GROWTH AND PRODUCTION OF ENTEROTOXIN BY STAPHYLOCOCCUS AUREUS GROWN IN ASSOCIATION WITH SELECTED LACTIC ACID BACTERIA

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
WILLIAM C. HAINES
1972

This is to certify that the

thesis entitled

Growth and Production of Entertoxin by
Staphylococcus Aureus Grown in Association
with Selected Lactic Acid Bacteria

presented by

William C. Haines

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Food Science

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Date April 3, 1972

O-7639

ABSTRACT

GROWTH AND PRODUCTION OF ENTEROTOXIN BY STAPHYLOCOCCUS AUREUS GROWN IN ASSOCIATION WITH SELECTED LACTIC ACID BACTERIA

By

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Experiments were conducted in APT ("All Purpose plus Tween") broth to determine the effects of several lactic acid culture bacteria upon the growth of *Staphylococcus aureus* and production of enterotoxin. Initial populations of *S. aureus* and effector organisms were approximately equal. Samples were agitated continuously during incubation. When strains of *Streptococcus lactis*, *Pediococcus cerevisiae*, *Streptococcus cremoris*, *Lactobacillus casei*, *Lactobacillus plantarum* and *Leuconostoc citrovorum* were utilized as effectors, the temperature of incubation was 30 C. The temperature of incubation was 41 C when *Lactobacillus bulgaricus* and Streptococcus thermophilus were used as effectors. The streptococci consistently inhibited both growth of *S. aureus* and production of enterotoxin. *P. cerevisiae* inhibited growth and production of enterotoxin to a lesser degree than the streptococci. The lactobacilli and *L. citrovorum* demonstrated only minor inhibitory effects upon

S. aureus. No enterotoxin was detected when the population of *S. aureus* did not reach at least 50 million cells per ml. Conversely, whenever the maximum population of *S. aureus* was 50 million cells per ml or greater, enterotoxin was detected.

Utilizing strains of *S. lactis* and *P. cerevisiae* as effectors, associative culture experiments were conducted which demonstrated that variations in the pH of the growth medium in the range of 6.0 to 7.0 did not greatly affect the ability of the effector organism to inhibit growth of *S. aureus* or production of enterotoxin. Similar experiments conducted at 25 C indicated that inhibition of *S. aureus* was slightly greater at a lower temperature of incubation than at 30 C. The relative proportion of *S. aureus* in the inoculum was found to have a great influence upon the growth of *S. aureus* in the mixed culture.

Comparison of several strains each of *S. lactis* and *P. cerevisiae* indicated that variations between the strains of a given specie of effector organism are of minor significance in determining the degree of inhibition of *S. aureus* in associative culture. Similarly, four enterotoxigenic strains of *S. aureus* were approximately equal in sensitivity to inhibition by *S. lactis* and *P. cerevisiae*.

Experiments in which *S. aureus* was grown in APT broth acidified with various quantities of lactic acid indicated that acid production is probably not the primary mechanism of antagonism by *S. lactis* and *P. cerevisiae*. Agar diffusion cup assays indicated that production of antibiotic-like substances is also not likely to be the major mechanism of antagonism.

Spent media were prepared by growing *S. lactis* and *P. cerevisiae* in APT broth for 18 hours at 30 C. Growth of *S. aureus* in the spent APT broth for 18 hours at 30 C. Growth of *S. aureus* in the spent APT broths was enhanced by additions μg/ml of biotin and 100 μg/ml of niacin. It was therefore concluded that production of hydrogen peroxide and competition for vital nutrients were important factors in the inhibition of *S. aureus* by *S. lactis* and *P. cerevisiae*.

Treatment of washed cells of *S. aureus* 243 with lysozyme and toluene, and disintegration by an acetone-dry powder procedure caused the release of small quantities of enterotoxin B. Disruption of the washed cells by sonication, grinding, and freeze-thaw procedures did not result in the release of detectable quantities of enterotoxin.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition

1972

ACKNOWLEDGEMENTS

The author is indebted to Dr. L. G. Harmon for his guidance during the program of study and for his assistance in preparing this manuscript.

Further expressions of gratitude are made to Dr. E. H. Marth of the University of Wisconsin, Dr. W. E. Sandine of Oregon State University, and to Mrs. Cecelia Marshall for providing some of the cultures of lactic acid bacteria used in this investigation.

The author acknowledges the assistance of Dr. H. A. Lillevik of the Department of Biochemistry, Dr. E. Sanders of the Department of Microbiology and Public Health, Dr. R. V. Lechowich of Virginia Polytechnic Institute, and Drs. C. M. Stine and K. E. Stevenson of the Department of Food Science and Human Nutrition, who served on the guidance committee.

Appreciation is offered to the Department of Food Science and Human Nutrition for use of the facilities and for the provision of financial assistance.

Special thanks should be given to Mrs. Marguerite Dynnik for her valuable assistance in the performance of many of the experiments.

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INTRODUCTION

Recent statistics tabulated by the Center for Disease Control (1970), indicate that staphylococci are implicated as the causative agent in more food poisoning outbreaks in the United States than any other microorganism. Although staphylococcal food poisoning is rarely fatal, the most notable symptoms of vomiting and diarrhea are extremely uncomfortable, and certainly have resulted in a definite inconvenience to great numbers of people.

Although the role of staphylococci in food poisoning has been recognized since 1914, and perhaps earlier, the lack of reliable and accurate assay procedures for the enterotoxin has limited scientific investigation in this important area until recent years. Thus, while a great deal is known about the morphology and physiology of the organism itself, much less information is available regarding the various environmental factors which might influence production of the enterotoxin by competent strains.

Competition of staphylococci with other food bacteria frequently results in repression of the growth of the staphylococci, and may delay or prevent enterotoxin production. One aspect of this study was to examine the effects which certain lactic acid bacteria (members of the family *Lactobacillaceae*) have upon the growth and production of toxin by enterotoxigenic strains of *Staphylococcus aureus*.

A second area of investigation in recent years has centered upon determining the age of the cells of *S. aureus* when synthesis of enterotoxin is initiated. There has been some evidence that toxin production is primarily associated with late log phase or early stationary phase of the growth cycle of the organism. However, the studies heretofore conducted in this area have relied on toxin assays of culture filtrates or supernatant fluids. It is conceivable that significant toxin production may occur before the release of detectable quantities into the growth medium. Previous experiments attempting to obtain toxin from washed cells have not generally been successful, but information on these experiments is incomplete. Thus, a study of the ability of several common procedures to disrupt cells and cause the release of toxin from washed cells was conducted. Development of a method for obtaining intracellular toxin should provide a means for future experiments to attempt to resolve the controversies concerning the age of the cells of staphylococci when actual toxin synthesis begins, and when the rate of synthesis is maximal.

REVIEW OF LITERATURE

According to Jay (1970), the role of staphylococci in food poisoning was first studied by Denys in 1894, and later by Barber in 1914. However, Dack (1956) related earlier descriptions, primarily by physicians, of food poisoning incidents in which staphylococci were apparently the etiological agents. Many of these cases, in fact, occurred prior to the discovery of staphylococci in pus by Pasteur in 1880.

Dack *et al*. (1930) showed that the symptoms of staphylococcal food poisoning could be produced by feeding culture filtrates of *S*. *aureus* strains isolated from incriminated foods to human volunteers; thus, the capacity of staphylococci to produce a true food poisoning was established.

The literature on staphylococcal food poisoning has been extensively reviewed, first by Dack (1956), and more recently by Angelotti (1969) and Bergdoll (1966 and 1970). Therefore, no attempt will be made here to provide a thorough review. Instead, a survey of the literature pertinent to the various aspects of this study is presented.

Staphylococcal Enterotoxins

Background

Lachica *et al*. (1969) estimated that as many as 50% of *S. aureus* isolates may be capable of producing enterotoxin under suitable conditions.

Bergdoll *et al*. (1959b) first observed the existence of more than one type of enterotoxin. A tentative nomenclature was proposed by Casman (1960) in which the two types of enterotoxin which had been identified at that time were called E and F. Enterotoxin F was associated with isolates of *S. aureus* from foods implicated in food poisoning outbreaks. Surgalla and Dack (1955) noted the occurrence of staphylococcal enteritis in patients being treated with antibiotics, and Casman proposed that the type of enterotoxin associated with isolates of *S. aureus* from these cases be called type E.

In 1962 a meeting was held for the purpose of establishing a permanent nomenclature for the enterotoxins at the Annual Meeting of the American Society for Microbiology. Since that time, enterotoxins are designated by capital Roman letters, beginning with A and proceeding in alphabetical order as recommended by Casman *et al*. (1963). Specific antigen-antibody reactions are the basis for differentiation. Enterotoxin A, originally called F, was first identified by Casman (1960). Enterotoxin B (Casman's type E) was identified by Bergdoll *et al*. (1965), D by Casman *et al*. (1967), and E by Bergdoll (1970).

Toxicity of the Enterotoxins

For obvious reasons estimates of the toxicity of the staphylococcal enterotoxins for humans have largely been based upon results from animal studies. According to Bergdoll (1970) the type of animal which may be used for toxicity studies is limited by the fact that only Primates are sensitive to oral administration of toxin. Surgalla *et al*. (1953) showed that the rhesus monkey was a suitable test animal for studying toxicity. Using this animal, an ED_{50} of 0.9 μg per Kg of body weight for orally administered enterotoxin B was obtained by Schantz *et al*. (1965), when either vomiting or diarrhea occurring within five hours of administration were considered positive reactions. Berdgoll (1970) reported that an approximate dose of 5 μg of enterotoxin B per 2-3 Kg of weight was an average toxic dose when only emesis was considered positive. Chu *et al*. (1966) determined that 5 μg of enterotoxin A per animal was also sufficient to cause emesis. Similar toxicity levels were determined for enterotoxin C by Borja and Bergdoll (1967).

Wilson (1959) observed that chimpanzees are much more sensitive to enterotoxin than rhesus monkeys. Thus, great differences in sensitivity to enterotoxin may exist between species of primates. Investigations attempting to estimate toxicity for humans based upon results from animal studies have had to consider this possibility.

There have been limited studies utilizing human volunteers which give some insight into the toxicity of enterotoxins for humans. Raj and Bergdoll (1969) reported typical food poisoning symptoms in three volunteers given 50 μg of enterotoxin B which had a purity of approximately 50%. In a second study, described by Bergdoll (1970), two volunteers failed to exhibit symptoms when given as much as 10 μg of pure toxin. Baird-Parker (1971) has indicated that the average emetic dose for types A, B, and C enterotoxins is approximately 0.2 μg per Kg of body weight. Several authors have expressed opinions that substantially smaller amounts of enterotoxin may cause food poisoning, on the basis of assays for the quantity of enterotoxin present in incriminated foods.

Purification of the Enterotoxins

Most scientists interested in studying the enterotoxins have recognized that pure preparations of the toxins are necessary for investigation. Jordan and Burrows (1933) reported the first attempts at purification and described limited success in effecting a concentration of enterotoxin by dialysis. Davidson and Dack (1939) reported attempts to purify the toxin by precipitation with ammonium sulfate. A combination of ammonium sulfate and ethanol precipitation was proposed by Hammon (1941). Several methods, including ammonium sulfate, hydrochloric acid, ethanol and methanol precipitation were studied by Bergdoll *et al.*

(1951). The first studies utilizing chromatographic procedures were reported by Bergdoll _et al_. (1952).

The first successful purification of an enterotoxin was reported by Bergdoll _et al_. (1959a) in which enterotoxin B was purified by precipitation with phosphoric acid, chromatography on alumina, ethanol precipitation, chromatography on Amberlite IRC-50, ethanol precipation, starch gel electrophoresis, and a final ethanol precipitation. Schantz _et al_. (1965) modified Bergdoll's procedure to enable purification of large quantities of enterotoxin B, utilizing chromatography twice on Amberlite CG-50, adsorption onto carboxymethylcellulose followed by elution with a linear gradient phosphate buffer (0.02 _M_ at pH 6.2 to 0.07 _M_ at pH 6.8), and dialysis. Schantz reported yields of up to 60% for this procedure, on the basis of toxin assays by gel diffusion.

Enterotoxin A is currently purified in the Food Research Institute laboratories by a procedure first described by Chu _et al_. (1966), involving the following steps: (1) concentration of the culture supernatant fluid by dialysis against carbowax 20 M, (2) dialysis against distilled water, (3) chromatography on carboxymethylcellulose, (4) gel filtration utilizing Sephadex G-100, (5) lyophilization and rehydration in distilled water, (6) gel filtration with Sephadex G-75, (7) dialysis and (8) lyophilization. Yields of 30 to 35% have been reported for this procedure.

Similar procedures for purification of enterotoxin C were discussed by Avena and Bergdoll (1967) and Borja and Bergdoll (1967).

Casman *et al*. (1967) attempted to purify enterotoxin D by the procedure used by Schantz *et al*. (1955) for enterotoxin B, but were only able to achieve yields of approximately 10%. According to Bergdoll (1970), work is underway at the Food Research Institute to develop specific procedures for purification of enterotoxins D and E.

