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Production of Enterotoxin A in Milk

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Enterotoxin A production in milk was studied by use of variables of milk quality, initial numbers of enterotoxigenic staphylococci, incubation temperature, and time. In both raw and pasteurized milks having a low total viable count, enterotoxin was detected in minimal incubation times of 6 to 9 hr at 35 C, 9 to 12 hr at 30 C, 18 hr at 25 C, and 36 hr at 20 C, after inoculation with 10^6 *Staphylococcus aureus* cells per ml. When similar milks were inoculated with 10^4 *S. aureus* cells per ml, enterotoxin was detected in 12 hr at 35 C, 18 hr at 30 C, 24 to 36 hr at 25 C, and 48 to 96 hr at 20 C. In high-count raw milk, enterotoxin was detected only in samples inoculated with 10^6 *S. aureus* cells per ml and incubated at 35 C. Generally, a concentration of 5×10^7 *S. aureus* cells per ml of milk was reached before enterotoxin A was detected.

Although some outbreaks of staphylococcal food poisoning have been attributed to consumption of fluid milk, most large-scale outbreaks ascribed to dairy products have involved dried milk (2, 3) or cheese (1, 5). In outbreaks attributed to dried milk and perhaps in some attributed to cheese, enterotoxin is believed to have been formed in the milk prior to conversion to the manufactured product. Because of this, knowledge of the kinetics of enterotoxin production in milk is essential in order to evaluate the potential of such things as poor refrigeration and post-pasteurization contamination for providing conditions conducive to enterotoxin production. The recent application of gel-diffusion methods, together with methods for the concentration of enterotoxin in milk, has made it possible to study the conditions under which staphylococci will produce enterotoxin in milk. In the studies reported here, we attempted to identify some of these conditions.

MATERIALS AND METHODS

Cultures. Several strains of enterotoxin A-producing *Staphylococcus aureus* were examined for their ability to produce this toxin in milk. A strain which we isolated (4) from market cheese (*S. aureus* MF-224) appeared to be most consistent in the production of enterotoxin A in milk and was selected as the test organism for this study. Suspensions of this organism were prepared by (i) harvesting the growth from Brain Heart Infusion slants, incubated for 24 hr at 35 C, in phosphate-buffered dilution water; (ii) washing, centrifuging, and resuspending the cells in dilution water; and (iii) adjusting the optical density to obtain the desired concentration of cells for inoculating the milk.

Milk. Milks of three different microbiological qualities were used to establish the effect of competing flora on the process of enterotoxin production. These were (i) milks having a standard plate count (SPC) of 10^4 to 3×10^4, or a relatively low count; (ii) milks with an SPC of 3×10^6 to 5×10^6/ml, or a relatively high count; and (iii) pasteurized milk that had an SPC of 3×10^6 and <3,000/ml before and after pasteurization, respectively. All growth studies were done in cotton-plugged 300-ml Erlenmeyer flasks with a milk volume of 100 ml.

Cultural examination. Total viable counts were determined by the SPC method, and *S. aureus* counts by the spreader plate technique on Staphylococcus Medium No. 110 (Difco) that had a plating efficiency of 100% for the test organism. Microbial counts were determined immediately after the sampling interval; the samples were then frozen and held in a food freezer until enterotoxin tests could be done. Enterotoxin is stable at freezer temperatures (6).

Examination for enterotoxin. To detect consistently the small quantities of enterotoxin produced in milk by *S. aureus* MF-224, it was necessary to extract the milk, in order to eliminate interfering substances that would mask a positive test, and to concentrate the extract. The milk samples were thawed and were concentrated by the method of Read et al. (6), which consists essentially of separating the whey from the proteins by acid precipitation, centrifugation, and filtration, and then extracting the whey with $CHCl_3$. The whey is concentrated to 1:20 volume by dialysis in polyvinylpyrrolidone, and the *p*H of the concentrate is adjusted to 7.2; it is then heated at 50 C for 10 min and centrifuged. The concentrated whey is extracted with $CHCl_3$, centrifuged, and the supernatant fluid, which constitutes the antigen, is removed and examined for enterotoxin by the double gel-diffusion tube technique. The enterotoxin A antiserum and the neutral (intermediate layer) agar were prepared with

