

DAIRY FOODS TECHNICAL NOTES

Carryover of Preformed Staphylococcal Enterotoxins and Thermostable Deoxyribonuclease from Raw Cow Milk to Khoa – A Heat-Concentrated Indian Milk Product

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ABSTRACT

Preformation of enterotoxins and thermostable deoxyribonuclease by *Staphylococcus aureus* in raw cow milk and their carryover to Khoa – a heat-concentrated (98°C for 15 to 20 min) Indian milk product – was evaluated. Detectable enterotoxins and deoxyribonuclease, as evidenced in the zone diameters of 10 to 16 mm, were preformed in 10 h incubation of milk samples inoculated with strains of *Staphylococcus aureus* at 1×10^6 cfu/ml of milk. Both preformed enterotoxins and thermostable deoxyribonuclease were carried over to Khoa from raw milk. The study implies the use of good quality raw milk free from pathogenic organisms for preparation of milk products such as Khoa.

INTRODUCTION

Khoa is a heat-concentrated (98°C/15 to 20 min) Indian milk product with 26 to 28% moisture; it is one of several indigenous milk products of importance. Khoa is used as a base in the preparation of several Indian milk sweets. Indian milk products and milk sweets often have been implicated in food poisoning outbreaks. In such cases, there have been no follow-up action, and causative agents have not been studied in detail. In most of these outbreaks, symptoms of gastroenteritis were noticed within 1 to 2 h in victims after ingestion of contaminated edibles, and victims have recovered within a day.

From symptoms, it is possible to trace these food poisoning outbreaks to staphylococcal enterotoxins. The hygienic conditions during

preparation of Khoa and subsequent handling by personnel enable staphylococcal contamination. Often raw milk used for Khoa manufacture contains large numbers of staphylococcal organisms including enterotoxigenic strains of *Staphylococcus aureus* (6, 7, 11, 13). In such cases, *S. aureus* cells grow and produce enterotoxins and thermostable deoxyribonuclease (TDNase).

Because staphylococci killed at or near pasteurization temperature (2, 9, 10, 19, 22, 23), the chance of these organisms surviving heat treatment of Khoa preparation is remote. However, staphylococcal enterotoxins and deoxyribonuclease (DNase), which are heat stable, may survive the process of Khoa preparation if once preformed in milk. Our study evaluates carryover of enterotoxins and TDNase preformed by *S. aureus* in raw cow milk to Khoa prepared from such milk.

MATERIALS AND METHODS

Cultures

Test cultures were four wild strains of *S. aureus* (K-283, K-192, K-213, and K-208) isolated from market Khoa, which produced enterotoxin(s) A (SEA), E (SEE), BE (SEB and E), and CE (SEC and E) and two standard strains of *S. aureus* (A₁₀₀ and B_{S-6}) that produced enterotoxins SEA and SEB were obtained from M. S. Bergdoll, Food Research Institute, Madison, WI. Test cultures were grown in brain-heart infusion (BHI) broth and screened for SEA, SEB, SEC, SED, and SEE individually by optimal sensitivity plate (OSP) method of Robbins et al. (17).

Inoculation of Milk and Preparation of Khoa

Raw cow milk in 1-L lots was inoculated individually with test cultures of *S. aureus* at 1

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$\times 10^6$ cfu/ml of milk. Inoculated milk samples were incubated at 37°C for 10 h, necessitating preformation of TDNase and enterotoxins. Incubated milk samples then were converted into Khoa as follows.

Milk samples in one liter lots were heated to boiling in an open pan (Karahi) with continuous stirring in a circular motion by the flattened end of the stirrer (Khunti). During the process, all parts of the pan contacted by the milk were scrapped. Vigorous stirring and desiccating was continued until the product reached a pasty consistency. The pan was removed from the fire and after scraped for a few minutes, contents were allowed to cool to room temperature. The final product was placed in previously sterilized glass-stoppered bottles and stored for 7 d at room temperature (RT) (25 to 35°C) and refrigeration temperature (RFGT) (4 to 5°C). Khoa prepared from uninoculated milk samples served as controls.

Extraction and Detection of Thermostable Deoxyribonuclease and Enterotoxins

Samples of raw and incubated milk and fresh and stored Khoa were analyzed qualitatively for TDNase and enterotoxins. Samples were not enumerated for staphylococcal counts.

The TDNase from milk and Khoa samples was extracted following the method of Read et al. (15, 16) for extraction of enterotoxins from cheese and milk with slight modification. For Khoa samples, 10^{-1} dilutions in 2% sodium citrate were centrifuged at 10,000 rpm for 30 min, and the resulting supernatant solutions were acidified to pH 4.5 with 6 N hydrochloric acid (HCl). Acidified samples of Khoa and milk then were centrifuged at 10,000 rpm for 30 min, followed by neutralization of the supernates to pH 7 with 1 N sodium hydroxide and steaming at 100°C for 15 min. Steamed supernates then were centrifuged at 10,000 rpm for 30 min and screened for TDNase by toluidene blue-deoxyribonucleic acid (TB-DNA) agar plate method of Lachica et al. (12).