Factors Affecting Growth and Production of Enterotoxin by Staphylococci

Effects of Temperature

Frazier (1967) stated that enterotoxin production is optimum between 21.1 and 36.1 C. According to Bergdoll (1970) most studies involving production of enterotoxin for research purposes have involved incubation at 35-37 C. Angelotti *et al*. (1961a,b) reported that food poisoning strains of staphylococci do not grow in foods at temperatures below 42 F, nor above 116 F. Seaglove and Dack (1941) demonstrated toxin production at 18 C after incubation for three days. Enterotoxin was detected after incubation at 16 C and 20 C by McLean *et al*. (1968), but in much smaller quantities than that produced at 37 C, though the total maximum population of the organism was nearly the same in all three cases. Genigeorgis *et al*. (1969) reported limited enterotoxin production at 10 C.

Effects of pH

Lechowich et al. (1956) found that anaerobic growth of staphylococci in ground pork did not occur at a pH of 4.8 to 5.0. In the same study, aerobic and anaerobic growth in broth were prevented at pH values of 5.0 and 4.8, respectively. Some growth of staphylococci in frozen pot pies was found at pH 4.5 by Dack and Lippitz (1962), but good growth was observed only at pH 5.0 and above.

Casman and Bennett (1963) reported that pH 5.3 gave highest yields of enterotoxin A on semisolid media, whereas an initial pH of 6.5 to 7.0 was optimum for toxin production in Brain Heart Infusion (BHI) broth. Very little production of enterotoxin B was found to occur at pH values below 5.0 by Peters (1966). Reiser and Weiss (1969) found that pH 6.8 was optimal for production of enterotoxins B and C, while a nearly uniform rate for production of enterotoxin A was observed at all pH values tested down to 5.3. Production of enterotoxin B did not occur at pH 9.0 and higher in an investigation performed by Marland (1967).

Baird-Parker (1971) noted that production of enterotoxin B in broth culture coincides with a rise in the pH value of the medium near the end of the log growth phase. Furthermore, he observed that the rate of toxin production is related to the rate of pH increase, and that toxin production is greatest in media in which the pH is not controlled.

Effects of Aeration

McLean *et al*. (1968) reported the quantity of enterotoxin B produced in aerated BHI broth at 37 C was at least twice that produced in static cultures. Baird-Parker (1971) indicated that after incubation for 7 days at 30 C approximately 10 times more enterotoxin B was produced in static cultures incubated in the presence of air than in cultures incubated in a 95:5 mixture of N_2 and CO_2. Marland (1967) studied the effects of aeration with pure O_2 upon enterotoxin production and noted that excessive aeration caused a decrease in enterotoxin production without adversely affecting growth. According to Bergdoll (1970) deep culture aeration results in decreased production of enterotoxin B compared to shake culture incubation.

Effects of Chemicals and Antibiotics

The effect of salts on production of enterotoxin has been extensively studied. Genigeorgis and Sadler (1966a) found that enterotoxin B could be produced in BHI broth containing up to 10% NaCl at pH 6.9 or in BHI broth containing 4% NaCl at pH 5.1. McLean *et al*. (1968) demonstrated that increasing the NaCl concentration had a greater effect on toxin synthesis than on growth. According to Baird-Parker (1971) NaCl concentrations up to 5% had no effect upon toxin synthesis when the temperature of incubation was 20-30 C. NaCl concentrations of 7-9% resulted in decreased toxin production and no enterotoxin was detected at concentrations of 10-14%.

The effects of $NaNO_2$ and $NaNO_3$ upon enterotoxin production were studied by McLean _et al_. (1968), who observed that these salts alone had no effect at the concentrations used in curing meats. It was found, however, that $NaNO_2$ and $NaNO_3$ did effect a decrease in toxin production when 2% NaCl was also included in the medium, suggesting a synergistic phenomenon.

Friedman (1966) studied the effects of several compounds upon production of enterotoxin B, and reported that K_2HPO_4, KCl, $CoCl_2$, NaF, acriflavine, phenethyl alcohol, streptomycin sulfate, chloramphenicol, spermine phosphate, spermidine phosphate and tween 80 inhibited toxin synthesis without noticeably affecting growth. Since the inhibition by the salts containing K+ ions and NaF could be reversed by increasing the concentration of Mg++ ions, Friedman suggested that the enzyme system controlling toxin synthesis requires Mg++, and that the other cations might be competitive inhibitors. Inhibition of enterotoxin B synthesis by streptomycin was also reported by Rosenwald and Lincoln (1966), who hypothesized that either incomplete coding for synthesis of the toxin itself, or alterations in the cell resulting in failure of toxin to be released, were responsible.

In a later study, Friedman (1968) observed that certain compounds which have an effect on bacterial cell walls, specifically tween 80, oleic acid, sodium deoxycholate, penicillin, D-cycloserine and bacitracin inhibited enterotoxin formation without adversely affecting growth. Production of αhemolysin and coagulase were not affected by any of these compounds, which gave additional support to Friedman's conclusion that the action of the inhibitory compounds was specifically directed toward the cell wall and associated products.

Markus and Silverman (1968) developed a technique for obtaining synthesis of enterotoxin B by nongrowing washed cells harvested from the late-exponential to the early stationary growth phase. Using this technique, they reported in a later paper (1969) that chloramphenicol, penicillin, streptomycin and $CoSO_4$ did not inhibit toxin synthesis in these so-called "committed" cells, and suggested that a toxin precursor pool accumulates in the cells during the logarithmic growth phase.

Repression of enterotoxin synthesis by glucose was observed by Morse *et al.* (1969), who suggested that toxin synthesis is regulated by catabolite repression.

Age of Cells When Toxin Production Occurs

The question of the age of the cells at the time when enterotoxin synthesis occurs has not yet been completely resolved, since conflicting reports have been made. The work of Markus and Silverman (1968, 1969) indicated that 95% of the total enterotoxin B produced in a given culture is released during the late logarithmic growth phase. A similar observation was made by McLean *et al*. (1968). Morse *et al*. (1969), however, reported no synthesis of enterotoxin B during the exponential growth phase, but rather during the early stationary phase. Recently Markus and Silverman (1970) suggested that synthesis of enterotoxin A might be quite distinct from synthesis of enterotoxin B, based upon their observation that enterotoxin A is synthesized throughout the entire logarithmic growth phase.

Nutrients Required by S. Aureus

Staphylococci require both nicotinic acid and thiamine for growth according to Knight (1937a,b) and Knight and McIlwain (1938). Porter and Pelczar (1940) noted that biotin stimulated growth of *S. aureus*. The first studies using defined media to study the nutritional requirements of *S. aureus* were reported by Surgalla (1947), who observed that growth and production of enterotoxin occurred in a medium consisting of arginine, cystine, nicotinic acid, thiamine, glucose, magnesium sulfate, ferrous ammonium sulfate and potassium dihydrogen phosphate. The growth requirements of enterotoxigenic strains were reported to be essentially the same as those of non-enterotoxigenic strains by Peters (1966). Mah *et al*. (1967) studied the nutritional requirements of *S. aureus* S-6, and concluded that the amino acids glycine, valine, leucine, threonine, phenylalanine, tryrosine, cysteine, methionine, proline, arginine and histidine, and the vitamins nicotinic acid and thiamine are required for optimal growth of the organism. Biotin was also a growth requirement in some situations. In a recent study Morse and Baldwin (1971) reported an increase in toxin production when thiamine was eliminated from the growth medium.

Competition with Other Bacteria

It has generally been recognized that staphylococci do not compete well with other bacteria in foods. Dack and Lippitz (1962) found that the natural flora of slurries prepared from frozen pot pies exhibited an inhibitory effect upon staphylococci. Since the organism which predominated after incubation was a lactobacillus which produced substantial acid, Dack and Lippitz speculated that a reduction of the pH of the slurries was responsible, at least in part for the inhibition. Similarly, Peterson *et al*. (1962a) observed inhibition of staphylococci by the natural flora of thawed frozen pot pies to which the staphylococci had been added. Growth of staphylococci in the pies was observed only after extended incubation under conditions which resulted in extreme spoilage. In a second study, Peterson *et al*. (1962b) reported that inhibition of staphylococci was more pronounced when the relative proportion of staphylococci in the population became smaller. Casman *et al.* (1963) observed that good growth of staphylococci in meats and production of enterotoxin A occurred only when the sample inoculated with *S. aureus* was aseptically taken from internal portions of meat, or when the meat was cooked. Presumably the staphylococci were unable to compete with other organisms in the raw meat. Donnelly *et al.* (1968), in studying the production of enterotoxin A in milk, noted that the flora of high count raw milk exerted an inhibitory effect upon staphylococci. In a study by Tatini *et al.* (1969) growth of staphylococci and production of enterotoxin were inhibited in commercial raw whole milk, but not in the same milk after pasteurization.

Various methods of evaluating the ability of organisms to compete with *S. aureus* have been utilized. Oberhofer and Frazier (1961) used spot-plate tests to screen 66 microorganisms for the ability to inhibit the growth of staphylococci, and found that streptococci and lactobacilli were the most consistently inhibitory of the organisms tested. Spot-plate tests were also used by Graves and Frazier (1963) to evaluate 870 isolates of food bacteria from a range of sources, and again members of the genera *Streptococcus* and *Lactobacillus*, as well as members of the genus *Leuconostoc* were most consistently inhibitory to staphylococci. Similarly, McCoy and Faber (1966) used spot plates to study the effect of 44 organisms isolated from foods on the growth of staphylococci.

Dahiya and Speck (1967) used an agar cup diffusion procedure to screen several strains of *Lactobacillus bulgaricus* and *Lactobacillus lactis* for inhibitory action toward staphylococci, but found only one strain of each which demonstrated any inhibitory effect.

Several workers have studied the effects of various bacteria upon growth of *S. aureus* in associative culture. Oberhofer and Frazier (1961) reported that certain strains of *Escherichia coli*, which gave no indication of inhibition upon spot plates, exhibited inhibitory tendencies when grown in association with *S. aureus*. Iandolo *et al*. (1965) found that *Streptococcus diacetilactis* was inhibitory to *S. aureus* strain MF 31 when the organisms were grown in association in 10% sterile skim milk medium. Certain coliform and *Proteus* species were inhibitory to staphylococci, according to DiGiacinto and Frazier (1965), in associative culture studies utilizing Trypticase Soy Broth as the growth medium. Kao and Frazier (1965), also using Trypticase Soy Broth, tested several lactic acid bacteria for their effects upon the growth of *S. aureus* in associative culture.

A number of workers have reported that several factors, including temperature of incubation, proportion of staphylococci in the inoculum, culture medium employed and its pH, variations between strains of staphylococci and variations between strains of effector organisms can affect the ability of an organism to compete with staphylococci.

The effects of variations in temperature have been studied by Oberhofer and Frazier (1961), Troller and Frazier (1962), Graves and Frazier (1963), DiGiacinto and Frazier (1965), Kao and Frazier (1965) and McCoy and Faber (1966). In general, these workers found that temperatures of 15 to 25 C resulted in greater inhibition of staphylococci than higher temperatures.

Peterson *et al*. (1962b), Troller and Frazier (1962), Kao and Frazier (1965) and DiGiacinto and Frazier (1965), indicated that the relative proportion of staphylococci was a very important factor in determining the degree of inhibition of staphylococci. In all cases, inhibition was greatest when the proportion of staphylococci was least.

Competition between *S. aureus* and several food microorganisms in a variety of culture media was examined by Oberhofer and Frazier (1962). It was found that the streptococci studied were inhibitory in all of the media utilized, while other species of organisms exhibited ability to inhibit *S. aureus* only in certain media. In the same study, variations between strains of *Staphylococci* were noted, with enterotoxigenic strains being more consistently inhibited by lactic acid bacteria than non-enterotoxigenic strains.

Peterson *et al*. (1962c) found that within a range of pH 6 to 8, no differences were detectable in the ability of saprophytic food organisms to compete with cultures of staphylococci. Any effects of pH they did observe appeared to be a direct result of the influence of the hydronium ion concentration upon staphylococcal growth rather than an indirect effect by virtue of an influence upon growth of the competing organisms. Troller and Frazier (1962) reported maximal inhibition of *S. aureus* by food bacteria within a pH range of 6.2 to 7.4.

Effects of Water Activity

There have been very few experiments to determine the effects of water activity upon growth and production of enterotoxin. Recently, however, Troller (1971) found that slight changes in water activity may have a marked effect upon production of enterotoxin. Utilizing two culture media, one in which the water activity was adjusted with added partially hydrolyzed protein (PHP) and NZ amine NAK, and a second in which glycerol was used for adjustment, he found that only very low levels of enterotoxin were produced at water activities of 0.97 and 0.98 in the two media, respectively. Abundant enterotoxin was produced at a water activity of 0.99 in both media. Little differences in growth of the organism were noted at these water activities.

Detection of Enterotoxin

A major problem which has confronted researchers attempting to study staphylococcal food poisoning, until fairly recently, has been the lack of a reliable assay procedure for the enterotoxin.

A great deal of work has centered upon attempts to correlate certain characteristics of staphylococci with ability to produce enterotoxin. Some of the procedures developed by this work, which may be referred to as indirect methods, are still widely used to evaluate potential toxigenicity of isolates of S. aureus.

