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Behavior of Enterotoxigenic Strains of *Staphylococcus aureus* in Milk Fermented with a Yogurt Starter Culture

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ABSTRACT. The ability of a yogurt starter culture formed by *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus* to inhibit the growth of four enterotoxin type A and B producers *Staphylococcus aureus* strains (ATCC 6538, S6, FRI-100 and a strain isolated from milk) during fermentation of milk and subsequent storage was investigated. Sterile skim milk was inoculated with about 10^6 CFU/ml of *S. aureus* and with about 10^6 CFU of starter culture, and incubated at 42°C during 8 h, followed by refrigeration at 4°C. Samples were taken every 2 h during fermentation and every 2 days during storage. Viable count of lactic acid bacteria and *S. aureus* as well as pH, acidity, thermostable deoxyribonuclease (TNase) and staphylococcal enterotoxin A (SEA) production were evaluated. Behavior of four strains was similar; *S. aureus* survived the 8 h fermentation with LAB, and its population began to decrease from the first day of storage, being completely inhibited at 9-10 days. TNase and SEA production were positive in all samples taken along the study. It was demonstrated that enterotoxigenic strains of *S. aureus* were able to survive the fermentation of milk with a yogurt starter culture and they were inhibited after several days during storage of the fermented product, contrary to the general belief which considered it very difficult due to the low pH. Even though *S. aureus* was inhibited, TNase and SEA were demonstrable along the storage. Therefore, fermented milks may play an important role in the transmission of this organism.

Key words: *Staphylococcus aureus*, yogurt, starter culture, lactic acid bacteria

RESUMEN. En este trabajo se determinó la capacidad de un cultivo iniciador para elaborar yogur, formado por una mezcla *de Streptococcus salivarius* subesp. *thermophilus* y *Lactobacillus delbrueckii* subesp. *bulgaricus* para inhibir el crecimiento de cuatro cepas enterotoxigénicas de *Staphylococcus aureus* (ATCC 6538, S6, FRI-100 y una cepa aislada de leche) en leche fermentada y almacenada. Cada cepa se inoculó en leche descremada estéril a una concentración de 10^6 UFC/ml junto con 10^6 UFC/ ml del cultivo iniciador. La leche inoculada se fermentó a 42°C durante 8 h y luego se refrigeró a 4°C. En las muestras tomadas cada 2 h durante la fermentación y cada 2 días durante el almacenamiento se determinó cuenta viable de bacterias lácticas y de *S. aureus*, así como el pH, la acidez y la producción de la enzima termonucleasa (TNasa) y de la enterotoxina estafilocócica tipo A (EEA). El comportamiento de las cuatro cepas de *S. aureus* fue similar; su población se mantuvo constante durante la fermentación con LAB, comenzó a decrecer desde el primer día de almacenamiento y fue completamente inhibida a los 9-10 días, aún cuando el pH del producto fermentado fue de 4.0-4.2. Las cepas de *S. aureus* sobrevivieron al proceso de fermentación de la leche, observándose inhibición en su desarrollo durante el almacenamiento del producto fermentado. Sin embargo, la producción de TNasa y de EEA fue positiva a pesar de que la cepa productora ya no se recuperó, por lo que se enfatiza la importancia que pueden tener los productos fermentados como el yogurt en la transmisión de este microorganismo y de sus metabolitos.

Palabras Claves: *Vibrio cholerae* O_1 tox^+; lemon industry chemical; bactericidal; bacteriostatic

INTRODUCTION

Contamination of dairy products with *S. aureus* persists as a serious public health problem in some underdeveloped and developed countries.[4] In Mexico, most of the outbreaks food poisoning were attributed to *S. aureus*, being milk, cheeses, cakes and dairy products the foods mainly responsible;[22] these foods were contaminated with over 10^6 CFU of *S. aureus* per gram of the food. This fact is due to poor hygienic conditions for milk obtention, inadequate preservation of the milk and the elaboration and consumption of non-pasteurized dairy products. These foods have

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been involved in a number of food poisoning outbreaks due not only to the presence and growth of the microorganism in the food, but also to enterotoxin production, which represents a risk for the consumer.[13]

S. aureus may be excreted from the udder of both mastitic and apparently healthy cows. If pasteurization and good practices of manufacture are not employed, the survival and growth of the pathogen in the milk is possible.[18]

Behavior of *S. aureus* during the manufacture, ripening and storage of some dairy products has been investigated by several authors, who have demonstrated that it depends on a number of factors, such as the use of either raw or pasteurized milk, type and concentration of the starter, concentration of the pathogen, processing procedures and ripening time.[3,7,9,14,17,19,29]

Lactic acid bacteria employed as starters in fermented dairy products have been shown to produce some substances, such as organic acids, hydrogen peroxide and bacteriocins, which were responsible of pathogenic and spoilage bacteria inhibition.[1,6,8,10,16,25,27,31]

The purpose of this work was to investigate the behavior of four enterotoxigenic *S. aureus* strains during the fermentation of sterile skim milk with a yogurt starter culture and subsequent storage. Thermostable deoxyribonuclease (TNase) and staphylococcal enterotoxin A (SEA) production were investigated too.