Enterotoxins were extracted from milk and Khoa by the method of Read et al. (15, 16) for extraction of enterotoxins from cheese and milk, except for the initial step in Khoa, wherein dilutions (10^{-1}) of Khoa in 2% sodium citrate were centrifuged at 10,000 rpm

for 30 min and the resulting supernates were acidified to pH 4.5 with 6 N HCl. Final supernates were screened for enterotoxins SEA, SEB, SEC, SED, and SEE individually as described by Robbins et al. (17).

RESULTS AND DISCUSSION

Of interest for public health, there was carryover of preformed staphylococcal enterotoxins and TDNase from raw milk to Khoa. Detectable amounts of the respective enterotoxins produced by the test cultures of *S. aureus* in raw cow milk incubated at 37°C for 10 h were carried over to Khoa (Table 1). However, there was no SEB in the Khoa sample prepared from milk inoculated with *S. aureus* K-213, even though this culture had produced SEB and SEE in milk before conversion into Khoa. Detectable SEA and SEB were not preformed in 10 h of incubation of milk samples inoculated individually with *S. aureus* strains A₁₀₀ and B_{S-6}. Hence, in these two samples there were no enterotoxins in the Khoa.

Staphylococcal enterotoxins are thermostable (1), with their pronounced heat stability observed early (4). Potency of enterotoxin is decreased gradually by prolonged boiling or autoclaving. Several investigations (3, 5, 8, 14, 18) on thermostability of different enterotoxins have shown some losses of activity during prolonged heating. The extent of such losses in terms of activity (reaction with antiserum) depended not only on initial concentrations but also on heating medium. Toxins are more heat resistant in foods than in buffer solutions. High concentration of proteins afford protection to toxins against heat treatment.

Failure to detect SEB in a sample of fresh Khoa prepared from milk containing preformed SEB and SEE may be attributed to reduction of concentration of preformed enterotoxin in milk to below detectable ($2 \mu\text{g}$) by the serological method of this study. The absence of SEA and SEB from milk samples inoculated with *S. aureus* A₁₀₀ and B_{S-6}, may be attributed to inability of these strains to produce enterotoxins in milk during minimum incubation times of 10 h, although strain B_{S-6} produces large amounts of SEB, especially when grown in media selective for its growth. In (21), detectable SEA and SEB produced by *S. aureus* A₁₀₀ and B_{S-6} in samples

TABLE 1. Carryover of preformed staphylococcal thermostable deoxyribonuclease (TDNase) and enterotoxins (Ent) from raw cow's milk to Khoa.

<i>Staphylococcus aureus</i> strains	K-283		K-192		K-213		K-208		A ₁₀₀		BS-6	
	TDNase ¹	Ent	TDNase	Ent	TDNase	Ent	TDNase	Ent	TDNase	Ent	TDNase	Ent
	(mm)		(mm)		(mm)		(mm)		(mm)		(mm)	
Raw cow milk	—	—	—	—	—	—	—	—	—	—	—	—
Incubated milk ²	15	A	16	E	10	BE	10	CE	pink line	—	pink	—
Age of Khoa, d												
Fresh	15	A	16	E	10	E	10	CE	pink line	—	pink line	—
7 at RT	12	A	18	E	14	E	12	E	—	—	—	—
7 at RFGT	14	A	16	E	10	E	10	E	—	—	—	—

¹ Zone diameter includes initial diameter of agar well (5 mm) on toluidene blue-deoxyribonucleic acid agar plates. RT = Room temperature (25 to 35°C), RFGT = refrigeration temperature (4 to 5°C), — = negative.

² Milk samples inoculated with test strains at levels of 1×10^6 cfu/ml and incubated for 10 h at 37°C.

of skim milk were in a minimum incubation time of 18 to 24 h at 37°C.

During storage of Khoa samples at RT and RFGT, the enterotoxin pattern remained unchanged. However, in one sample (strain K-208), SEC was not detected as it was in fresh Khoa.

Test cultures of *S. aureus* produced TDNase (zone diameter of 10 to 16 mm) (Table 1) in samples of raw cow's milk in an incubation time of 10 h. The same preformed TDNase was carried over from milk to Khoa in all samples. An increase of TDNase, as evidenced by the zone diameter of 2 to 4 mm, was observed in 3 RT stored Khoa samples prepared from milk inoculated with strains of *S. aureus* K-192, K-213, and K-208. However, there was a decrease of 3 mm of the zone diameter of TDNase during storage of Khoa prepared from milk inoculated with *S. aureus* K-283.

The resistance of DNase to the heat treatment in making Khoa is not surprising, as the test procedure itself involves heating the material at 100°C for 15 min. Actual measurements during Khoa preparation have shown temperature ranges from 62 to 98°C; hence, the actual thermal effect encountered during Khoa making is less than the treatment involved in screening TDNase. In (20), TDNase, as evidenced in the zone diameter of 10 to 12 mm, was detected in samples of market Khoa. Such TDNase in Khoa might have been the result of carryover of preformed TDNase from

raw milk to Khoa or may have been due to staphylococcal organisms that gained entry into the milk product as postprocessing contaminants. Cell numbers of contaminants in such cases have to be low.

Although staphylococci themselves may not survive Khoa processing, the use of milk containing preformed enterotoxins and TDNase for preparation of Khoa can be a serious danger to public health.

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