A second group of procedures for detecting toxin involves administration of suspect foods or culture filtrates to test animals. While these methods have been plagued by limitations, they do presumably give direct evidence of toxigenicity, and hence may be referred to as direct biological methods.

Demonstration of the ability of the enterotoxin to elicit in certain animals specific antibodies which can give precipitation reactions with the antigen has led to the development of a variety of immunological methods for detection of enterotoxin. It is these procedures which have been widely used in research laboratories interested in studying the phenomenon of enterotoxigenesis.

Indirect Methods

The ability of certain strains of staphylococci to produce the enzyme coagulase, which causes the coagulation of citrated (or oxalated) rabbit plasma has generally been accepted as the most useful indirect evidence of pathogenicity. In fact, according to recent taxonomy Breed *et al.* (1957), coagulase production is an important characteristic for classification of a strain as a member of the specie *S. aureus*. Davis *et al.* (1967) has suggested that this enzyme may be related to the invasive ability of strains possessing it, and may therefore be directly related to pathogenicity. All coagulase positive staphylococci do not produce enterotoxin, but nearly all enterotoxigenic strains are coagulase positive. Thus, for lack of a direct assay for enterotoxin, the isolation of a coagulase positive staphylococcus from an incriminated food has traditionally been accepted as presumptive evidence for staphylococcal food poisoning.

The test for the production of coagulase involves the mixing of 0.5 ml of citrated rabbit plasma with approximately 0.1 ml of a BHI broth culture of the organism followed by incubation at 35 C. Presence of coagulase is evidenced by the formation of a clot within three hours (Sharf, 1968).

A number of studies have been conducted which attempted to relate ability to produce enterotoxin to phage type. Munch-Peterson (1963) summarized the results of these studies, and reported that while the majority of isolates of *S. aureus* from food poisoning cases belong to phage group III, it is not possible to reliably predict whether an isolate is capable of producing enterotoxin by phage typing.

Recent work by Brandish and Willis (1970), Stickler (1970) and Lachica *et al*. (1971) has indicated a high degree of correlation between production of deoxyribonuclease and pathogenicity. The latter authors demonstrated that the deoxyribonuclease of *S. aureus* differs from deoxyribonucleases of other micrococci and staphylococci in termal stability.

Grossegbauer *et al*. (1968) suggested that production of staphylococcal lysozyme may in fact be a better indication of pathogenicity than coagulase production.

Production of several other metabolites, including phosphatase, lipase, hemolysins, gelatinase and pigment have been investigated at various times as indicators of potential pathogenicity. However, Genigeorgis (1966) showed that all of these characteristics have a high degree of incidence among non-enterotoxigenic strains.

Direct Biological Methods

A variety of animals have been studied for susceptibility to the enterotoxins in hope of finding a suitable specific test animal. Bergdoll *et al*. (1970) summarized the literature concerning this work. Among the animals tested, monkeys and kittens have been the most reliable for detection of enterotoxin.

The kitten test was proposed by Dolman *et al*. (1936) and Dolman and Wilson (1940). A sample to be assayed by the kitten test is first heated for 20 to 30 min at 100 C to destroy hemolysins, which may give false positive results, and then centrifuged. The supernatant fluid is injected into 350 to 700 gram kittens, and enterotoxigenicity is confirmed by observation of emesis. A complicating factor in the use of kittens for detection of enterotoxin has been the occurrence of a number of non-specific reactions according to Bergdoll (1970). Dolman (1943) suggested neutralization of the hemolysins with specific antisera, which would eliminate some of the non-specific reactions. The use of trypsin to digest other proteins responsible for non-specific reactions was reported by Denny and Bohrer (1963).

The use of rhesus monkeys (*Macaca mullata*) to assay for enterotoxin was first described by Jordan and McBroom (1931) and later by Surgalla *et al*. (1953). Assays have been performed with this animal by feeding solutions of the toxic material to the monkeys by means of a catheter to the stomach and observing for vomiting within five hours, which would be considered a positive reaction to enterotoxin. Normally, six animals are used per test, and at least two must give positive results for a sample to be considered toxic. A complicating factor in the monkey assay has arisen from the fact that animals gradually become resistant to enterotoxin when used repeatedly.

Immunological Methods

Demonstration of the ability of the enterotoxins to elicit specific antibodies when injected into certain animals was utilized in the development of a number of specific serological assay procedures for the toxins. Basically, five categories of such assays have been reported: gel diffusion procedures, agglutination procedures, immunofluorescence techniques, quantitative precipitation assays and radioimmunoassays.

A complete review of gel diffusion procedures was made by Crowle (1961). Some of these procedures have been adapted for use in detection of enterotoxin.

The single gel diffusion, or Oudin, technique involves the layering of an enterotoxic solution upon an agar column containing specific antiserum. Reaction of the enterotoxin with the antiserum results in the formation of a precipitate band. The length of the band, after a given time interval, may be measured and compared with a standard reference curve to estimate toxin concentration. The first description of the use of this technique was made by Bergdoll (1962). Weirether et al. (1966) discussed modifications of the procedure, including incubation at 30 C rather than room temperature, which shortened the time of incubation to as little as 24 hours. The limit of sensitivity for single diffusion assays appears to be of the order of one to two μg of enterotoxin per ml.

The double gel tube diffusion procedure, as developed by Oakley and Fulthorpe (1955), was first applied to the assay of enterotoxin by Bergdoll _et al_. (1959a). To perform this technique, a layer of agar containing the antiserum is placed in the bottom of a glass tube. A layer of neutral agar is then placed in the tube, followed by a layer of the sample to be assayed. The reactants diffuse into the neutral layer and lines of precipitate form. Hall _et al_. (1965) reported being able to detect as little as 0.05 μg/ml of enterotoxin by this technique. The use of a comparator cell was described by Bergdoll _et al_. (1959b) as a modification of this procedure which allowed observation of coalescence with a reference line of known enterotoxin.

Bergdoll _et al_. (1956a) used the Ouchterlony plate double diffusion technique in their work on the identification of enterotoxin C. This type of assay is conducted by placing the antiserum and sample in separate wells cut in agar in a petri dish. Normally a central well containing antiserum is surrounded by several peripheral wells containing antigens. Lines of precipitate form in the agar matrix between the wells. Reference standard toxins may be placed in some of the sample wells. This technique can be used for resolution of multiple antigen-antibody systems. A major disadvantage which has limited use of agar plate diffusion has been the requirement for relatively large quantities of reagents.

Wadsworth (1957) developed a micro slide modification of the Ouchterlony technique which required much smaller quantities of reagents than the plate procedure. In this method a thin agar film is layered on a microscope slide and a Plexiglass template immediately placed upon the agar. The antigens and antisera are placed in holes drilled in the template. Normally, lines of precipitation form within 24 to 72 hours. Casman and Bennett (1965) described the use of this technique for assay of staphylococcal enterotoxins, reporting a limit of sensitivity of one μg per ml.

A hemagglutination inhibition procedure for detection of enterotoxin was reported by Morse and Mah (1967), which utilized formalinized sheep red blood cells treated with tannic acid and sensitized with enterotoxin. Dilutions of the sample tube assayed were mixed with standardized antitoxin, and the mixtures added to the sensitized erythrocytes. The titer of toxin the sample was then taken as the reciprocal of the highest dilution of the sample which prevented agglutination of the sensitized erythrocytes.

Silverman _et al_. (1968) described a reversed passive hemagglutination procedure which involved sensitization of tanned erythrocytes with antitoxin, followed by reaction with toxin solutions and observation for agglutination. Sensitivity for this procedure was claimed to be 0.0015 μg of enterotoxin per ml. The use of latex particles in place of erythrocytes for reversed passive agglutination was described by Salomon and Tew (1968).

Conjugation of antitoxin with fluorescein isothiocyanate was described by Genigeorgis and Sadler (1966b,c). The conjugates were used by the authors to stain smears of *S. aureus*, which fluoresced under ultraviolet light. A similar immunofluorescent assay was reported by Friedman and White (1965).

Quantitative precipitation was first applied by Silverman (1963) as a means of assay for enterotoxin. In this assay, equal volumes of toxin and antiserum were reacted and the precipitate which formed was assayed for nitrogen content by a micro-Kjeldahl procedure. Enterotoxin concentration was estimated by comparing the nitrogen content with a standard reference curve constructed from purified toxin-antitoxin preparations.

Johnson *et al*. (1971) recently reported an innovative radioimmunoassay employing I^{125} labeled enterotoxin and polystyrene tubes coated with specific antibody. In performing the assay for enterotoxin, unlabeled antigen in the sample being assayed was allowed to compete with the labeled antigen for antibody sites. Uptake of labeled antigen by the coated tube was a function of enterotoxin concentration in the sample.

Detection of Enterotoxins in Foods

Detection of the enterotoxins in actual foods has generally been performed using double diffusion tube and micro slide assay according to Bergdoll (1970). Since these assays are incapable of detecting less than one μg of toxin per ml of fluid, it has been necessary to concentrate the extracts from incriminated foods, which often contain much lower quantities of enterotoxin. Also, many foods contain substances which can interfere with the serological assays. Therefore, procedures developed for assay of toxin from foods have incorporated steps for removal of these interfering substances.

The extraction procedure developed by Casman and Bennett (1965) has been used more extensively than any other. The principal features of this procedure are chloroform extractions for the removal of interfering substances, and concentration by (1) dialysis against polyethylene glycol 20,000 and (2) chromatography upon carboxymethylcellulose. Zehren and Zehren (1968a) used this technique in assaying samples from 2112 vats of cheese representing more than four million pounds of cheese suspected of being contaminated with enterotoxin.

Read _et_ _al_. (1965a,b) developed similar procedures for the assay of enterotoxins from milk and from cheese. Basic features of their procedures are (1) precipitation by acidification, (2) precipitation by heating, (3) chloroform extraction to remove interfering substances and (4) dialysis against polyvinylpyrolidone to effect a concentration. The authors claimed to be able to detect as little as 0.02 to 0.05 µg of toxin per gram of sample.

Antagonistic Action by Lactic Acid Bacteria

Seven theories on the nature of antagonism of one organism towards another were listed by Waksman (1947) as follows: (1) exhaustion of nutrients, (2) physio-chemical changes in the medium, (3) pigment action, (4) action at a distance, (5) spore antagonism, (6) enzyme action and (7) production and liberation of antibiotic substances. Some of these mechanisms are involved in the antagonistic actions directed by lactic acid bacteria towards other microorganisms. In particular, production of acid, antibiotic substances and hydrogen peroxide, as well as competition for essential nutrients have been suggested as applicable mechanisms for antagonism by lactic acid bacteria.

The lactic acid bacteria produce relatively large quantities of acid, principally lactic. Other acids produced in lesser quantities include acetic and propionic. Dack and Lippitz (1962) observed that the growth of staphylococci and enteric microorganisms in slurries prepared from frozen pot pies was inhibited, apparently as a result of lowering the pH by a lactobacillus which was the predominant organism of the natural flora of the slurries.

Several researchers have studied the effect of various organic acids at low pH upon growth of microorganisms, noting that many organisms are inhibited by undissociated acids which exist at lower pH levels. Sorrells and Speck (1970) found that inhibition of *Salmonella gallinarum* by *Leucouostoc citrovorum* was principally due to the production of acetic acid, and to a lesser extent, lactic acid, both of which exerted distinct inhibitory action, disproportionate to effects of pH, upon growth of the *Salmonella*. Zehren and Zehren (1968b) following their assay of a large quantity of cheese for staphylococcal enterotoxin, reported that enterotoxin production had occurred only when low quantities of acid were produced during the manufacture of the cheese.

The production of antibiotic substances by lactic acid bacteria was reviewed by Hirsch and Grinstead (1951) and Hirsch and Wheater (1951). Two of these substances have received a degree of fame. Nisin, first described by Mattick and Hirsch (1944 and 1947), is produced by some Group N streptococci which includes strains of *Streptococcus lactis*. Diplococcin, first described by Oxford (1944), is produced by strains of *Streptococcus cremoris*. Both have been shown to exert antibiotic action toward a variety of microorganisms.

Production of hydrogen peroxide has been suspected at various times as being a factor in the antagonistic action of lactic acid bacteria. Dahiya and Speck (1967) demonstrated that hydrogen peroxide was the active factor in culture filtrates of strains of *L. bulgaricus* and *L. lactis* which were inhibitory to *S. aureus*.

Mossel and Ingram (1955) presented a discussion of the ability of one microorganism, by virtue of a rapid growth rate and consumption of vital nutrients, to retard the growth of another microorganism. Iandolo *et al.* (1965) found that depletion of vital nutrients, especially nicotinamide, was the major factor in the inhibition of the growth of *S. aureus* by *S. diacetilactis*.

METHODS AND MATERIALS

Cultures

Four enterotoxigenic strains of *S. aureus* were used in this investigation, and were identified as (1) strain 265-1 which produces type A enterotoxin, (2) strain 243 which produces type B enterotoxin, (3) strain 137 which produces type C enterotoxin, and (4) strain 361 which produces type D enterotoxin. All were obtained through the courtesy of the late Dr. E. P. Casman of the United States Food and Drug Administration, Washington, D. C.

