized 0.1% K_2TeO_3, and 50 ml 10 kGy γ-irradiated 10% egg yolk suspension in 0.85% NaCl respectively, per 940 ml of basal medium. Incubation 2 days at 37°C. All black colonies with or without white margin and clear halo were counted as *S. aureus*. Occasionally, confirmations were performed by carrying out coagulase tests (Bacto coagulase plasma EDTA, Difco, 083-46-5).

R. oligosporus: surface counts on Rose Bengal Chloramphenicol Chlortetracyclin Agar with increased rose bengal concentration according to Baggerman (1981). Incubation 2–3 days at 30°C.

Lactobacilli: pour plate counts with overlay in Rogosa agar (LBS agar BBL No. 11327, Becton Dickinson & Co.) supplemented with 0.2% natamycin (Delvocid, Gist-Brocades, Delft, The Netherlands) to suppress the growth of yeasts. Incubation 3 days at 30°C. Counts were corrected by microscopic confirmation of $\sqrt{n}$ randomly chosen colonies.

Reporting of counts: as previously described (Nout et al., 1987a).

Staphylococcal enterotoxins: the amounts of enterotoxins A and B were determined using a direct sandwich ELISA procedure, as described by Notermans et al. (1983a). Microtiter wells were coated with 0.1 ml immunoglobin (IgG) solution in phosphate buffered saline (PBS: mix 0.07 M NaH_2PO_4 solution in 0.15 M NaCl with 0.07 M Na_2HPO_4 in 0.15 M NaCl to obtain pH 7.2, sterilize 15 min at 121°C) for 17 h at ambient temperature on an orbital shaker at 60 rpm. IgG-anti-SEA: 3–5 μg/ml PBS; IgG-anti-SEB: 10 μg/ml PBS (monoclonal as well as polyclonal sets of IgGs and conjugates were used). Rinse 3 times with PBST. Add to each well 0.1 ml sample, diluted in PBST. Incubate at ambient temperature at 60 rpm for 1.5 h. Rinse 3 times with PBST. Add to each well 0.1 ml IgG-peroxidase conjugate (IgG = Po), dissolved in PBST (optimum concentration was determined by checkerboard titration). Incubate as with sample. Rinse as above, 3 times. Add 0.1 ml enzyme substrate (0.08% 5-aminosalicylic acid in dist. water, pH 6.0, to which 20 μl 30% H_2O_2 was added) and read extinction at 450 nm after 30 min using an ELISA-reader (EAR 400, SLT-Labinstruments, Austria). Each microtiter plate

included 6 blank wells and 6 wells containing known concentrations of pure enterotoxin. Quantification was based on interpolations on a dose-response calibration curve which was re-adjusted for each plate. Detection levels (99% significant difference from mean of blanks) of both enterotoxins A and B was 0.8 ng/ml.

Biological activity of enterotoxin A: two cynomolgus monkeys (*Macaca fascicularis*) of 2–2.5 kg body weight were each fed 20 ml supernatant of heat treated tempe mash as described by Notermans et al. (1987).

Results and Discussion

During a non-sterile traditional tempe manufacturing process, the first operation is the soaking of the beans. Variations notwithstanding, each newly started soak will be dominated by Enterobacteriaceae and later by lactic acid bacteria; the pH will decrease from approx. 6.5 to values between 4 and 6, usually between 4.5 and 5.5. However, pH values ⩽ 4.0 are obtained during soaking with accelerated souring, achieved by recycling of soak water (Nout et al., 1987b). Table I shows that *S. aureus* is able to survive in newly started soaks, whereas it dies during soaks with accelerated souring. This is in line with the earlier finding (Notermans and Heuvelman, 1983b) that *S. aureus* can grow at pH ⩾ 4.6.

After boiling, draining, air-drying and cooling of the soaked beans, their microbiological content depends very much on processing and handling conditions. During our laboratory experiments we could not detect the presence of *S. aureus* in cooked beans using surface plate count on Baird-Parker medium.

