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PAPER

Microbiological safety of kinema, a fermented soya bean food

M.J.R. Nout*, D. Bakshi and P.K. Sarkar

Kinema is a fermented soya bean food of Nepal and the hilly regions of North-eastern States of India. Generally, the fermentation is dominated by Bacillus spp. that often cause alkalinity and desirable stickiness in the product. The present study was undertaken in a limited number of commercial (market) kinema samples to test for the presence of foodborne pathogens and their properties. Bacillus cereus was present in numbers exceeding 10^4 cfu/g product in five of the tested 15 market samples. Enterobacteriaceae and coliform bacteria exceeded 10^5 cfu/g in 10 of the 15 samples. Escherichia coli exceeding 10^5 cfu/g was found in two samples. Staphylococcus aureus was not detected in any of the tested samples. Of 31 isolated typical and atypical strains of B. cereus, 18 representative strains were tested qualitatively for the ability to produce diarrhoeal type enterotoxin (BCET) using an Oxoid BCET-RPLA test kit. Overall, BCET was formed by 12 strains in BHIG (brain heart infusion broth +1% glucose), by seven strains on sterilized cooked rice, and by five strains on sterilized cooked soya beans. Semi-quantitative tests on BCET revealed that levels exceeding 256 ng/g soya beans, produced by single pure culture inoculation with the isolated B. cereus strains, were reduced to ≤ 8 ng/g by frying kinema in oil, a common procedure when making kinema curry. It was also shown in a mixed pure culture experiment that a kinema strain, B. subtilis DK-W1, is able to suppress growth and BCET formation by a selected toxin producing strain (BC7-5) of B. cereus. It is concluded that the traditional way of making kinema and its culinary use in curries is safe. However, for novel applications of kinema, safety precautions are advisable. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: kinema; alkaline fermented food; *Bacillus cereus*; *B. subtilis*; *Staphylococcus aureus*; Enterobacteriaceae; enterotoxin; safety

INTRODUCTION

Kinema is a solid-substrate soya bean food, obtained by bacterial fermentation. Dominant bacteria in tradi-

tional kinema include *Bacillus subtilis* and *Enterococcus faecium*. Production processes are not uniform and depend on locally available materials, fuel, and tradition. A traditional process observed near Gangtok in Sikkim, also described by Sarkar *et al.* (1994), consists of overnight soaking of small-sized 'local yellow' variety of soya beans, followed by a 4-h cooking on a wood fire, draining of the water, transfer of cooked beans into a wooden mortar followed by moderate mashing with addition of small quantities of wood ash, transfer of the grits into bamboo baskets lined with jute-bags and a layer of

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fresh fern (*Athyrium* sp.) leaves. At this stage the beans are still quite hot (50–60°C). A package is made by folding closed the ferns and sack-cloth, and fermentation is allowed to take place for 1–2 days depending on ambient temperature and habit. A combination of the porous fern leaves and the dimensioning of the bean mass are of importance in ensuring adequate aerobic condition during the fermentation. In Kalimpong (Darjeeling district, West Bengal) we observed that modernized versions of the process are used, including omission of soaking, use of household-scale pressure cookers to cook the beans, mashing of cooked beans in the cooking vessel rather than a wooden mortar, omission of ash addition, different leaf linings (eg *Macaranga pustulata* King, Fam. Euphorbiaceae, Nepali: malata; *Ficus hookeriana* Corner, Fam. Moraceae, Nepali: nevara; or *Bauhinia vahlii* Wt. and Arnott, Fam. Caesalpinaceae, Nepali: bhorla) and different dimensioning of the bean package.

Traditional kinema is characterized by its neutral to alkaline pH, stickiness and pungent ammoniacal odour. It is customarily used to prepare kinema curry, by frying fresh kinema in vegetable oil for 3–5 min, and addition of tomato, onion, spices, salt and a little amount of water. Although its pH may vary from neutral to alkaline, kinema can in principle be classified as an alkaline fermented food (Steinkraus, 1995), like West African dawadawa, Japanese natto or Thai thua-nao. Studies including investigation of the process and its optimization, and major chemical and microbiological compositional changes during the fermentation, have been carried out earlier (Sarkar and Tamang, 1995; Sarkar *et al.*, 1996, 1997a,b).