Three strains of *S. lactis*, designated as A62, A64 and A254, as well as *S. cremoris* E8 and *Lactobacillus casei* strain 54, were obtained from the culture collection of the Department of Food Science and Human Nutrition, Michigan State University. *Pediococcus cerevisiae* strains FBB-39 and FBB-63, *Lactobacillus plantarum* strain FBB-12 and *S. lactis* G18 were provided by the Department of Microbiology and Public Health, Michigan State University. *P. cerevisiae* "accel" was obtained from Merck and Co., Rahway, New Jersey. *S. lactis* 11454 and *P. cerevisiae* 10791 were obtained from the American Type Culture Collection, Rockville, Maryland. Dr. E. H. Marth of the University of

Wisconsin provided *S. lactis* W and a strain each of *Streptococcus thermophilus*, *L. bulgaricus* and *L. citrovorum*. *S. lactis* C24 was provided by Dr. W. E. Sandine of Oregon State University.

S. aureus cultures were maintained on ATP agar (Difco) slants and were subcultured at 30-day intervals, incubated 24 hours at 37 C and stored at 4 C. All lactic cultures were maintained in APT agar stabs with calcium carbonate added and were likewise subcultured at 30-day intervals, incubated for 24 hours and stored at 4 C. All lactic subcultures were incubated at 30 C with the exception of *S. thermophilus* and *L. bulgaricus* strains, which were incubated at 37 C. Prior to inoculation into broth for associative culture and spent medium experiments, active broth cultures of the appropriate organisms were prepared by transferring once daily in APT broth for 3 days. Populations of the active broth cultures were determined for use in estimating required volumes of inoculum for the experiments.

Associative Culture Experiments

Culture Medium

APT broth (Difco) was utilized in all associative culture experiments. This medium contains the following ingredients: 0.75% Bacto-yeast extract, 1.25% Bacto-tryptone, 1.0% Bacto-dextrose, 0.5% sodium chloride, 0.5% dipotassium phosphate, 0.014% manganese chloride, 0.08%

magnesium sulfate, 0.004% ferrous sulfate and 0.02% sorbitan monoleate complex (Tween 80). APT is a suitable medium for both growth and production of enterotoxin by *S. aureus* and for growth of the lactic acid bacteria.

Enumeration of Organisms

Populations of lactic acid bacteria and total populations of mixed cultures were determined by means of APT agar pour plates incubated for 48 hours at 30 C. Staphylococcal populations were determined by means of surface smear plates upon Mannitol Salt Agar (Difco) as described by Scharf (1966) with the exception that the plates were incubated at 37 C for 48 hours.

Preliminary studies were conducted to compare estimates of the population of *S. aureus* obtained on APT pour plates, and on MSA smear plates. The population as determined on APT pour plates was an average of 2/3 of that obtained on MSA smear plates. This factor was utilized in estimating the population of effector organism in mixed culture by the following equation:

$$E = T - \frac{2}{3} S$$

where E is the population of the effector organism, T is the total population (APT pour plate) and S is the population of *S. aureus* (MSA smear plate).

Assay for Enterotoxin

The method chosen for assay of enterotoxin was the micro slide double gel-diffusion procedure described by Casman (1965). Types A, C and D reference toxins and corresponding antisera were obtained from Dr. Casman. Type B reference toxin and anti-enterotoxin B were obtained from Makor Chemicals Ltd., Jerusalem, Israel. All toxins and antisera were supplied as lyophilized preparations, and were rehydrated with a sterile diluent consisting of 1 part Brain Heart Infusion Broth (BHI, Difco) and 9 parts 0.85% NaCl solution. Reference toxins were diluted to give approximate concentrations of 1 μg per ml. Antisera were diluted as follows: Anti-A, 1:90, Anti-B, 1:10, Anti-C, 1:500, and Anti-D, 1:20.

When the presence of toxin was determined the sample was assayed directly and in dilutions of 1:10 and 1:100, 1 dilution per slide, to avoid the occurrence of false negative reactions which can occur when toxin and antiserum concentrations differ greatly. To determine toxin titer, two-fold serial dilutions of the samples in 0.85% NaCl solution were assayed, the titer being the reciprocal of the highest dilution giving a precipitin line which coalesced with that of the reference enterotoxin.

In certain instances samples were concentrated approximately 10-fold prior to being assayed. This was accomplished by lyophilization of 1 ml of the sample followed by rehydration with 0.1 ml of 0.85% saline. When concentrated samples were assayed, some clouding of the diffusion agar around the sample wells was noted; however, precepitin lines were still visible.

Survey Experiments with Associative Culture

A survey was conducted to observe the effects which representative strains of several common lactic acid culture bacteria have upon growth and enterotoxin production by *S. aureus*. For this purpose, one strain each of *S. lactis*, *S. cremoris*, *P. cerevisiae*, *S. thermophilus*, *L. plantarum*, *L. casei*, *L. bulgaricus* and *L. citrovorum* were selected as effector organisms. Experiments were conducted whereby each effector organism was grown in association with *S. aureus* 243 in APT broth, under conditions of incubation conducive to good growth of both organisms, as described below.

APT broth was prepared and 300 ml dispensed into 1-liter screw cap flasks. The flasks were autoclaved for 15 minutes at 121 C. Three flasks were required per experiment. One flask was inoculated with an active broth culture of *S. aureus*, a second with an effector organism, and a third with both organisms. Initial populations of both organisms were approximately 100,000 per ml. The flasks were incubated for 48 hours on a rotary shaker-incubator (New Brunswick Scientific Co.) at 175 rotations per minute.

The incubation temperature was 30 C in all experiments except those in which *S. thermophilus* and *L. bulgaricus* were the effector organisms, where the temperature of incubation was 41 C. Populations were determined by the methods previously described, at intervals of approximately 3 hours during the first 12 hours of incubation, and at 6 to 10 hour intervals thereafter. Fifteen ml samples were taken at the same time intervals, and centrifuged at 12,000 x *g* for 15 min in a Sorvall RC-2 refrigerated (0-5C) centrifuge. The pH of a 1 ml aliquot of the supernatant fluid was determined by means of a glass electrode Beckman research pH meter. The remainder of the supernatant fluids of the samples taken from flasks containing *S. aureus* were frozen at -23 C and held for subsequent assay for toxin. All such samples taken at 48 hours of incubation were titrated to estimate enterotoxin concentration. When toxin was detected in a 48 hour sample, subsequent assays for the presence of toxin were performed on all samples in which the staphylococcal population was determined to be at least 1,000,000 organisms per ml.

To conserve reagents and supplies only one complete determination for growth and toxin production of *S. aureus* alone was performed at each incubation temperature. In subsequent experiments, the flask inoculated with *S. aureus* alone served only as a control and as such was sampled at 0, 12, 24, and 48 hours for population, and assayed for toxin at 48 hours.

Variations in Environmental Conditions

Experiments were conducted to determine the effect which variations in certain environmental conditions would have had upon the results of the survey experiments. The experimental procedure for these investigations was basically the same as that of the survey experiments with the exception that samples were taken at 6-hour intervals throughout the period of incubation.

A preliminary study was conducted in which the pH of several APT broth samples was measured before and after autoclaving. Six flasks containing 300 ml of APT broth were then prepared. The pH of 3 of the flasks was adjusted with 1.0 *N* HCl so that the pH after sterilization was approximately 6.0. The remaining 3 flasks were similarly adjusted with 1.0 *N* NaOH to a pH after sterilization of approximately 7.0. One flask at each pH value was inoculated with *S. aureus* alone, a second with *S. lactis* A64 and a third with both organisms. The remainder of the experimental procedure, including conditions of incubation and determinations of population, pH and enterotoxin was the same as that of the survey experiments. The entire procedure was repeated with *P. cerevisiae* 10791 as the effector organism. The results were compared with those obtained in the survey investigations in which the pH was approximately 6.5.

The effect which a lower incubation temperature would have had was examined by repeating the survey procedure, but with incubation at 25 C.

To study the effect of the proportion of staphlyococci in the inoculum, 2 flasks of sterile APT broth were prepared and inoculated with *S. aureus* to give an initial population of approximately 100,000 cells per ml. Each of the flasks was then inoculated with *S. lactis* A64, but with initial populations of approximately 10,000 and 1,000,000 cells per ml, respectively, to give relative proportions of staphylococci initially of about 90% and 10%. The flasks were then incubated in the same manner as in the survey studies. The entire procedure was repeated with *P. cerevisiae* 10791 as the effector organism.

Variations in Strains of Bacteria

In order to determine the extent of the variations between strains of both effector organisms and *S. aureus* with respect to interactions in associative culture, two series of studies were conducted. In the first series, several strains each of *S. lactis* and *P. cerevisiae* were utilized as effector organisms, with *S. aureus* 243 serving as the test organism. In the second series, *S. aureus* strains 265-1, 137, and 361 were used as test organisms and one strain each of *S. lactis* and *P. cerevisiae* were used as effectors. The experimental procedure was the same as that of the survey studies with the exception that samples were taken at 6-hour intervals throughout the incubation period.

Studies on the Role of Acid Production in the Inhibition of S. aureus by the Lactic Acid Bacteria

Determination of Lactic Acid Production by S. lactis and P. cerevisiae

One flask containing 300 ml of sterile APT broth was inoculated with *S. lactis* A64, and a second with *P. cerevisiae* 10791. The initial population in both flasks was approximately 100,000 cells per ml. Both flasks were incubated at 30 C and 175 gyrations per min on the shaker-incubator and 15-ml samples taken at 0, 3, 6, 9, 12, 18, 24, and 30 hours. The samples were centrifuged for 15 min at 12,000 x *g*, and a 10-ml aliquot of the supernatant fluid titrated with 0.1 N NaOH to the phenolpthalein end point. The total titratable acidity for each sample was then calculated as lactic acid according to the equation:

$$\text{Percent acid} = \frac{\text{ml of } 0.1\text{ N NaOH} \times 0.009 \times 100}{\text{wt. of sample in grams}}$$

Growth of S. aureus in Acidified Broth

APT broth was prepared and 100 ml dispersed into each of nine-500 ml screw cap Erlenmeyer flasks. Reagent grade 85% lactic acid (Malinckrodt Chemical Works) was added to

8 of the flasks to give final concentrations of 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.40, and 0.50 percent, respectively. The ninth flask served as a control. The flasks were autoclaved at 121 C for 15 min and cooled. The pH of the contents of the flasks was then determined by means of the glass electrode Beckman research pH meter. Each of the flasks was inoculated with 1 ml of an active broth culture of _S. aureus_ 243, and incubated on the rotary shaker-incubator at 175 gyrations per minute and at 30 C. Samples were taken at intervals of 1 hour, and the absorbance was measured at 620 nm by means of a Spectronic 20 Colorimeter (Basuch and Lomb). Each sample was measured against a blank consisting of APT broth plus lactic acid at a concentration corresponding to that of the sample. The results were plotted as log absorbance versus time for each culture, and the specific growth rate constant, k, calculated from the maximum slope according to the equation:

$$k = \frac{(2.3)(\log N_2 - \log N_1)}{t_2 - t_1}$$

where N_1 and N_2 represent numbers of cells at times t_1 and t_2 (generally, 5 and 7 hours of incubation). Generation time (g) was then calculated for each culture by:

$$g = 0.69/k$$

Agar Diffusion Cup Assays

APT agar (Difco) was prepared, dispensed into 8 oz prescription bottles, 100 ml per bottle, and sterilized at 121 C for 15 min. The medium was held in a water bath at 48 C until used. One ml of an active broth culture of *S. aureus* 243 was added to each bottle. The contents of the bottles were well mixed, and dispensed into sterile petri dishes, 20 ml per dish. The agar was allowed to harden, and a well approximately 1/4 inch in diameter cut into the agar, near the center of the plate, with a sterile spatula. APT agar suspensions of the effector organisms prepared in the same manner as the *S. aureus* suspensions were pipetted into the holes and allowed to harden. The plates were incubated for 24 hours at 30 C and examined for zones of clearing around the wells.

Investigations Using Spent Broth

Preparation of Spent Broth

Two 1-liter flasks containing 600 ml of sterile APT broth were prepared as previously described. One of the flasks was inoculated with 6 ml of an active broth culture of *S. lactis* A64, the other with 6 ml of an active broth culture of *P. cerevisiae* 10791. Both flasks were incubated for 18 hours at 30 C on the rotary-shaker incubator at 175 revolutions per minute. The contents of the flasks were then centrifuged at 8,000 x g for 30 minutes in a Sorvall RC-2 refrigerated centrifuge. The supernatant fluids, referred to hereafter as spent broth, were adjusted to a pH of 6.5 with 40% NaOH, and divided into 100 ml fractions. Additions were made to the fractions as follows:

Fraction	Spent broth produced by growth of	Additive
1	*S. lactis*	0.03% catalase
2	*S. lactis*	1.0% glucose
3	*S. lactis*	1.25% trypton
4	*S. lactis*	0.75% yeast extract
5	*S. lactis*	none (control)
6	*P. cerevisiae*	0.03% catalase
7	*P. cerevisiae*	1.0% glucose
8	*P. cerevisiae*	1.25% tryptone
9	*P. cerevisiae*	0.75% yeast extract
10	*P. cerevisiae*	none (control)

All fractions were then sterilized by millipore filtration, and collected in sterile 250 ml Erlenmeyer flasks fitted with cotton plugs.