MATERIAL AND METHODS

***S. aureus* strains.** Four strains of *S. aureus* were used, which previously have demonstrated their ability for enterotoxin production:, *S. aureus* FRI-100, *S. aureus* S6 (these strains were provided by M. S. Bergdoll and Amy C. L. Wong of the Food Research Institute, University of Wisconsin, Madison, USA), *S. aureus* ATCC 6538[2] and a *S. aureus* isolated from milk in a previous work. Cultures were maintained in brain heart infusion agar (Oxoid) slants at 4°C. Counts of *S. aureus* in brain heart infusion broth (Oxoid) suspensions, were measured by optical density and corroborated by viable count on Baird Parker agar (Difco).

Starter culture. A lyophilized starter culture obtained in our laboratory, formed by a 1:1 mix of *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* was used. Working lactic cultures were prepared transferring 0.1 g of this starter into a tube containing 10 ml of sterile skim milk (Difco; SSM), and incubated 8 h at 42 °C. From this coagulated milk, a 2% (v/v) inoculum was transferred into an Erlenmeyer flask containing 200 ml of SSM and incubated in the same conditions. This procedure was performed three times to determine a known final population of *Streptococcus* and *Lactobacillus* achieved in milk.

Inhibition assays. For each assay, one set of five 500 ml Erlenmeyer flasks containing 200 ml of SSM were used; two flasks were inoculated with about 10^6 CFU of *S. aureus* per ml of milk and with the yogurt starter culture (10^6 CFU per ml), the third one was inoculated only with *S. aureus* (pathogen control), the fourth one only with the yogurt starter culture (starter control) and the fifth one was not inoculated (negative control). Flasks were incubated at 42°C during 8 h for fermentation, then they were stored at 4°C. Samples were taken at 2 h and 2 days intervals during fermentation and storage, respectively.

For continued monitoring of bacterial populations, samples were diluted and plated on *Lactobacillus Streptococcus* differential agar for viable count of LAB, and on Baird Parker agar for viable count of *S. aureus*; all plates were incubated 48 h at 37°C.

Determination of pH and acidity. The pH of the milk samples was measured with a Corning model 220 pHmeter equipped with a combined electrode. Titrable acidity was determined in 1.0 ml samples of milk, diluted 1:10 with sterile water, using 0.01N NaOH and 1 % phenolphthalein as indicator. Acidity was expressed as % of lactic acid.

TNase determination. 3 ml of toluidine blue-deoxyribonucleic acid were spread on the surface of slides; when agar was solidified, wells of 2 mm diameter were made. About 0.01 ml of the heated sample (15 min in boiling water bath) were added to each well. The slides were incubated in a moist chamber during 4h at 37°C. Development of bright pink zones extending at least 1 mm from the edge, was considered a positive reaction.

SEA extraction and determination.[20] The SEA from yogurt samples was extracted by a partial procedure. 25 ml of distilled water was added and blended. The pH was adjusted to 4.5 with 6N HCl and centrifuged at 23,000 x *g* for 30 min at 4°C. The supernatants previously adjusted to pH 7.5 with 5N NaOH were assayed by ELISA. Briefly the ELISA assay was made in the following manner. 100 µl aliquots of the extracts were added to prewashed anti-SEA coated microtiter wells and incubating for 2.0 h at 37°C. The wells were washed (3 times) with PBS and treated with 100 µl of anti-SEA and incubated for 1 h at 37°C. The wells were washed (3 times) and 100 µl of anti-IgG horseradish peroxidase conjugate were added and incubated 1 h at 25°C. After the wells were washed (5 times), 100 µl of substrate (OPD-H_20_2) were added (30 min at room temperature), and the reaction was stopped with 20µl of 6N H_2SO_4. Absorbance was read in a microtiter reader. Samples with an absorbance >0.2 were considered positive for enterotoxin A.