B. subtilis strains are always present in high numbers in the freshly fermented product. This species has also been associated with incidents of foodborne disease (Kramer and Gilbert, 1989) related to unheated food such as ropy bread. As no cases of illness have been associated with the consumption of kinema curry we assume that, in principle, the process of curry preparation by frying in oil yields a safe product.

Earlier investigations of neutral-to-alkaline fermented foods, eg tempe, revealed the presence of considerable levels of potentially objectionable microorganisms such as *Bacillus cereus*, *Staphylococcus aureus* and members of Enterobacteriaceae (Samson *et al.*, 1987). In dawadawa, the presence of *B. cereus* was observed by Antai and Ibrahim (1986). This organism was encountered in a wide range of Indian food items (Varadaraj *et al.*, 1992; Shah *et al.*, 1996), but kinema was not tested. *Bacillus cereus* is associated with emetic and diarrhoeal types of food poisoning (Granum, 1994; Andersson *et al.*, 1995).

Kinema-making provides cash income to rural hill populations. It is of interest to upgrade the status of traditional kinema in order to increase its market and profitability. It is conceivable that kinema could also be used as a novel food enrichment or flavouring

ingredient. The aim of the present work is to contribute towards the understanding of the agents limiting the consumer safety of traditional kinema. In particular, the investigation was focused on the occurrence and properties of *B. cereus*, *S. aureus* and Enterobacteriaceae. The investigation was of limited scope and was not designed to compare the impact of different process modifications.

MATERIALS AND METHODS

Organism

The kinema strain, *Bacillus subtilis* DK-W1 (MTCC1747), is available from the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. It was grown and maintained on nutrient agar (NA) (Cat. No. MM012, HiMedia Laboratories Pvt Ltd, Mumbai, India).

Sampling and sample treatment

At market sites of Kalimpong and Darjeeling in West Bengal, samples (100–150 g) of kinema were purchased, and collected in sterile sampling bags. These were transported to the laboratory in an ice-box, and analyzed as soon as possible (within 24 h). Representative 10 g portions were aseptically weighed and blended (Seward Stomacher, type 400) with 90 ml peptone–physiological saline (0.1% neutral peptone, 0.85% NaCl). Serial decimal dilutions were prepared with the same diluent, and in all cases, duplicate counting plates were prepared of all appropriate dilutions.

Microbiological analysis

Bacillus cereus: Selective enumeration was carried out on spread-plates of BCSA (*Bacillus cereus* selective agar base, HiMedia M833; with appropriate additions of polymyxin B, Oxoid SR099E and egg yolk emulsion, HiMedia FD045). The inoculated plates were incubated at 30°C for 24–48 h. Characteristic turquoise to peacock blue colonies surrounded by a zone of precipitate of the same colour were regarded as presumptive *B. cereus*. A representative number (usually five per plate counted) were isolated and purified on BCSA, followed by NA. Confirmation was on the basis of endospore formation (4 day-old culture on NA), fermentation of glucose (+), xylose (–) and arabinose (–) in stab cultures of purple agar base (Difco, Detroit, USA, Cat. 0228-17-7) to which 1% w/w had been added of, respectively, D(+)-glucose (Merck, Darmstadt, Germany, Cat. 1.08346), D(+)-xylose (Merck 8689) and D(–)-arabinose (Merck 1494). Ability to grow (–) at 50 ± 0.5°C was tested on slants of NA.

Staphylococcus aureus: Selective enumeration was carried out on spread-plates of BP (Baird-Parker agar base, HiMedia M043; with appropriate additions of

potassium tellurite 3.5%, Oxoid SR030J; and egg yolk emulsion). After incubation at 30°C for 24–48 h, presumptive and/or dubious colonies were confirmed using Staphytest Plus agglutination kit (Oxoid DR850M).