A second series of spent broth fractions was prepared as above but with additions as follows:

Fraction	Spent broth produced by growth of	Additive
1	*S. lactis*	100 μg/ml thiamine HCl
2	*S. lactis*	100 μg/ml nicotinic acid

Fraction	Spent broth produced by growth of	Additive
3	*S. lactis*	100 µg/ml biotin
4	*S. lactis*	none (control)
5	*P. cerevisiae*	100 µg/ml thiamine HCl
6	*P. cerevisiae*	100 µg/ml nicotinic acid
7	*P. cerevisiae*	100 µg/ml biotin
8	*P. cerevisiae*	none (control

The catalase and vitamins used were obtained from Nutritional Biochemicals Corporation. The glucose, tryptone, and yeast extract used were obtained from Difco Laboratories.

Growth in Spent Broth

Two growth experiments were conducted utilizing the two series of spent broth fractions plus additives. The following procedure was used in both studies:

All flasks were inoculated with 1 ml of an active broth culture of *S. aureus* 243 and incubated at 30 C on the rotary shaker at 175 revolutions per minute. The absorbance at 620 nm was determined on the contents of each flask at 1 hour intervals, in a Spectronic 20 Colorimeter.

Cell Disruption Experiments

Preparation of Washed Cells

Five hundred-ml quantities of sterile BHI broth in 1 liter screw cap Erlenmeyer flasks were inoculated with 1% of an active broth culture of _S. aureus_ 243. The flasks were incubated for 24 hours on the rotary shaker-incubator at 37 C and 175 gyrations per minute. The cells were harvested by centrifugation at 7,900 x _g_ for 30 min in a Sorvall RC-2 refrigerated (0-5 C) centrifuge. The cells were then washed by resuspension in a quantity of 0.85% NaCl solution approximately equal to the original volume of broth, and then recentrifuged. A second washing was performed in the same manner. A third washing was similarly performed but with suspension in a volume of saline approximately 1/50 of the original volume of broth. Samples of the supernatant fluids from the growth medium and wash solutions were frozen at -23 C and held for toxin assay. The packed, washed cells were stored at 4 C in 4-g portions.

Grinding

A 4-g portion of the cells was mixed with 20 g of sterile washed sand. The mixture was ground in a mortar and pestle for approximately 15 minutes. Grinding was performed at approximately 4 C in a cold room. The mixture was then suspended in 20 ml of sterile de-ionized water prior to separation of the extract from cellular debris and sand.

Freezing and Thawing

A 4-g portion of the cells was suspended in 20 ml of sterile de-ionized water. The suspension was placed in a -23 C freezer until frozen. The sample was then placed in a 25 C incubator until completely thawed. The cycle of freezing and thawing was repeated a total of 10 times. The total elapsed time was 24 hours.

Sonic Treatment

A 20-ml suspension of 4 g of the packed cells in de-ionized water was placed in a 28-mm x 100 mm stainless steel centrifuge tube. The tube was placed in a mixture of alcohol and ice to maintain a temperature of -10 C or lower. Sufficient dry ice was added to the alcohol-ice mixture to maintain the desired temperature. The contents of the tube were subjected to sonic treatment by a Branson L 575 Sonifier (Heat Systems-Ultrasonics Inc., Plainview, N.Y.) operated at 9 amp for 15 minutes.

Acetone Powder Procedure

Four g of the packed cells were suspended in 20 ml of sterile de-ionized water. The suspension was slowly added to 200 ml of cold acetone (-10 C or colder) with constant stirring. The cells were removed by vacuum filtration using a Büchner funnel, placed in an aluminum foil moisture dish, and dried in a desiccator for 24 hours. The dried cells were ground in a mortar and pestle and mixed with 20 ml of sterile de-ionized water.

Toluene Treatment

A suspension of a 4-g portion of the cells in 20 ml of sterile de-ionized water was mixed with 10 ml of toluene in a 100 ml beaker. The mixture was incubated, with frequent agitation, in a water bath at 37 C for 15 min, and then at 45 C for an additional 15 min. The mixture was transferred to a test tube, allowed to stand for 10 min and the toluene layer was aspirated off with a Pasteur pipet.

Lysozyme Treatment

Four g of the packed cells were suspended in 20 ml of sterile de-ionized water. Four mg of crystalline egg white lysozyme (Nutritional Biochemicals Corp.) was mixed with the suspension, and the mixture incubated for 2 hours at 30 C.

Separation of Cell Extracts from Cellular Debris

After application of the above methods of disintegration, cellular debris and unbroken cells were removed by centrifugation in a Sorvall RC-2 centrifuge operated at 12,000 x *g* for 30 min. Merthiolate (Eli Lilly and Co.) was added to the supernatant fluids to a final dilution of approximately 1/10,000. The supernatant fractions were held at 4 C until assayed for enterotoxin.

Determination of Relative Efficiency of Disintegration

Estimates of the comparative ability of each of the disintegration procedures to release proteinaceous material from the cells were made as follows:

Samples of the supernatant fractions were diluted 1/20 in de-ionized water. The absorbancies of the diluted specimens at 280 nm were then determined on a Beckman DBG Spectrophotometer.

Toxin Assay

All supernatant fractions were assayed for quantity of enterotoxin by the method previously described.

Effects of Disintegration Upon Enterotoxin

A sample of supernatant fluid from the original BHI broth was assayed for quantity of enterotoxin. Twenty milliliter fractions of the fluid were then subjected to treatment by the disintegration procedures which appeared not to have caused release of toxin from the cells and then reassayed.

RESULTS

Effect of Lactic Acid Bacteria Upon Growth and Production of Enterotoxin by S. aureus

Survey of Experiments with Associative Cultures

Figures 1-8 illustrate the data obtained in experiments in which *S. aureus* 243 was grown alone and in association with the strains of lactic acid bacteria selected as effector organisms. Also illustrated in the same figures are the results of assays for the presence of enterotoxin, and data indicating changes in the pH of the growth medium during incubation. These figures show that all of the streptococci used as effectors inhibited growth of *S. aureus* 243 and prohibited production of enterotoxin. *P. cerevisiae* 10791 also inhibited growth of *S. aureus* 243, and effected delay in enterotoxin production. Inhibition of the growth of *S. aureus* 243 by the lactobacilli and *L. citrovorum* was only slight when compared to inhibition by the streptococci and *P. cerevisiae.* All of the lactobacilli caused a delay in the production of detectable quantities of enterotoxin. Production of enterotoxin by *S. aureus* 243 when grown in association with *L. citrovorum* appeared to be unaffected.

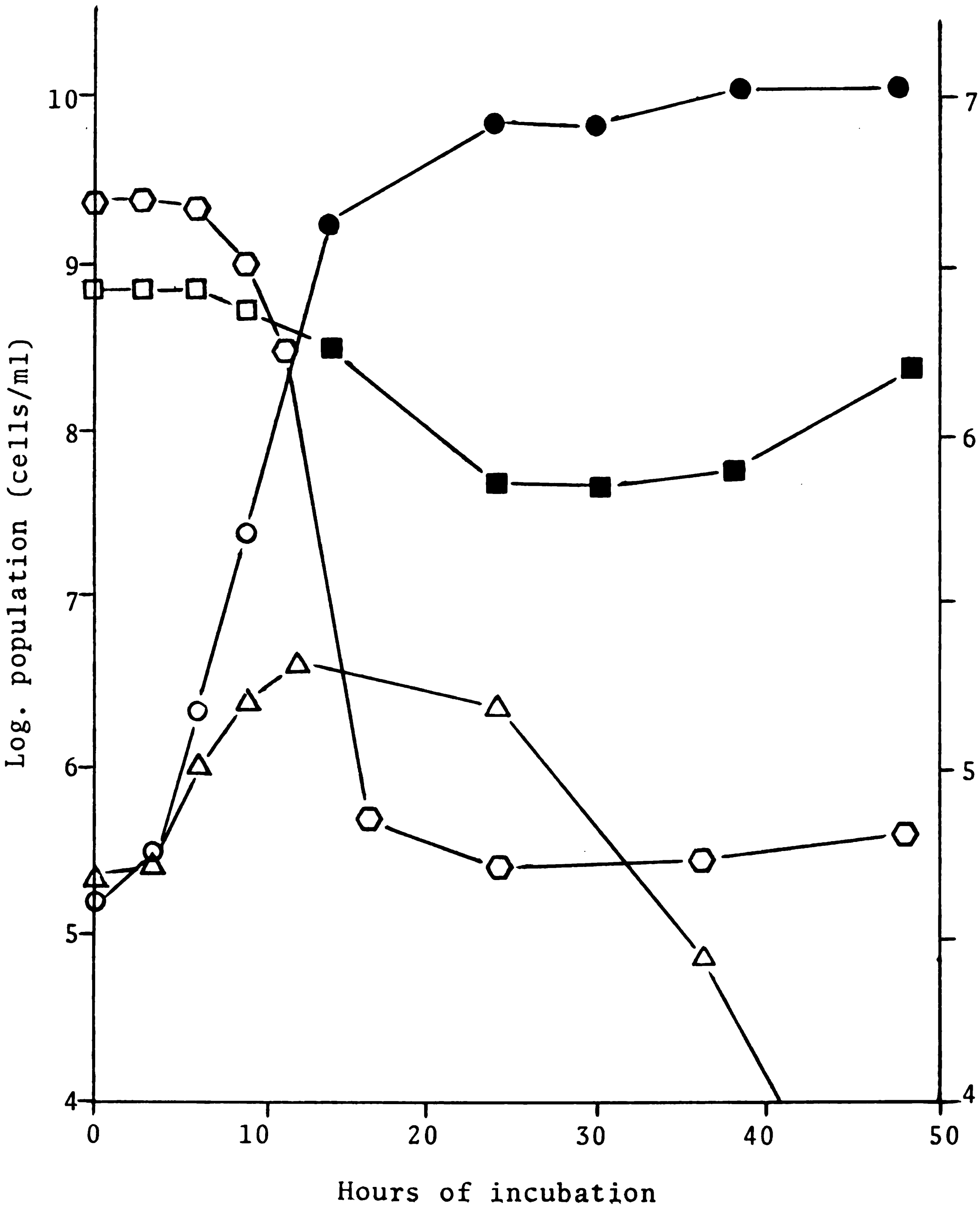

Figure 1.--Effect of association with S. lactis A64 upon growth of S. aureus 243 and production of enterotoxin in APT broth at 30 C. Initial populations were equal. Legend: ○ population of S. aureus alone; △ population of S. aureus in mixed culture; □ pH of S. aureus alone; ⬡ pH of mixed culture. Shaded symbols indicate enterotoxin was detected.