During the inoculation, mixing and moulding stages of the manufacturing process, the practically sterile cooked beans are subjected to chance contaminations from equipment, handling, substandard inoculum, etc. In addition, during the fungal fermentation phase the growth and enterotoxin production of *S. aureus* could be expected to be influenced by several factors, i.e. the initial pH and organic acids

TABLE 1

Growth of *Staphylococcus aureus* strain 1 during soya-bean soaking (soya-beans 1 kg + tapwater 3 kg; soaking 18 h at 25 °C)

Initial contamination with *S. aureus* strain 1 (log CFU/g)	Final pH of soak water	*S. aureus* in soaked beans (log CFU/g)
Freshly started soak with spontaneous fermentation:		
not inoculated (control)	4.9	2.86
6.76	4.7	7.10
Soak with accelerated souring using recycling [a]:		
not inoculated (control)	4.0	< 2.70
4.52	4.0	< 2.70

[a] Nout et al. (1987b).

present in the beans; the presence of competing micro-organisms (Notermans et al., 1984); and the reduced availability of oxygen (Barber and Deibel, 1972). In order to eliminate growth limitations which might arise from the use of beans soaked with accelerated souring, we used beans from freshly started soaks in all following experiments, unless stated otherwise.

The production of staphylococcal enterotoxins (SEA and SEB) of *S. aureus* strains 196E (SEA), FRI913 (SEA), S6 (SEA and SEB), FRI722 (SEB) and 14458 (SEB) was tested and confirmed in BHI cultures and on chicken meat. On cooked soya-beans, strains 196E and FRI913 did not produce enterotoxin. Strain S6 was selected for further experiments because of its production of both SEA and SEB on soya-beans.

Table II shows that there is no significant effect of the rate of contamination with *S. aureus* strain S6 on its growth in beans without or with *R. oligosporus*. Also the growth of *R. oligosporus* is not influenced by the presence of growing *S. aureus*. Storage (5 days at 7 °C) of the 48 h fermented samples did not significantly affect the numbers of viable *S. aureus* and *R. oligosporus* (data not shown). Staphylococcal enterotoxins A and B produced by *S. aureus* strain S6 could be detected already after 24 h. Their quantities were quite variable. From the results, no significant effect could be observed of initial *S. aureus* contamination rate or presence of *R. oligosporus*. In most samples the enterotoxin A and B content was highest after 48 h fermentation. Six fermented samples were analyzed after 5 days storage at 7 °C; 3 samples had significantly lower SEA levels after storage (8, 3 and 128 ng/g) compared to their SEA levels before storage (208, 74 and 670 ng/g, respectively). Other SEA or SEB levels were not affected.

Table III summarizes the effect of *Lactobacillus brevis* and *L. plantarum* during the fungal fermentation stage. Both strains had been isolated earlier (Nout et al., 1987b) from soaks with accelerated souring; *L. plantarum* was shown to contribute to the quality of fresh tempe. The presence of *S. aureus* strain S6 and *R. oligosporus* alone (Table III, treatment B) resulted in staphylococcal growth and enterotoxin production comparable to the data in Table II. The additional presence and growth of *L. brevis* (Table III, treatment C) and *L. plantarum* (Table III, treatment D) did not significantly influence staphylococcal growth; the pH of the product was slightly reduced after 48 hours. Nevertheless, the presence of the lactobacilli resulted in lower enterotoxin A and B levels. As yet, insufficient data are available to speculate on the role of the lactobacilli in the reduction of enterotoxin levels in fermented tempe.