Enterobacteriaceae: Sample dilutions in TSB (tryptone soya broth, HiMedia M011) were allowed resuscitation on thin TSA (tryptone soya agar, HiMedia M290) plates for 1–2 h at room temperature (27°C), followed by a thick overlay of VRBG (violet red bile glucose agar w/o lactose, HiMedia M581) and incubation at 30°C for 20 h. Representative colonies (three per counted plate) were isolated and purified on NA. Confirmation was done on 24 h-old NA-cultures by testing for cytochrome oxidase (Identification Sticks Oxidase, Oxoid BR64) and fermentation of glucose in stab culture (same as for *B. cereus*).

Coliforms: Confirmed strains of Enterobacteriaceae were inoculated on BGGB (brilliant green bile broth 2%, HiMedia M121) with inverted Durham tubes. Incubation at 30°C for 24–48 h was followed by examination for growth and gas formation.

Escherichia coli: Same as for coliforms, but incubated at $43.8 \pm 0.3^\circ\text{C}$.

Chemical analysis

Dry matter: Total and water-soluble dry matter were determined by conventional oven-drying (105°C, 48 h) of 10 g fresh sample, or 10 g of supernatant obtained by centrifugation (20 min at 900g) and filtration (Schleicher and Schuell 595 $\frac{1}{2}$) of a homogenate of 10 g sample in 50 ml distilled water that had soaked for 10 min.

pH and free amino nitrogen: An accurately known weight of about 5 g sample was homogenized with distilled water to a total volume of 20 ml. Of this suspension, the pH was measured using a calibrated conventional glass/calomel electrode (accuracy 0.05 pH units). On a magnetic stirrer, the pH was subsequently increased to pH 8.50 using 0.1 M NaOH. After addition of 10 ml 35% formaldehyde (Merck, Mumbai, India, Art. 17808) the suspension was stirred for 2 min, and the released protons were titrated using 0.1 M NaOH until an end-point of

pH 8.50. Free amino nitrogen was calculated on the basis of stoichiometric equivalence of 1 NaOH and 1 amino group.

Enterotoxin

B. cereus diarrhoeal enterotoxin was detected using the BCET-RPLA (reversed passive latex agglutination) kit (Oxoid TD950) as directed in the manual. For toxin production tests, the following substrates were used: BHIG (brain heart infusion broth, HiMedia M210 with 1% w/v glucose), cooked polished rice and cooked soya beans (small-sized, local yellow variety). The BHIG was used as a shaking culture (75 ml in 250 ml conical flask; approx. 200 rpm). The previously cooked rice and soya beans were deposited separately in 250 ml Erlenmeyer flasks (25 g per flask) and autoclaved (20 min, 121°C).

Incubation for toxin production was during 24 h at room temperature for BHIG, and during 1–2 days at 30°C for rice and soya beans. Experiments and analyses were done in duplicate.

Statistical analysis

Bivariate correlation coefficients were calculated using SPSS for Windows, release 6.0.

RESULTS AND DISCUSSION

Factors such as water activity (affected by dry matter content and level of solutes) and pH (affected by acids as well as ammonia, amines etc.) can determine chances of survival or proliferation of microbial food contaminants (Sutherland *et al.*, 1996). We observe from *Table 1* that the chemical composition of the tested samples shows wide ranges of variation, especially for water-soluble dry matter, pH and free amino nitrogen. Some six market kinema samples had pH values below that of cooked soya beans, indicating that acidification, rather than alkalification, had dominated the biochemical modification. Possibly lactic acid bacteria eg *Enterococcus faecium*, can dominate when the growth of *Bacillus* spp. is

Table 1 Kinema and its processing stages: chemical characteristics of ecophysiological relevance*