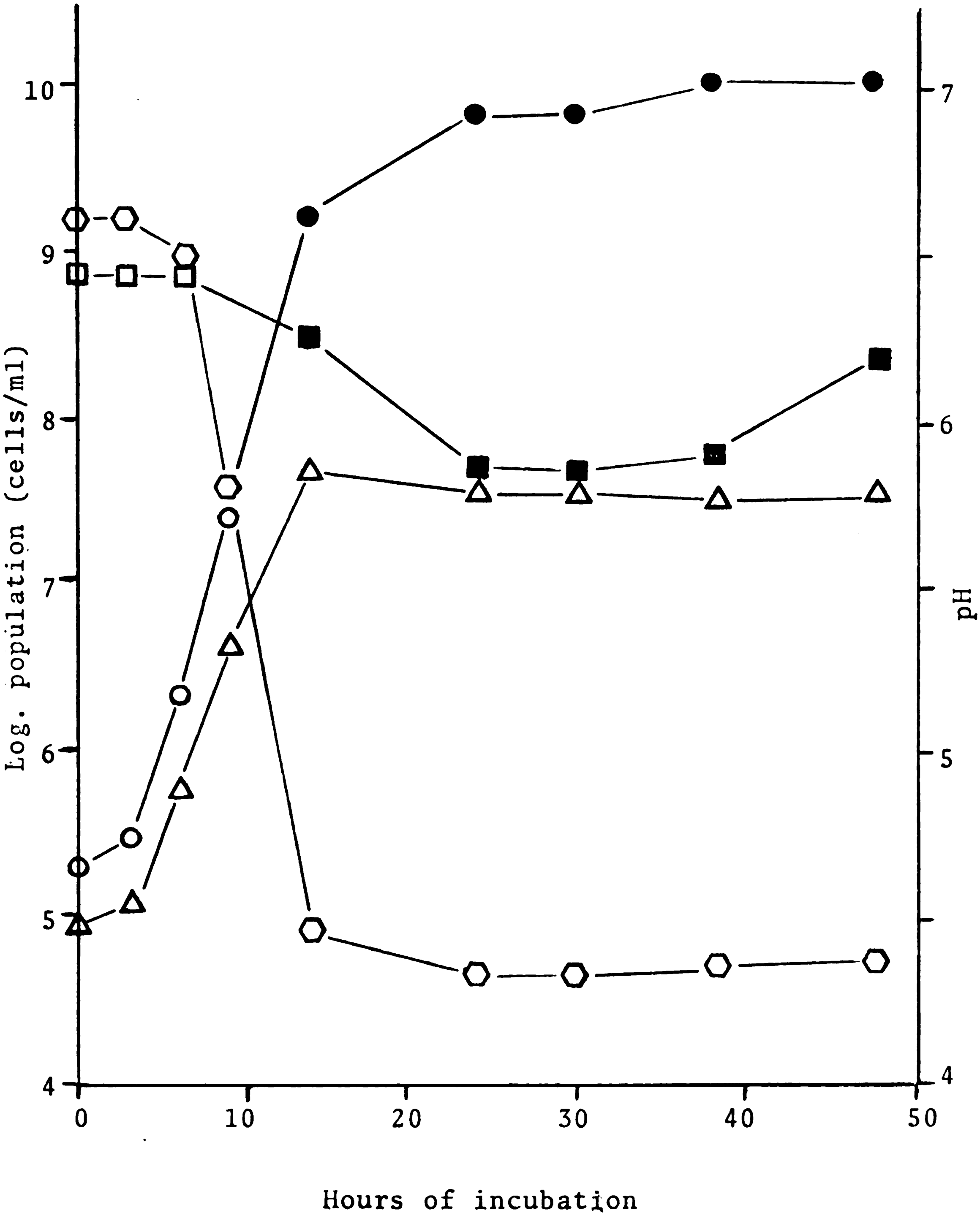

Figure 2.--Effect of association with S. *cremoris* E8 upon growth of S. *aureus* 243 and production of enterotoxin in APT broth at 30 C. Initial populations were equal. Legend: ○ population of S. *aureus* alone; △ population of S. *aureus* in mixed culture; □ pH of S. *aureus* alone; ⬡ pH of mixed culture. Shaded symbols indicate enterotoxin was detected.

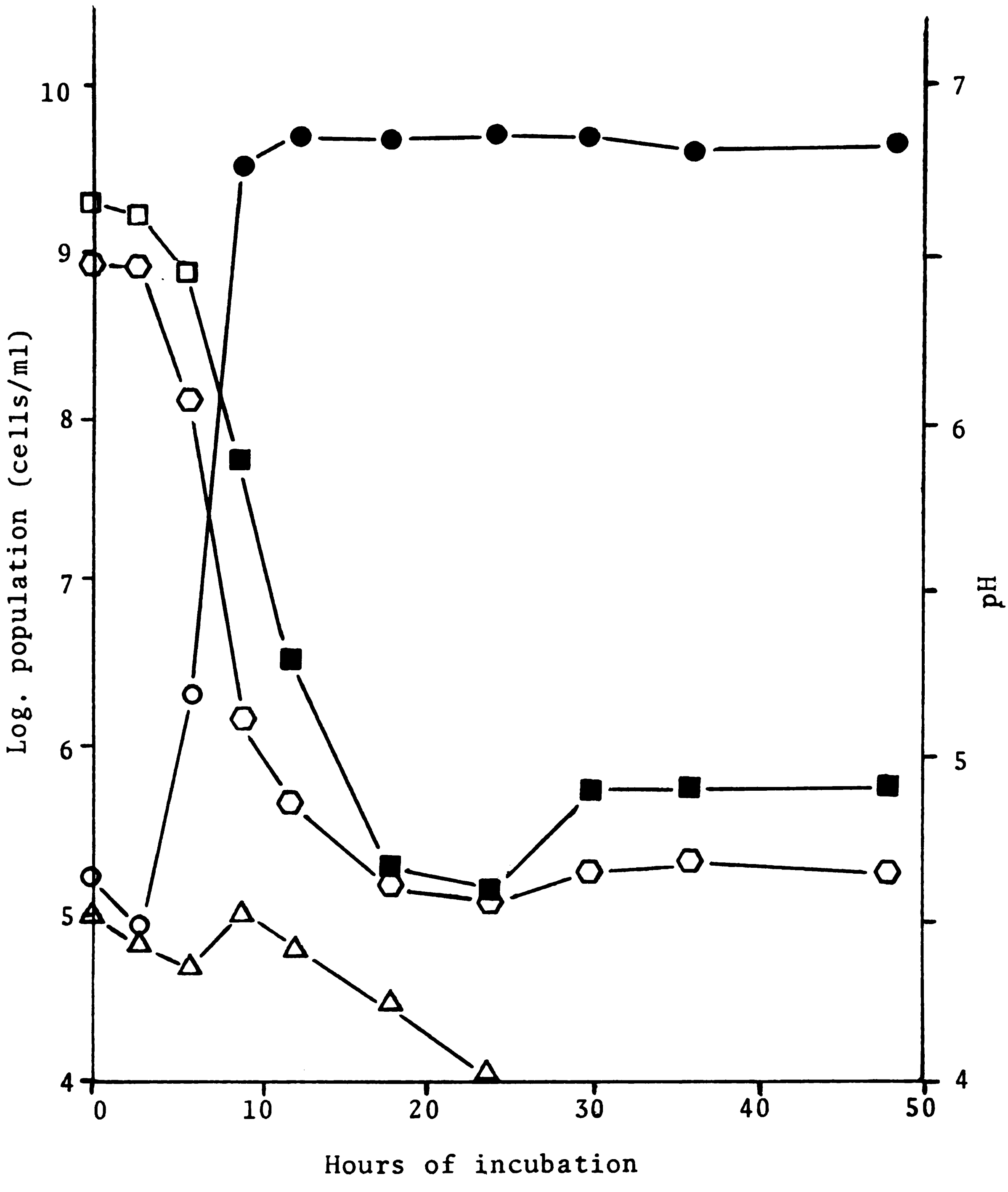

Figure 3.--Effect of association with *S. thermophilus* W upon growth of *S. aureus* 243 and production of enterotoxin in APT broth at 41 C. Initial populations were equal. Legend: ○ population of *S. aureus* alone; △ population of *S. aureus* in mixed culture; □ pH of *S. aureus* alone; ⬡ pH of mixed culture. Shaded symbols indicate enterotoxin was detected.

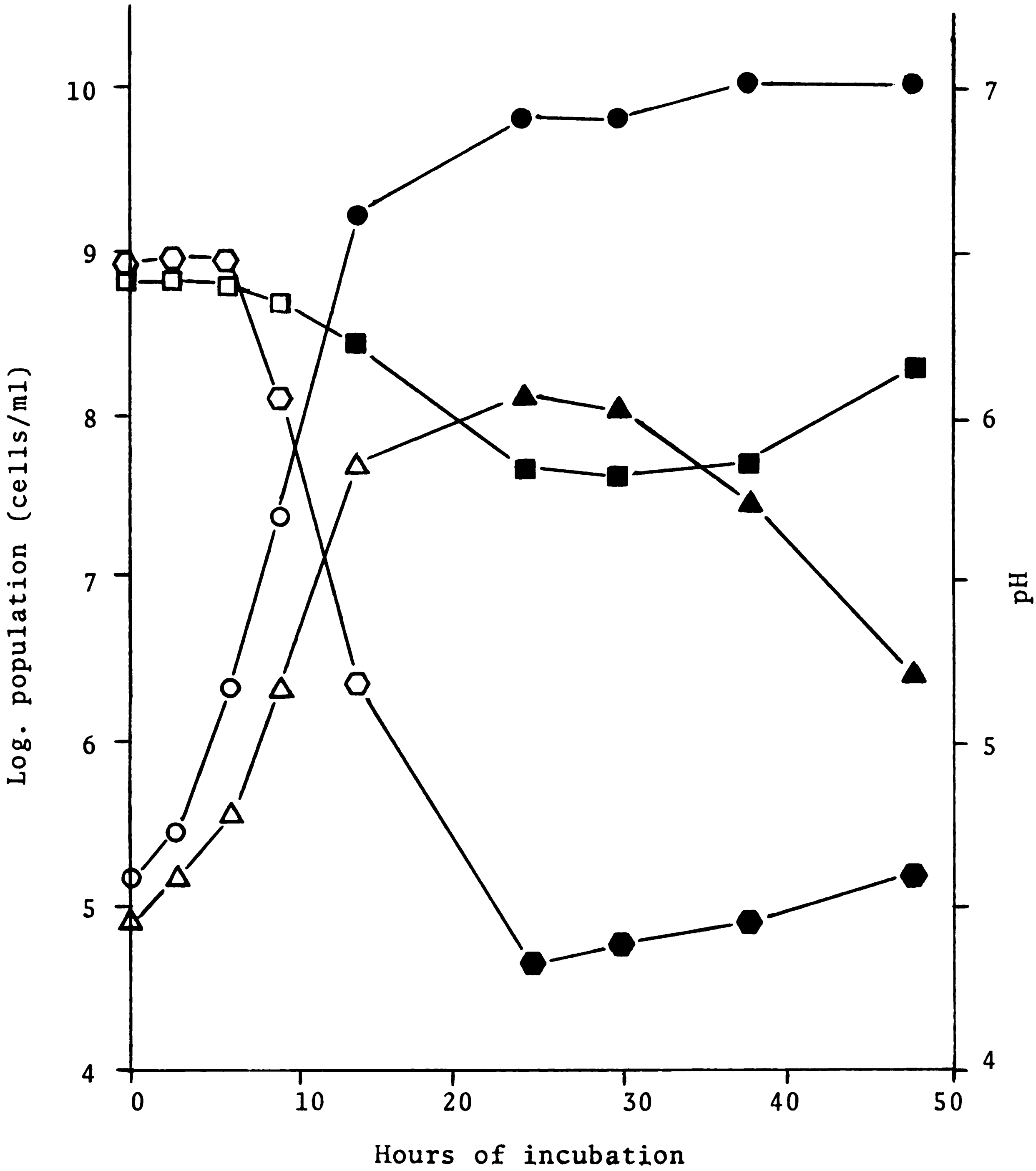

Figure 4.--Effect of association with P. cerevisiae 10791 upon growth of S. aureus 243 and production of enterotoxin in APT broth incubated at 30 C. Initial populations were equal. Legend: ○ population of S. aureus alone; △ population of S. aureus in mixed culture; □ pH of S. aureus alone; ⬡ pH of mixed culture. Shaded symbols indicate enterotoxin was detected.

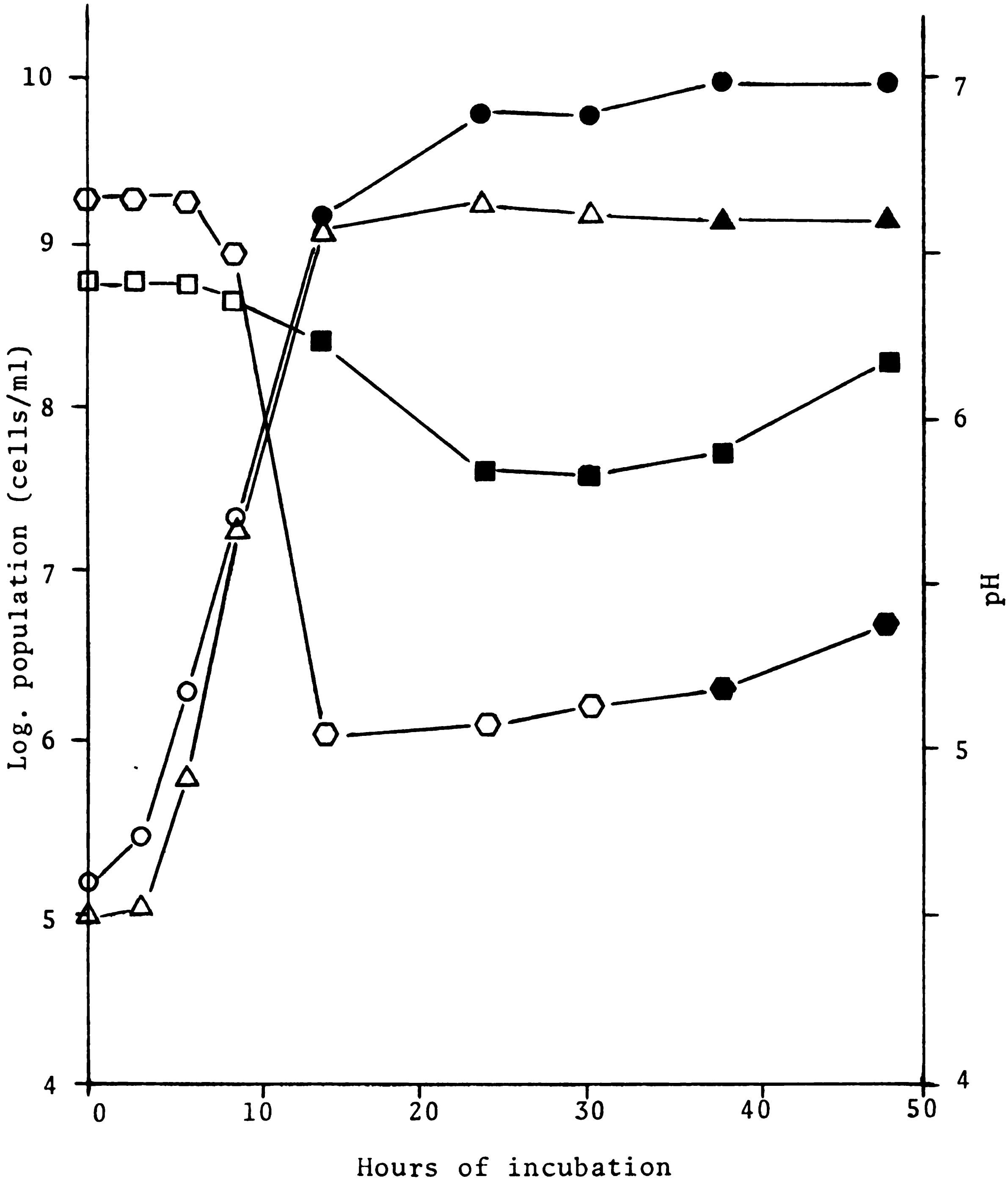

Figure 5.--Effect of association with *L. casei* 54 upon growth of *S. aureus* 243 and production of enterotoxin in APT broth at 30 C. Initial populations were equal. Legend: ○ population of *S. aureus* alone; △ population of *S. aureus* in mixed culture; □ pH of *S. aureus* alone; ⬡ pH of mixed culture. Shaded symbols indicate enterotoxin was detected.