TABLE 1. *Effect of competition on staphylococcal counts and enterotoxin A production in two samples of low-count raw milk inoculated with 10^4 Staphylococcus aureus cells per ml*

Time (hr)	Incubation temp											
	25 C				30 C				35 C			
	1st sample		2nd sample		1st sample		2nd sample		1st sample		2nd sample	
	SPC[a]	SAC[b]	SPC	SAC	SPC	SAC	SPC	SAC	SPC	SAC	SPC	SAC
6	0.7[c]	0.02	0.02	0.02	1	0.03	0.04	0.06	8	0.3	2	2
9	5	0.1	0.06	0.03	20	0.2	1	1	40	5	30	30
12	50	0.4	1	0.3	100	2	20	10	400	20	90	*70*
18	200	5	50	20	700	20	200	*80*	1,000	*50*	700	*200*
24	600	7	200	60	1,000	30	600	*200*	2,000	*60*	2,000	*300*
36	2,000	10	700	*90*[d]	3,000	30	2,000	*400*	3,000	*50*	2,000	*400*
48	2,000	20	1,000	*100*	3,000	30	2,000	*300*	3,000	*40*	1,000	*200*
72	3,000	23	1,000	*200*	4,000	20	2,000	*300*	3,000	*10*	600	*100*
96	4,000	10	2,000	*200*	3,000	9	800	*200*	1,000	0.2	80	*50*

[a] Standard plate count: 1st sample, 3×10^4/ml; 2nd sample, 10^4/ml.
[b] Staphylococcal counts.
[c] Results are expressed as counts ($\times 10^6$) per ml.
[d] Enterotoxin A detection indicated by italicized staphylococcal counts.

0.04 M Veronal buffer (6). Each antigen was examined by duplicate double-diffusion tests; a short intermediate agar layer (approximately 6 mm long) was used for one of the tests and a longer intermediate agar layer (approximately 13 mm) was used for the replicate test. With the concentration technique and the double-diffusion test, levels of enterotoxin as low as 0.01 to 0.02 μg could be detected in 1 ml of the original milk.

In the initial phases of this investigation, duplicate flasks were examined by SPC and staphylococcal count methods, and for enterotoxin at each incubation temperature and at each sampling interval. Since there appeared to be no significant differences between the duplicate flasks, duplicate determinations were not used for the remainder of the study.

RESULTS

Two studies were done with low-count raw milk inoculated with 10^4 and 10^6 *S. aureus* cells per ml. With the lower inoculum and incubation temperatures of 25 and 30 C, enterotoxin production appeared to depend on the rate at which the competing flora multiplied. When multiplication was rapid, growth of *S. aureus* was suppressed, and detectable levels of enterotoxin were not found (Table 1). With less rapid growth of the competing microorganisms, enterotoxin was detected in incubation times as short as 12 hr (Fig. 1). When the raw milk was inoculated with 10^6 *S. aureus* cells per ml, detectable levels of enterotoxin were produced in incubation times as short as 6 hr (Fig. 2). In a second experiment, in which low-count raw milk was inoculated with 10^6 *S. aureus* cells per ml and was incubated at 35 C, enterotoxin was detected at all sampling intervals from 6 to 72 hr; however, it was not detected at the 96-hr sampling interval.

In two studies of high-count raw milks, staphylococcal growth differed very little and no enterotoxin was detected in samples inoculated with 10^4 *S. aureus* cells per ml. In the milks inoculated with 10^6 *S. aureus* cells per ml, the results were so similar that only those from one study are reported in Fig. 3, which shows that enterotoxin was detected only in those samples incubated at 35 C.

Pasteurized milk supported the rapid growth of *S. aureus*, and enterotoxin was produced after relatively short incubation times (Fig. 4 and 5). When incubated at 25, 30, and 35 C, the milk inoculated with 10^6 *S. aureus* cells per ml contained detectable levels of enterotoxin 3 to 6 hr sooner than did those inoculated with 10^4 *S. aureus* cells per ml. In milk inoculated with 10^6 and 10^4 *S. aureus* cells per ml and incubated at 20 C, enterotoxin was detected after 36 and 48 hr, respectively. After 7 days (168 hr) of incubation at 35 C, enterotoxin was still detectable in milk inoculated with 10^4 *S. aureus* cells per ml, but was no longer detectable in milk inoculated with 10^6 *S. aureus* cells per ml and incubated under the same conditions.