Type of sample	Number of samples tested	Total dry matter (% w/w)	Water-soluble dry matter (% of total dry matter)	pH	Free amino nitrogen (mm/g dry matter)
Raw, rinsed soya beans (processing stage)	1	39.9	28	6.55	0.25
Cooked soya beans (processing stage)	2	36.8 (35.4–38.1)	19	6.45 (6.40–6.50)	0.23 (0.19–0.26)
Fresh kinema (market samples)	12	39.1 (35.8–44.0)	25.6 (14.0–34.0)	6.25 (4.90–7.25)	1.19 (0.40–1.74)
Sun-dried kinema (market samples)	3	84.0 (80.2–87.2)	20.3 (16.0–23.0)	6.80 (5.70–7.50)	0.25 (0.17–0.33)

*Data represent the means, with ranges in parentheses.

Table 2 Occurrence of *B. cereus*, *S. aureus* and Enterobacteriaceae in 18 samples of kinema

Micro-organisms	Log cfu/g fresh weight							
	<2	2-3	3-4	4-5	5-6	6-7	7-8	8-9
<i>B. cereus</i>	13 ^{a,b}			2		1	1	1
<i>S. aureus</i>	18							
Enterobacteriaceae	4	1	1	2	2	2	5	1
Coliforms	1	1		1	2	1		1
<i>E. coli</i>	3				1			1

^a Number of samples containing less than 10² *B. cereus*/g, etc.

^b All data refer to confirmed counts.

restricted due to, for example, lack of oxygen. There is an urgent need to understand the mechanisms of these variations, in order to facilitate control of quality and safety. In particular, it will be of interest to evaluate the impact of some of the modifications of the traditional process that were observed.

Table 2 summarizes the distribution of the tested micro-organisms. Whereas *S. aureus* could not be detected in any of the kinema samples, *B. cereus* was present in relatively high numbers in five of the 18 samples. Also, Enterobacteriaceae (coliforms as well as non-lactose fermenting species) occurred widely in market samples of kinema. Even though *S. aureus* was not detected, it would be of interest to understand the reason for its absence. Possibly the lack of initial contamination, or the impact of competition and/or antagonistic reactions would have prevented proliferation. Earlier work with *S. aureus* in the tempe fermentation would suggest that *S. aureus* will not produce significant levels of its enterotoxins in the presence of a competitive microflora (Nout *et al.*, 1988). The presence of high numbers of Enterobacteriaceae in fresh kinema could be considered as undesirable: kinema could serve as a vehicle for contamination of other foods by cross-contamination routes.

Statistical analysis revealed no significant correlations between any two of the following variables: total dry matter content, water-soluble dry matter content, pH, free amino nitrogen, length of fermentation period that the product had undergone, and levels of *B. cereus* and Enterobacteriaceae, respectively. This indicates that other parameters determine the observed levels of *B. cereus* and Enterobacteriaceae. Probably, the initial contamination as well as the chances for proliferation (competition, oxygen, and inhibitory substances) are key aspects. It follows that directing the course of the process towards lower *a_w* or higher pH values would not significantly contribute to the suppression of these groups of bacteria.

Presumptive *B. cereus* isolates obtained from BCSA (distinctive turquoise to peacock blue colonies surrounded by a zone of precipitate of the same colour) were differentiated according to Kramer and Gilbert (1989). Gram-positive rod-shaped bacteria, able to form endospores and to ferment glucose, but

unable to ferment xylose and arabinose and unable to grow at 50°C were considered *B. cereus*. Of 29 presumptive strains, 10 were thus confirmed as *B. cereus*. Qualitative tests on *B. cereus* enterotoxin (BCET) production showed that most of the confirmed *B. cereus* strains were able to produce it in BHIG and on cooked rice. A lower number of strains was also able to produce BCET on cooked soya beans. The relatively low water-soluble carbohydrate level in cooked beans may prevent some strains from producing BCET; a stimulatory effect of maltodextrin on enterotoxin formation was observed elsewhere (Rowan and Anderson, 1997). All but one strain (BC7-3) producing BCET on soya beans did so on rice. From these data it is concluded that, in principle, *B. cereus* is able to produce BCET on cooked soya beans, ie in kinema. It should, however, be borne in mind that the BCET-RPLA test does not specifically react with the diarrhoeal toxin, but with an indicator protein. In addition, this test cannot detect the *B. cereus* emetic toxin. Consequently, *in vivo* toxicity tests should be carried out in case of doubt of toxicity of samples having high BCET levels.