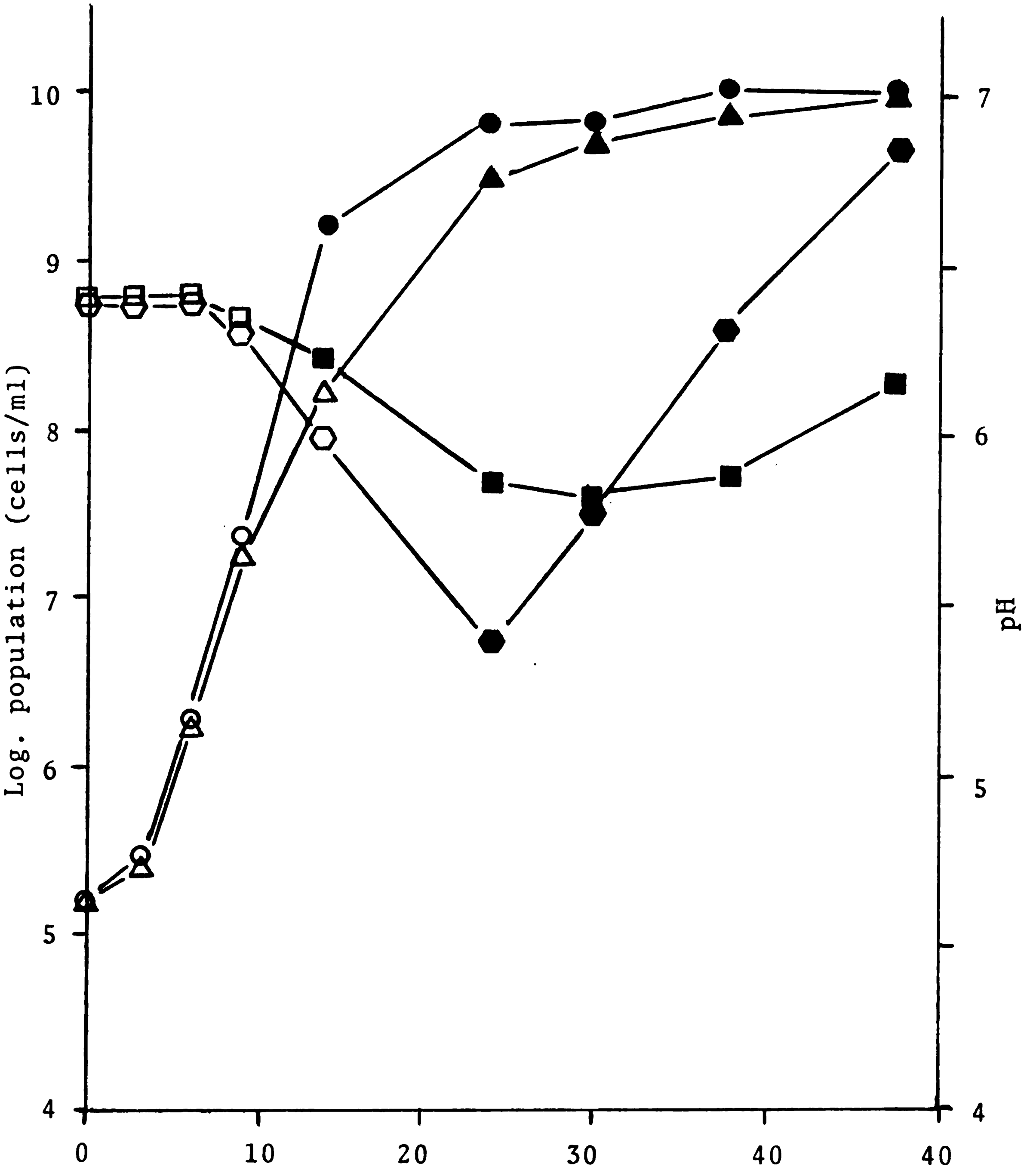

Figure 6.--Effect of association with L. plantarum FBB 12 upon growth of S. aureus 243 and production of enterotoxin in APT broth at 30 C. Initial populations were equal. Legend: ○ population of S. aureus alone; △ population of S. aureus in mixed culture; □ pH of S. aureus alone; ⬡ pH of mixed culture. Shaded symbols indicate enterotoxin was detected.

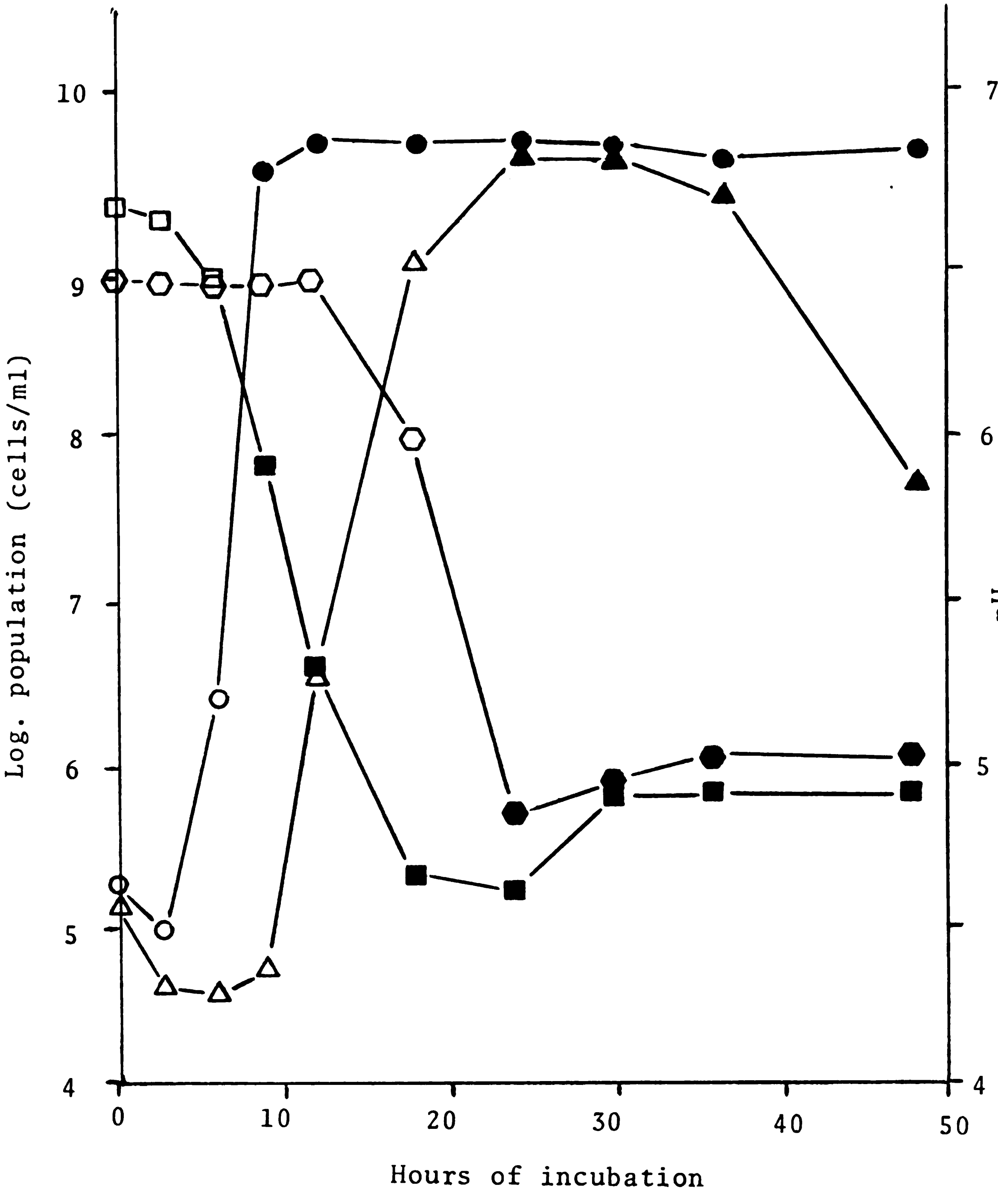

Figure 7.--Effect of association with L. bulgaricus W upon growth of S. aureus 243 and production of enterotoxin in APT broth at 41 C. Initial populations were equal. Legend: ○ population of S. aureus alone; △ population of S. aureus in mixed culture; □ pH of S. aureus alone; ⬡ pH of mixed culture. Shaded symbols indicate enterotoxin was detected.

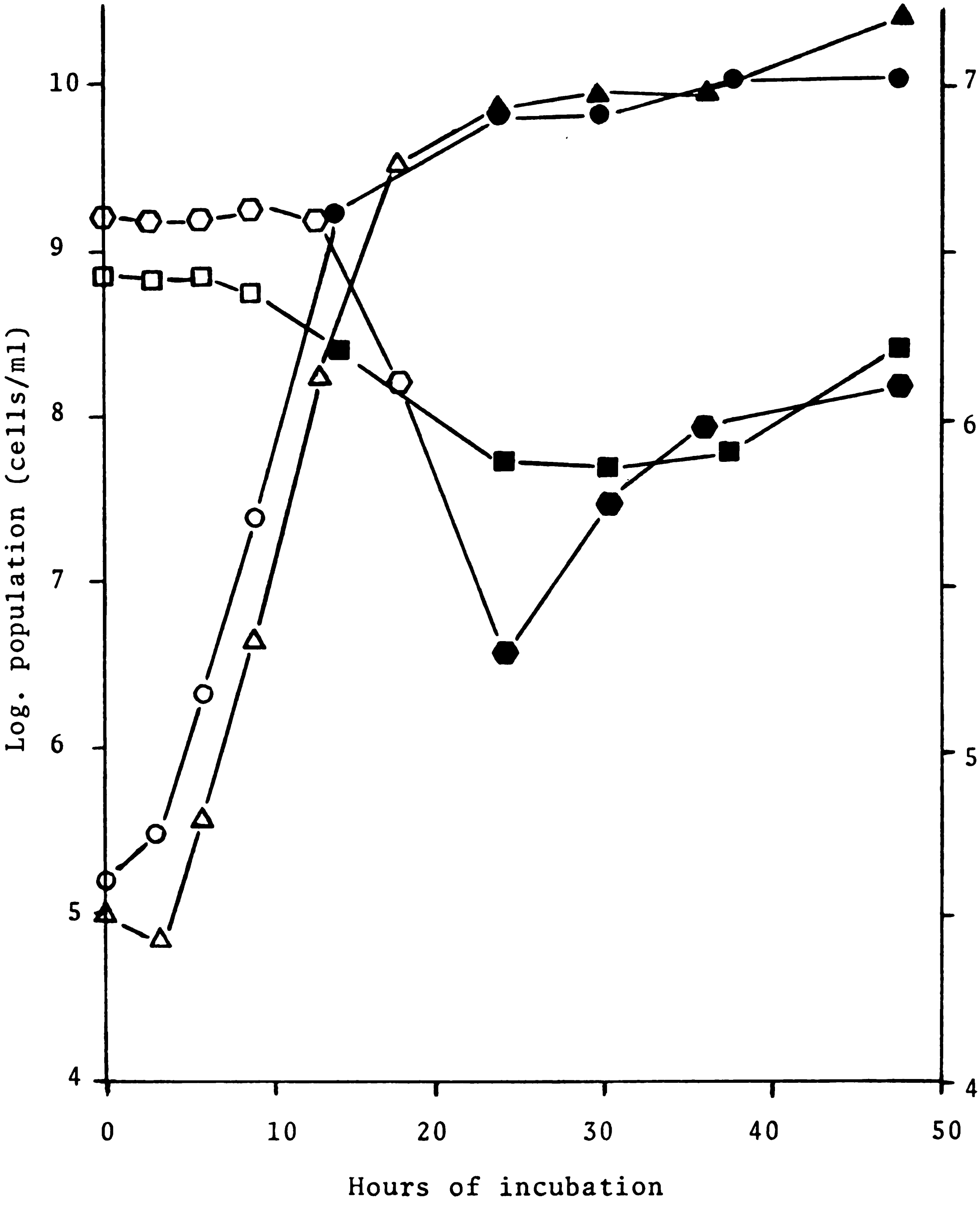

Figure 8.--Effect of association with L. citrovorum W upon growth of S. aureus 243 and production of enterotoxin in APT broth at 30 C. Initial populations were equal. Legend: ○ population of S. aureus alone; △ population of S. aureus in mixed culture; □ pH of S. aureus alone; ⬡ pH of mixed culture. Shaded symbols indicate enterotoxin was detected.