Growth and enterotoxin production determinations on *S. aureus* in high-count milk before and after pasteurization showed that pasteurized milk, with its reduced viable count, makes an excellent vehicle for rapid multiplication of

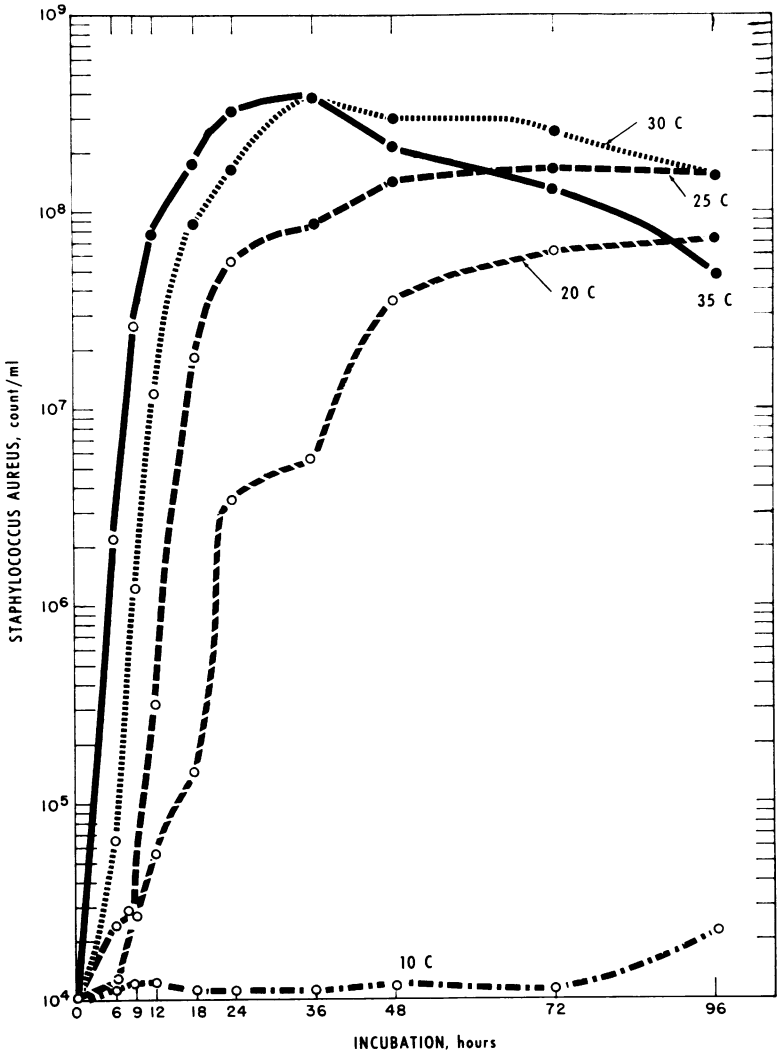

FIG. 1. *Staphylococcal growth and enterotoxin A production in low-count raw milk inoculated with 10^4 Staphylococcus aureus cells per ml. Symbols: ●, enterotoxin detected; ○, entertoxin not detected.*

S. aureus with the early production of enterotoxin. The inability of the test culture to grow rapidly and to produce enterotoxin in the same milk before pasteurization further supports the well-documented inability of *S. aureus* to grow in a competitive environment (Table 2).

DISCUSSION

We chose *S. aureus* MF-224 as the culture to be used in these studies for two reasons. First, it grew well and produced enterotoxin in milk more readily than most of the cultures screened; secondly, it demonstrated that a culture of *S. aureus* isolated from *market* cheese was capable of producing enterotoxin A in milk. The milk, inoculum levels, and incubation temperatures were chosen so that these data would be applicable to enterotoxin production in milk under a variety of conditions.