Whereas the above experiments had been carried out under aerobic atmospheric conditions, the influence of reduced oxygen availability occurring in the centre of fermenting tempe mass (Rathbun and Shuler, 1983) was investigated in an incubator flushed with 98% N_2 + 2% O_2 gas mixture. Table IV shows that under conditions of reduced oxygen pressure, growth of *S. aureus* strain S6 was not inhibited, but considerably less enterotoxin A was produced. This is in line with the findings of Barber and Deibel (1972). From the above it is concluded that the presence of lactobacilli and a reduced oxygen transfer during the tempe fermentation will result in reduced enterotoxin levels in *S. aureus* contaminated tempe.

TABLE II

Growth and production of enterotoxins A (SEA) and B (SEB) by *Staphylococcus aureus* strain S6 on cooked soya-beans, as influenced by its initial inoculation rate and the presence of *Rhizopus oligosporus*

Initial inoculation with *S. aureus* (S6) (log CFU/g)	Presence of *R. oligosporus*	Incubation Period at 30 °C							
		24 h				48 h			
		R. oligosporus (log CFU/g)	*S. aureus* (log CFU/g)	SEA (ng/g)	SEB (ng/g)	*R. oligosporus* (log CFU/g)	*S. aureus* (log CFU/g)	SEA (ng/g)	SEB (ng/g)
2.00		– [a]	7.03	ND [b]	ND	–	8.29	3	ND
2.00	+ [c]	3.00	5.92	–	56	5.63	6.48	26	–
2.81	+	3.00	8.44	102	124	5.65	8.08	208	–
2.88		–	9.40	64	88	–	9.99	350	72
3.00	+	3.13	9.21	269	166	4.72	8.74	74	2
3.80		–	5.00	48	24	–	10.32	54	170
3.96	+	4.00	5.00	54	118	5.88	8.68	101	78
4.54		–	9.68	104	36	–	9.96	670	111
5.91		–	9.66	85	69	–	10.07	–	216
6.02	+	4.00	9.52	169	131	5.44	9.32	203	158
7.77	+	3.00	9.31	280	135	5.72	9.36	394	415
8.01		–	9.98	104	109	–	10.11	192	598

[a] – = not determined.

[b] ND = not detectable (<1 ng/g).

[c] + = inoculation rate 5.00 log CFU/g beans.

TABLE III

Influence of lactobacilli on the growth and enterotoxin A (SEA) and B (SEB) production of *Staphylococcus aureus* strain S6 on cooked soya-beans in the presence of *Rhizopus oligosporus*

Inoculum	Incubation Period at 30 °C									
	24 h					48 h				
	Lactobacilli (log CFU/g)	*S. aureus* (log CFU/g)	pH beans	SEA (ng/g)	SEB (ng/g)	Lactobacilli (log CFU/g)	*S. aureus* (log CFU/g)	pH beans	SEA (ng/g)	SEB (ng/g)
A. *R. oligosporus* [c]	– [a]	–	6.35	ND [b]	ND	–	–	6.50	ND	ND
B. *R. oligosporus* + *S. aureus* [d]	–	9.21	6.35	43	88	–	8.74	6.50	80	72
C. *R. oligosporus* + *S. aureus* + *L. brevis* [e]	7.69	8.57	6.37	8	72	8.36	8.50	6.40	29	3
D. *R. oligosporus* + *S. aureus* + *L. plantarum* [f]	7.45	8.59	6.30	6	77	8.96	8.66	6.12	27	4

[a] – = not determined.
[b] ND = not detectable (<1 ng/g).
[c] *R. oligosporus* 5.00 log CFU/g beans.
[d] *S. aureus* 2.91 log CFU/g beans.
[e] *L. brevis* 3.54 log CFU/g beans.
[f] *L. plantarum* 3.61 log CFU/g beans.

TABLE IV

Influence of atmospheric oxygen concentration on growth and enterotoxin A (SEA) production by *Staphylococcus aureus* strain S6 on cooked soya-beans in the presence of *Rhizopus oligosporus* (incubation 30 ° C, 48 h).