A strain of *B. subtilis* (DK-W1; MTCC1747) that gives good quality kinema, was tested simultaneously and was unable to produce BCET on either of the three tested substrates.

We also analyzed the market samples (Nos 5, 7 and 8) from which confirmed *B. cereus* strains had been isolated that produce BCET on soya beans. No detectable levels (ie ≥ 4 ng/g sample) of BCET were found.

Table 3 *B. cereus* enterotoxin (BCET) production on cooked soya beans, and effect of frying in mustard seed oil

<i>B. cereus</i> strain	BCET (ng/g wet weight)	
	After 24 h incubation at 30°C	Followed by frying (3 min, 180–200°C)
BC5-1	32	nd ^a
BC7-3	> 256	4
BC7-5	> 256	8
BC8-4	8	nd
BC12-3	> 256	nd

^and: not detectable (< 4 ng/g wet weight).

Table 4 Growth and BCET production of single *B. cereus*, and mixed culture with *B. subtilis* on sterile cooked soya beans at 30°C

Inoculum, and incubation period (h)	<i>B. subtilis</i> DK-W1 (log cfu/g wet weight)	<i>B. cereus</i> BC7-5 (log cfu/g wet weight)	BCET (ng/g wet weight)
BC7-5 alone			
t ₀		2.81	
t ₄₈		10.13	> 256
DK-W1 + BC7-5			
t ₀	6.51	2.18	
t ₄₈	10.51	5.93	nd ^a

^and: not detectable (< 4 ng/g wet weight).

The BCET levels on soya beans after 24 h incubation are presented in Table 3. There are some apparent differences among the strains. Most of them produced high levels that went beyond the dilution range of the test. The BCET was quite sensitive to temperatures during normal frying in oil (180–200°C). Kramer and Gilbert (1989) specify that BCET is inactivated at 56°C after 5 min, whereas the *B. cereus* emetic toxin resists 90 min heating at 126°C. Buchanan and Schultz (1994) observed that BCET-RPLA tests in boiled cell-free culture filtrates still gave positive results. Our data in Table 3 indicate that heating at high temperatures in oil will lead to inactivation, resulting in negative BCET-RPLA tests.

The result of a mixed culture experiment is shown in Table 4. The single pure culture of *B. cereus* BC7-5 produced a high level of BCET, confirming the previous experiment shown in Table 3. On the other hand, the mixed culture shows strongly reduced growth and BCET production by *B. cereus* when dominated by *B. subtilis*. The data in Table 4 suggest a competition or antagonistic effect of *B. subtilis* on *B. cereus*. In this experiment, initial *B. cereus* levels were intentionally low to simulate a contamination of the major functional starter. However, the single pure culture experiment indicates that higher initial *B. cereus*: *B. subtilis* ratios will increase the risk of *B. cereus* growth and BCET formation.

With regard to the safety of kinema, we suggest to distinguish two different scenarios. First, if prepared as a curry according to tradition, low levels of BCET that might have been formed despite inhibitory effects caused by dominating *B. subtilis* will be inactivated during frying in oil. Obviously, Gram-negative bacteria will be destroyed as well during this preparation. Second, if we consider the utilization of kinema in novel applications, eg as a dried powdered ingredient for protein or flavour enrichment of starchy foods, adequate measures must be taken to avoid the carry-over of enterotoxin or *B. cereus* endospores, that might germinate, proliferate and produce enterotoxin in fabricated foods.

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