The bacteriological quality of the milk was that of better grade A supplies or of a quality commonly used for manufacturing dairy products, such as cheese. The inoculum levels were representative of the range of staphylococci found in some raw milk. The incubation temperatures chosen included those unfavorable to the growth of staphylococci (10 and 20 C) and those more nearly optimal (35, 30, and 25 C). The latter are

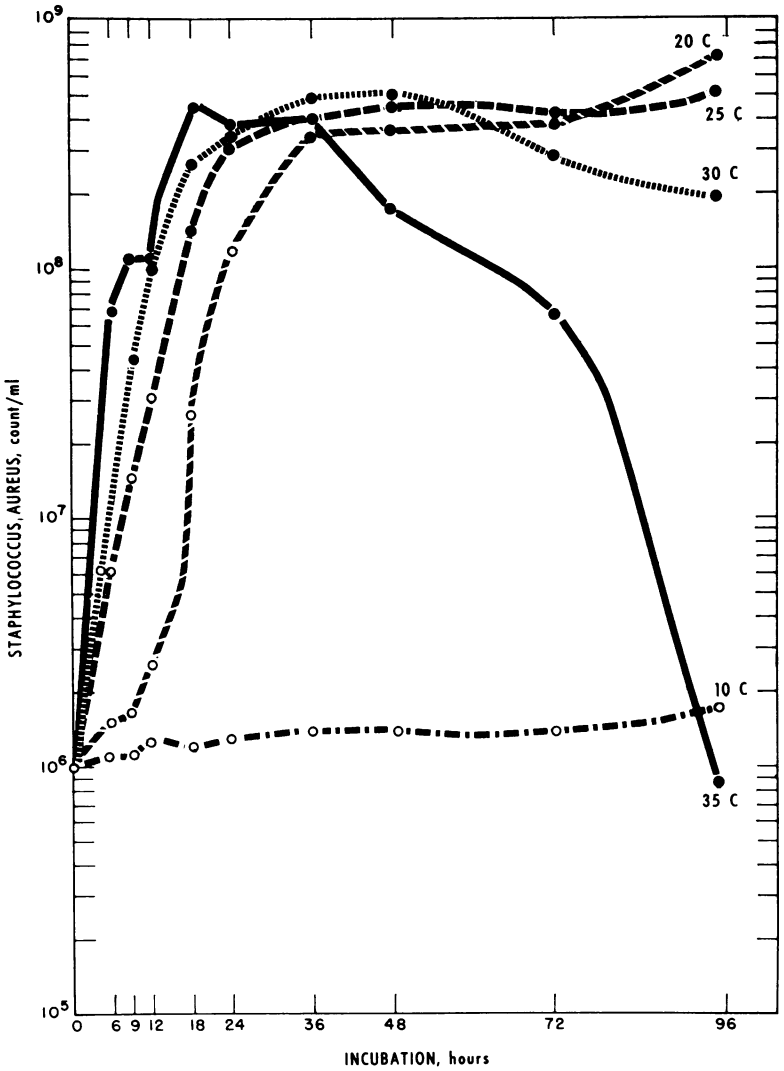

FIG. 2. *Staphylococcal growth and enterotoxin A production in low-count raw milk inoculated with 10^6 Staphylococcus aureus cells per ml. Symbols: ●, enterotoxin detected; ○, enterotoxin not detected.*

also representative of the temperatures at which cheddar cheese is made.

Staphylococcus medium 110 was chosen over the many other selective media for enumerating staphylococci because of our long experience in using it, its plating efficiency of *S. aureus* MF-224, and the characteristic appearance of this particular strain of staphylococcus on it, which enabled us to estimate fairly accurately the numbers of test organisms recovered from the milk despite the indigenous staphylococci.

The technique for concentrating enterotoxin in milk was found to be satisfactory for the preparation of antigens for enterotoxin A assay, even after the milk had been incubated at 35 C for 96 hr or longer. The double-diffusion test, which was used because of its sensitivity, frequently detected nonspecific reactions. These reactions were distinguishable from those of enterotoxin A, since they were less dense and less sharply defined.