Inoculum	21% O_2 (air)		2% O_2	
	S. aureus (log CFU/g)	SEA (ng/g)	*S. aureus* (log CFU/g)	SEA (ng/g)
R. oligosporus [c]	– [a]	ND [b]	–	ND
R. oligosporus + *S. aureus* [d]	9.15	360	9.17	30

[a] – = not determined.
[b] ND = not detectable (<1 ng/g).
[c] *R. oligosporus* 5.00 log CFU/g beans.
[d] *S. aureus* 3.71 log CFU/g beans.

Staphylococcal enterotoxins have been reported to be rather heat stable (Tatini, 1976). Therefore, the effect of heat treatments such as applied during tempe preparation prior to consumption was investigated. Table V shows the thermal resistance of 100 ng/ml pure enterotoxin A added to BHI and heated in the same medium. The medium was buffered to have pH values comparable to those occurring in fresh tempe (Nout et al., 1985). Higher pH levels tended to increase SEA thermal resistance.

The thermal resistance of similar initial levels of enterotoxins A and B produced by *S. aureus* strain S6 was examined in tempe (final pH 6.75). Enterotoxin B had a higher thermal resistance than enterotoxin A; as was reported elsewhere (Tibana et al., 1987). However, we observed a drastic effect of the heating medium on the thermal stability. In tempe, 1 min at 80 ° C was sufficient to inactivate enterotoxin A fully and enterotoxin B for 92%. After heating for 1 min at 100 ° C the enterotoxins were not detectable (< 1 ng/g).

The above enterotoxin determinations had been carried out using monoclonal ELISA antibody-sets (Mab). If heat-induced artefacts would have occurred at epitope level, the use of Mab might result in erroneously low enterotoxin results. To eliminate such chances, the experiments were repeated with mashed tempe to which

TABLE V

Influence of pH on thermal stability of enterotoxin A added to BHI; SEA levels as ng/ml

Heat treatment	pH		
	6.0	6.5	7.4
None (control)	100	100	100
80 ° C, 10 min	25	35	47
100 ° C, 10 min	22	34	40
125 ° C, 10 min	1	8	– [a]

[a] – = not determined.

TABLE VI

Influence of 10 min heating at 100 °C on 5000 ng/ml enterotoxin A (SEA) homogenized in tempe mash

	SEA (ng/ml) [a]	
Before heating:		
tempe mash	4860	recovery 97.2%
supernatant of tempe mash	4860	
After heating:		
tempe mash	12	residual enterotoxin 0.3%
supernatant of tempe mash	10 [b]	

[a] Determined by polyclonal ELISA antibody-sets.
[b] 20 ml supernatant fed gastro-intestinally, failed to cause vomiting in enterotoxin A-sensitive *M. fascicularis*.

5000 ng/ml pure enterotoxin A had been added. Enterotoxin A was now determined using monoclonal as well as polyclonal ELISA antibody-sets. Table VI summarizes the results of the polyclonal test which were identical to those obtained with Mab. Similar extensive heat inactivation of enterotoxin A was observed as previously.

In order to rule out other artefacts which might have interfered with the functioning of both monoclonal and polyclonal ELISA tests, the supernatant of the heated tempe mash was administerd to two monkeys. No vomiting occurred whereas they vomited approx. 2 h after subsequent administration of 20 μg enterotoxin A.

We regard these findings as strongly indicative for the efficient heat inactivation of enterotoxins in tempe. In view of possible application in other food systems, further research on the mechanism(s) of this inactivation continues and is focused on the influence of proteolytic activities of *R. oligosporus* and accompanying microflora (e.g. lactobacilli) in tempe. In particular, it might be possible that the inactivation of enterotoxins as a result of lactobacilli (Table III) is accelerated during the initial warming-up phase of the heat treatment of fresh tempe.

In conclusion, it has been shown that although staphylococcal enterotoxins can be produced during the manufacture of tempe, environmental conditions i.e. accompanying microflora and reduced oxygen concentration inhibit their formation, whereas heat treatments prior to consumption can be expected to adequately inactivate their toxicity.

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