RESULTS AND DISCUSSION

pH and acidity values of the milk samples taken at different times during fermentation and during storage are shown in table 1. During fermentation, pH decreased from 6.2 to 4.4., and during storage it was maintained at about 4.0; this pH was inhibitory for *S. aureus*, reducing its growth when compared to the control, as shown in fig. 1

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Table 1. pH and acidity values of milk samples taken during fermentation with a yogurt starter culture at 42 °C and subsequent storage at 4 °C

	Fermentation (h)					Storage (days)							
	0	2	4	6	8	1	2	4	6	8	9	10	12
pH	6.4	6.1	5.4	5.0	4.4	4.1	4	3.9	3.9	4.0	3.9	4	4.1
acidity*	0.23	0.35	0.54	0.72	0.98	1.13	1.19	1.22	1.24	1.27	1.31	1.31	1.32

* expressed as % of lactic acid.

and 2.

The behavior of four *S. aureus* strains when they grew in association with the yogurt starter culture during fermentation and during storage, is shown in figures 1 and 2, respectively. During the 8 h fermentation, *S. aureus* grew about 1 logarithm cycle in the presence of the starter, but about 2 logarithm cycle in the absence of it; therefore, there was a significant reduction in the pathogen growth rate during fermentation phase. Through the storage of fermented milk, *S. aureus* population decreased from the first day, and after 9 or 10 days it was not recovered on agar plates any more, in presence of starter culture; while in its absence *S. aureus* population remains almost constant within 1 logarithm.

Even though the pathogen population tended to decrease and was inhibited during storage, TNase and SEA were demonstrable along the experiments (tables 2 and 3). Previous studies have shown that *S. aureus* concentrations more than 106 CFU/ml per gram of food are normally considered enough to produce detectable amounts of Tnase.[1,15,30] The results showed that levels of over 10^6 UFC/g are required for enterotoxin production and its detection in yogurt samples. Although SEA was detected in almost all samples, there was a significant difference in absorbance lectures compared with *S. aureus* cultures grew in the absence of starter culture (data non shown), particularly in those samples inoculated with strain S6 in presence of starter culture whose lectures were just above 0.25 compared with control ones (above 0.5). As it was expected, no SEA production was detected by strain 6538.

The inhibition of enterotoxin production of *S. aureus* will depend on the *Lactobacillus* strain, and the food sample analyzed, as shown our results and the results obtained by Sameshima et al., who found a total inhibition of enterotoxin production when use *L. rhamnosus* FERM P-15129 and *L. paracasei* subsp. *paracasei* in fermented sau-

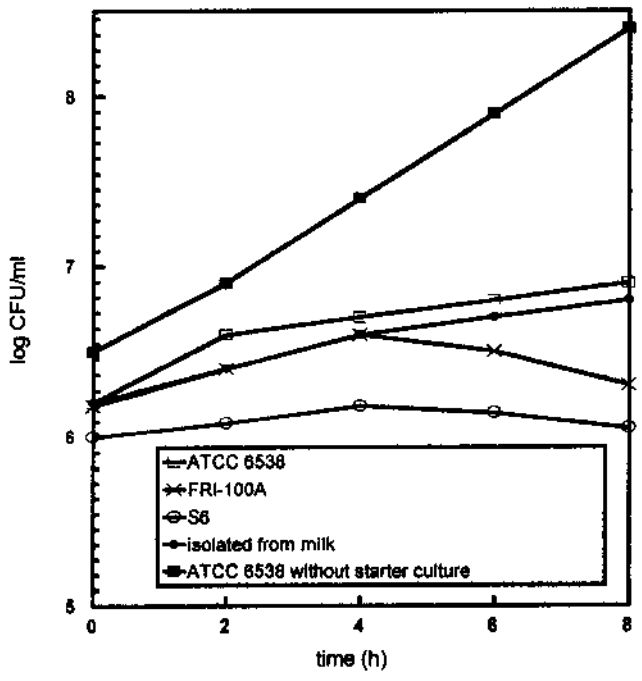

Fig. 1. Behavior of four enterotoxigenic *S. aureus* strains during fermentation at 42 °C, in the presence of a starter culture of lactic bacteria.

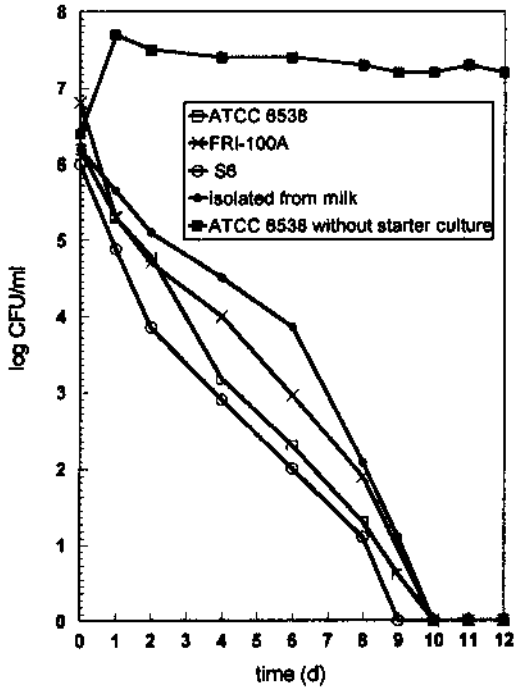

Fig. 2. Behavior of four enterotoxigenic *S. aureus* strains during storage at 4 °C, in the presence of a starter culture of lactic bacteria.