Double-diffusion tube tests with both a short (6 mm) and longer (13 mm) intermediate reaction layer, used in examining each sample for enterotoxin, were compared experimentally. This provided useful data, as it demonstrated that tests with the shorter reaction layer were more sensitive; enterotoxin A reactions which were better

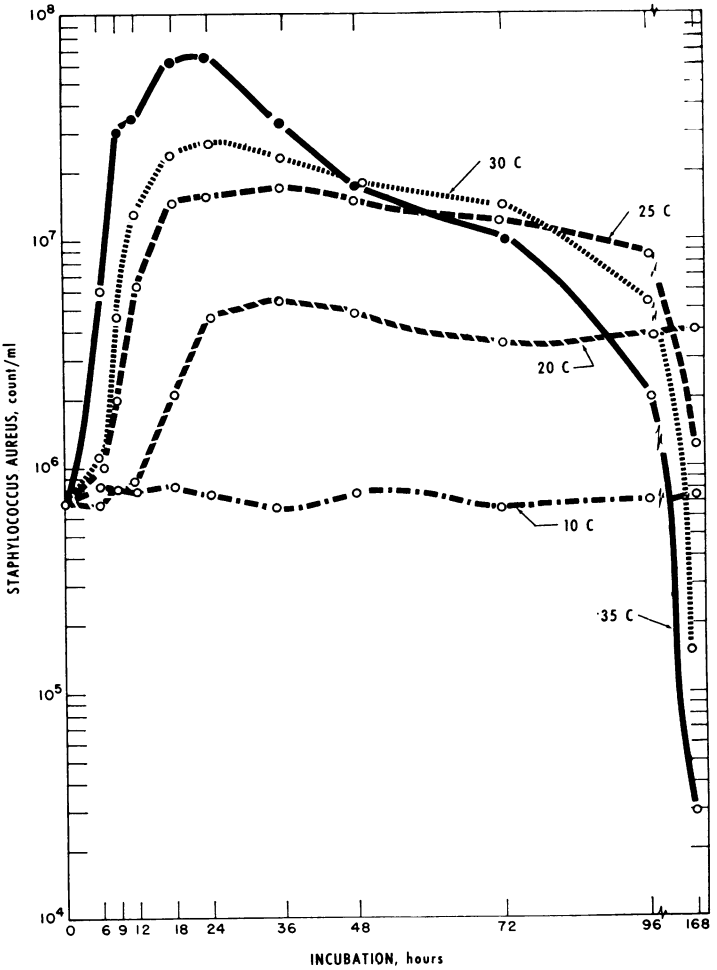

FIG. 3. *Staphylococcal growth and enterotoxin A production in high-count raw milk inoculated with 10^6 Staphylococcus aureus cells per ml. Symbols: ●, enterotoxin detected; ○, enterotoxin not detected.*

defined were usually observed earlier in these tests. On the other hand, tests with the shorter reaction layer showed nonspecific reactions more frequently.

With the exception of the first study with low-count raw milk inoculated with 10^4 *S. aureus* cells per ml, staphylococcal growth and enterotoxin production in low-count raw and pasteurized milk were similar and occurred in both types of milk at all incubation temperatures except 10 C. Not unexpectedly, enterotoxin was detected earliest in samples incubated at 35 C, followed in order by detection in samples incubated at 30, 25, and 20 C. Generally, enterotoxin was first detected in the samples when staphylococcal counts ranged from 50 to 100 million. As expected, enterotoxin was detected in those samples inoculated with 10^6 *S. aureus* cells per ml earlier than in those inoculated with 10^4 *S. aureus* cells per ml.

As the production of enterotoxin in milk is correlated with the staphylococcal counts, the failure of the staphylococci to produce enterotoxin in the high-count milk, except at 35 C, can be explained by their failure to grow and compete with the normal flora of the milk. In the same milk, after pasteurization, the staphylococci were able to compete and produce enterotoxin at 25, 30, and 35 C. Competition with the normal flora of the milk also accounts for the different results in the two low-count raw milks of similar quality inoculated with 10^4 *S. aureus* cells per ml. In the first low-count raw milk studied, the normal bacterial flora grew much more rapidly than did the staphylococci, and enterotoxin was detected only in samples incubated at 35 C. In the second

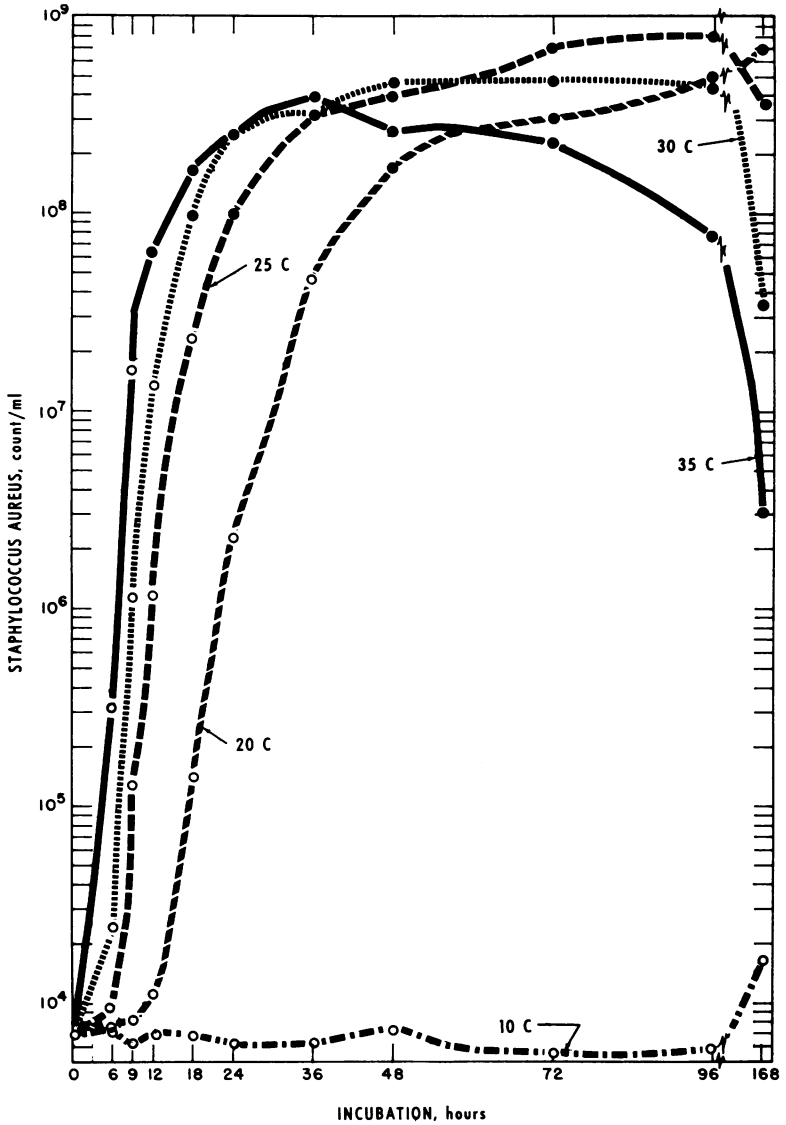

FIG. 4. *Staphylococcal growth and enterotoxin A production in pasteurized milk inoculated with 10^4 Staphylococcus aureus cells per ml. Symbols: ●, enterotoxin detected; ○, enterotoxin not detected.*

milk, the staphylococci were able to compete and produced enterotoxin at 25 and 30 as well as at 35 C.

An interesting field for investigation is suggested by the disappearance of enterotoxin from some of the samples incubated at 35 C. In one study of low-count raw milk inoculated with 10^6 *S. aureus* cells per ml, enterotoxin A, detected from the 6- through the 72-hr sampling intervals, was not detected at 96 hr, and in samples inoculated with 10^4 *S. aureus* cells per ml, it was not detected after the 48-hr sampling interval. In high-count raw milk inoculated with 10^6 *S. aureus* cells per ml, enterotoxin was not detected after the 72-hr sampling interval. The same phenomenon was also observed in pasteurized milk inoculated with 10^6 *S. aureus* cells per ml, in which case enterotoxin detected at 96 hr was not detected at 168 hr.

Our studies show that low-count milk, either raw or pasteurized, provides a better medium for staphylococcal growth and enterotoxin produc-