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Table 2. Results of the TNase test of milk samples taken during fermentation with a yogurt starter culture at 42 °C and subsequent storage at 4 °C

S. aureus strain	Fermentation (h)					Storage (days)							
	0	2	4	6	8	1	2	4	6	8	9	10	12
ATCC 6538	+	+	+	+	+	+	+	+	+	+	+	+	+
FRI-100A	-	+	+	+	+	+	+	+	+	+	+	+	+
S6	-	-	+	+	+	+	+	+	+	+	+	+	+
Isolated from milk	+	+	+	+	+	+	+	+	+	+	+	+	+

+ : positive, -: negative

Table 3.- Demonstration of staphylococcal enterotoxin A (SEA) in fermentation with a yogurt starter culture at 42°C and subsequent storage at 4°C.[a]

S.aureus strain	Fermentation (hours)					Storage (days)							
	0	2	4	6	8	1	2	4	6	8	9	10	12
ATCC 6538	--	--	--	--	--	--	--	--	--	--	--	--	--
FRI 100A	--	--	+	+	+	+	+	+	+	+	+	+	+
S6	--	--	+	+	+	+	+	+	+	+	+	+	+
Isolated from milk	--	+	+	+	+	+	+	+	+	+	+	+	+
FRI 100A[b]	--	+	+	+	+	+	+	+	+	+	+	+	+

(--), SEA non detected; [a], the determinations were made by triplicate; [b], without starter culture

sage, but a partial inhibition with *L. acidophilus* FERM P-15119.[26]

Contrary to our results, other studies have shown that staphylococcal TNase and enterotoxins synthesis were inhibited by a consequence of the starter in foods fermented with other LAB.[5,11,12,21] This fact has not been demonstrated using yogurt starter cultures.

Employing several experimental models to investigate post-pasteurization or post-manufacture contamination of fermented milks, the ability of the yogurt bacteria for the inhibition of some foodborne pathogens has been demonstrated[1,6,10,25,27,31]. Particularly, the behavior of *S. aureus* in fermented milks has been studied by Minor and Marth, which inoculated yogurt with 10^2 CFU/ml of *S. aureus*, and the fermented milks were free of viable *S. aureus* at the end of 1 day, but in yogurt inoculated with 10^5 CFU/ml, the pathogen survived for 2-4 days of storage.[17] Attaie et al. observed that when *S. aureus* was inoculated into milk base at 10^5 CFU/ml and then fermented to yogurt and acidophilus yogurt, the pathogen grew initially during the fermentation, but it died rapidly towards the end of fermentation.[3]

The global inhibitory effect depends not only of the drastic low pH, but also of various compounds produced by the yogurt starter bacteria, mainly hydrogen peroxide and bacteriocins. Dahiya et al. demonstrated that hydrogen peroxide produced by *L. bulgaricus*, was inhibitory to *S. aureus*.[6] Pulusani et al. reported antimicrobial activity of a methanol-acetone extract of milk fermented with *S. thermophilus*, against some pathogenic and spoilage bacteria.[23] Reddy and Shahani isolated an antibiotic substance from *L. delbrueckii* subsp. *bulgaricus* termed bulgarican, which was active against *S. aureus* and other bacteria.[24] Sikes and Hilton showed the inhibitory effect of a methanol-acetone extract of *S. thermophilus* fermented milk on *S. aureus*.[28]

Substances such as those mentioned above may be responsible in part for the inhibition of *S. aureus* growth in milks fermented with *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*.

Differences observed in our results on the survival time of *S. aureus* in fermented milks compared with those of previous works, may be due to conditions in experimental assays, varying strain and inoculum level of the pathogen, inoculation of the pathogen before or after fermentation, inoculum levels of the starter and starter culture type.

Results of this study stress that fermented dairy products, including yogurt, cannot be automatically considered free of *S. aureus* as a consequence of the action of the starter culture. There must be a high level of hygiene in the factory during production and handling of these foods to

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ensure they will be pathogens-free when they reach the consumer.

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