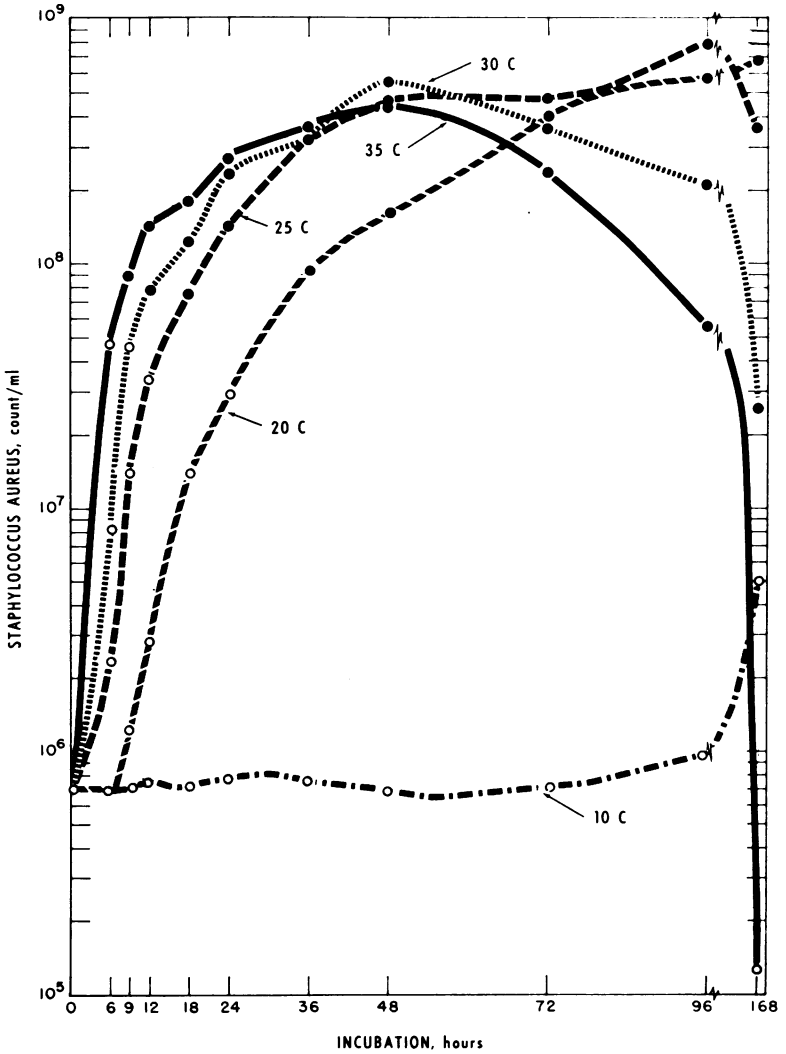

FIG. 5. *Staphylococcal growth and enterotoxin A production in pasteurized milk inoculated with 10^6 Staphylococcus aureus cells per ml. Symbols: ●, enterotoxin detected; ○, enterotoxin not detected.*

TABLE 2. *Effect of competition on staphylococcal counts and enterotoxin A production in high-count raw and pasteurized milk inoculated with 10^6 Staphylococcus aureus cells per ml*

Time (hr)	Incubation temp											
	25 C				30 C				35 C			
	Raw		Pasteurized		Raw		Pasteurized		Raw		Pasteurized	
	SPC[a]	SAC[b]	SPC	SAC	SPC	SAC	SPC	SAC	SPC	SAC	SPC	SAC
6	80[c]	1	3	2	100	1	8	8	100	6	5	50
9	200	2	10	10	200	5	40	50	200	*30*	100	*90*
12	400	6	30	30	600	10	70	*80*	500	*30*	100	*100*
18	2,000	20	90	*70*[d]	1,000	20	100	*100*	1,000	*60*	200	*200*
24	2,000	20	100	*100*	1,000	30	300	*200*	1,000	*60*	300	*300*
36	2,000	20	400	*300*	2,000	20	500	*300*	1,000	*30*	2,000	*400*
48	1,000	20	600	*500*	900	20	1,000	*500*	700	*20*	2,000	*400*
72	1,000	10	600	*500*	500	10	600	*400*	300	*10*	2,000	*300*
96	600	9	3,000	*800*	500	5	300	*200*	100	2	2,000	*50*
168	500	1	2,000	*400*	300	0.2	50	*30*	600	0.03	0.1	0.2

[a] Standard plate count: raw milk, 3×10^6/ml; pasteurized milk, 3×10^3.
[b] Staphylococcal counts.
[c] Results are expressed as counts ($\times 10^6$) per ml.
[d] Enterotoxin A detection indicated by italicized staphylococcal count.

tion than high-count raw milk, and that both of these low-count products are potential sources of staphylococcal food poisoning if they are not handled properly. High-count raw milk can also be a source of staphylococcal food poisoning under certain conditions. This milk, pasteurized, provides an excellent medium for staphylococcal growth and enterotoxin production.

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