Data given in Table 1 illustrate the effects of association with the lactic acid bacteria upon the quantity of enterotoxin B produced by *S. aureus* 243. No enterotoxin was detected when *S. aureus* 243 was grown in association with the streptococci. The quantity of enterotoxin detected was considerably lower than the control when *P. cerevisiae* 10791 was used as the effector organism. Association with *L. casei* and *L. plantarum* FBB-12 resulted in some reduction of the quantity of enterotoxin produced by *S. aureus* 243. Neither *L. bulgaricus* nor *L. citrovorum* appeared to have any effect on the quantity of enterotoxin produced.

Effects of Variations in Certain Environmental Conditions Upon Associative Culture Experiments

Table 2 represents a summary of the data obtained from the experiments conducted to determine the effect of minor changes in the initial pH of the growth medium upon growth and production of enterotoxin by *S. aureus* 243 in association with *S. lactis* A64 and *P. cerevisiae* 10791. In general, the effects observed were slight. It was noted, however, that the maximum population of *S. aureus* appeared to increase slightly as the initial pH increased. Similarly, production of enterotoxin was greater at an initial pH of approximately 7.0 than at 6.0 or 6.5.

Data obtained from experiments conducted to determine the effects of incubation at 25 C upon growth and production

Table 1. Production of enterotoxin B by *S. aureus* 243 in APT broth after 48 hours incubation when grown alone and in association with various lactic acid bacteria at the temperature indicated.

Effector Organism	Incubation Temperature (Degrees C)	Toxin Titer
None	30	16
None	41	32
S. lactis A64	30	N.D.[a]
P. cerevisiae 10791	30	1
S. cremoris E8	30	N.D.
S. thermophilus W	41	N.D.
L. bulgaricus W	41	32
L. casei	30	2
L. plantarum FBB12	30	8
L. citrovorum W	30	16

[a] None detected in direct assay or in sample concentrated 10-fold after incubation for 48 hours.

Table 2. Effect of initial pH upon growth and production of enterotoxin by *S. aureus* 243 grown alone and in association with *S. lactis* A64 and *P. cerevisiae* 10791 in APT broth at 30 C.

Effector Organism	Approximate initial pH	Maximum population of *S. aureus* observed (cells/ml)	Minimum pH observed	Time required for production of measurable toxin (hr.)	Total toxin (titer)
None	6.0	7.3×10^{9}	5.29	18	8
S. lactis	6.0	1.7×10^{6}	4.21	--	N.D.[b]
P. cerevisiae	6.0	4.0×10^{7}	4.24	--	N.D.
None	6.5[a]	1.1×10^{10}	5.85	14	16
S. lactis	6.5	3.7×10^{6}	4.47	--	N.D.
P. cerevisiae	6.5	1.7×10^{8}	4.34	24	1
None	7.0	1.3×10^{10}	6.42	12	32
S. lactis	7.0	6.6×10^{6}	4.56	--	N.D.
P. cerevisiae	7.0	7.3×10^{8}	4.55	24	1

[a] Approximate pH of APT (non-adjusted).

[b] None detected in direct assay or in sample concentrated 10-fold.

of enterotoxin by *S. aureus* 243 in association with *S. lactis* A64 and *P. cerevisiae* 10791 are summarized in Table 3. Also included in Table 3 are corresponding data obtained when incubation was at 30 C. Both growth and enterotoxin production by *S. aureus* 243 in associative culture were inhibited to a greater degree at 25 C than at 30 C. Temperatures significantly higher than 30 C would have been unrealistic in terms of conditions of handling of foods in which the majority of the lactic culture bacteria are used. Therefore, no experiments were conducted to determine the effects of higher incubation temperatures.

Data in Table 4 represent a comparison of the results from the investigations to determine the effect of the relative proportion of staphylococci in the inoculum upon growth and production of enterotoxin by *S. aureus* when grown in association with other organisms. When either *S. lactis* A64 or *P. cerevisiae* 10791 was used as the effector organism, inhibition of both growth and production of enterotoxin was greatest when the relative proportion of staphylococci in the inoculum was least. It was noted, however, that the effect of the proportion of staphylococci was more pronounced when *S. lactis* A64 was the effector organism than when *P. cerevisiae* 10791 was the effector organism.

Table 3. Comparison of growth and production of enterotoxin by *S. aureus* 243 at 25 C and 30 C when grown alone and in association with *S. lactis* A64 and *P. cerevisiae* 10791 in APT broth.

Effector Organism	Incubation Temperature (degrees C)	Maximum population of *S. aureus* observed (cells/ml)	Minimum pH observed	Time required for production of measurable toxin (hr.)	Total toxin (titer)
None	25	$1.1\text{x}10^{10}$	5.33	18	8
S. lactis	25	$4.1\text{x}10^{5}$	4.31	--	N.D.[a]
P. cerevisiae	25	$6.0\text{x}10^{5}$	4.32	--	N.D.
None	30	$1.1\text{x}10^{10}$	5.85	14	16
S. lactis	30	$3.7\text{x}10^{6}$	4.47	--	N.D.
P. cerevisiae	30	$1.7\text{x}10^{8}$	4.34	24	1

[a] None detected either in direct assay or in sample concentrated approximately 10-fold.

Table 4. Effect of ratio of staphylococci to effector organism upon growth and production of enterotoxin by S. aureus 243 grown in association with S. lactis A64 and P. cerevisiae 10791 in APT broth at 30 C.

Effector Organism	Percent of effector in inoculum	Maximum population of S. aureus observed (cells/ml)	Minimum pH observed	Time required for production of measurable toxin (hr.)	Total toxin (titer)
None	--	$1.1 x 10^{10}$	5.85	14	16
S. lactis	10	$1.0 x 10^{10}$	5.19	24	8
S. lactis	50	$3.7 x 10^{6}$	4.47	--	N.D.[a]
S. lactis	90	$2.3 x 10^{5}$	4.51	--	N.D.
P. cerevisiae	10	$4.7 x 10^{8}$	4.50	24	2
P. cerevisiae	50	$1.7 x 10^{8}$	4.44	24	1
P. cerevisiae	90	$1.6 x 10^{7}$	4.34	--	N.D.

[a] None detected either in direct assay or in sample concentrated approximately 10-fold.

Variations Between Strains of Organisms

A summary of the data obtained from studies conducted to determine the extent of variation between strains of effector bacteria, with respect to the antagonism of staphylococci, are given in Table 5. Only small differences in both the growth of *S. aureus* 243 and production of toxin were detected as a result of variations between strains of effector organisms. The variations observed between strains of either species were not as great as the variation between *S. lactis* and *P. cerevisiae*. It is noteworthy, however, that one of the strains of *P. cerevisiae*, FBB-39, did apparently prohibit production of a detectable quantity of enterotoxin by the *Staphylococcus*, while the other *Pediococcus* strains did not.

Data in Table 6 show a summary of the results obtained from investigations into the extent of variations between strains of enterotoxigenic staphylococci in susceptibility to antagonism by lactic acid bacteria. The range of maximum populations observed when the staphylococci were grown alone was of the same order of magnitude as the range observed when the staphylococci were grown with either of the effector organisms. Thus, only small differences between strains of *S. aureus* in ability to grow in association with a given effector organism were observed. Differences in the quantities of enterotoxin produced by the different strains were great. However, the strains of

Table 5. Comparison of the effects of several strains of *S. lactis* and *P. cerevisiae* upon growth and production of enterotoxin by *S. aureus* 243 when grown in association in APT broth at 30 C.

Effector Organism	Maximum staphylococcus population (cells/ml)	Minimum pH observed	Time required for production of measurable toxin (hr.)	Total toxin (titer)
S. lactis A62	2.9×10^{6}	4.38	--	N.D.[a]
S. lactis A64	3.7×10^{6}	4.47	--	N.D.
S. lactis A254	5.7×10^{6}	4.85	--	N.D.
S. lactis G18	3.8×10^{6}	4.23	--	N.D.
S. lactis W	4.1×10^{6}	4.39	--	N.D.
P. cerevisiae 10791	1.7×10^{8}	4.34	24	1
P. cerevisiae FBB 13	8.1×10^{7}	4.43	24	1
P. cerevisiae FBB 39	3.2×10^{7}	4.30	--	N.D.
P. cerevisiae "accel"	9.2×10^{7}	4.24	24	1
None	1.1×10^{10}	5.85	18	16

[a] None detected in direct assay or in sample concentrated 10-fold.

Table 6. Comparison of susceptibility of four enterotoxigenic strains of *S. aureus* to antagonism by *S. lactis* A64 and *P. cerevisiae* 10791 when grown in association in APT broth at 30 C.

Strain of staphylococcus	Enterotoxin serotype	Effector organism	Maximum population observed (cells/ml)	Minimum pH observed	Time required for production of measurable toxin (hr.)	Total toxin (titer)
265-1	A	None	1.5×10^{10}	5.34	12	2
265-1	A	*S. lactis*	3.1×10^{6}	4.31	--	N.D.[a]
265-1	A	*P. cerevisiae*	5.2×10^{8}	4.55	24	0.1[b]
243	B	None	1.1×10^{10}	5.85	18	16
243	B	*S. lactis*	3.7×10^{6}	4.47	--	N.D.
243	B	*P. cerevisiae*	1.7×10^{8}	4.34	24	1
137	C	None	7.2×10^{9}	5.64	18	8
137	C	*S. lactis*	1.7×10^{6}	4.42	--	N.D.
137	C	*P. cerevisiae*	1.6×10^{8}	4.53	24	0.4[b]
361	D	None	8.8×10^{9}	5.84	18	1
361	D	*S. lactis*	3.0×10^{5}	4.39	--	N.D.
361	D	*P. cerevisiae*	3.1×10^{8}	4.56	--	N.D.

[a] None detected in direct assay or in sample concentrated 10-fold.

[b] Samples concentrated 10-fold.

S. *aureus* which produced the least amount of enterotoxin in associative culture, 265-1 and 315, also produced comparatively small amounts when grown alone. It is perhaps significant that none of the *Staphylococcus* strains was able to produce detectable enterotoxin when grown in association with S. *lactis* A64.

Mechanisms of Inhibition of S. aureus by the Lactic Acid Bacteria

An initial study was conducted to determine the quantities of acid produced by both S. *lactis* A64 and P. *cerevisiae* 10791 at 30 C after various incubation times. The results are given in Tables 7 and 8. Since both species are homofermentative and produce predominantly lactic acid, the total titratable acidities were calculated as lactic acid.

Data in Table 9 illustrate the ability of S. *aureus* 243 to grow in APT broth containing various amounts of lactic acid. The addition of lactic acid in amounts of up to 0.20% resulted in an increase in the growth rate of the *Staphylococcus* compared to the control culture. Reduced growth rates occurred with additions of 0.25% and 0.30% lactic acid. Growth of S. *aureus* 243 was completely inhibited by the addition of 0.40% and 0.50% lactic acid. Significantly the initial pH of the medium was less than 4.8 when 0.40% and 0.50% lactic acid were added.

Table 7. Production of lactic acid by _S. lactis_ A64 in APT broth at 30 C.

Inoculation time (hr.)	Percent acid (as lactic)	pH
0	0.000	6.56
3	0.000	6.61
6	0.005	6.62
9	0.168	6.21
12	0.459	4.81
18	0.658	4.49
24	0.683	4.38
30	0.677	4.45

Table 8. Production of lactic acid by *P. cerevisiae* 10791 in APT broth at 30 C.

Incubation time (hr.)	Percent acid (as lactic)	pH
0	0.000	6.58
3	0.000	6.62
6	0.000	6.62
9	0.003	6.61
12	0.061	6.03
18	0.544	4.87
24	0.659	4.46
30	0.665	4.60

Table 9. Growth of *S. aureus* 243 in APT broth containing added lactic acid. Incubation temperature was 30 C.

Percent lactic acid	pH	Growth rate constant[a] (hr. $^{-1}$)
0.00	6.46	1.15
0.05	6.36	1.80
0.10	5.98	1.73
0.15	5.86	1.72
0.20	5.72	1.68
0.25	5.45	0.94
0.30	5.29	0.83
0.40	4.71	N.G.[b]
0.50	4.46	N.G.

[a] From plot of log. population vs. time of incubation, slope x 2.3.

[b] No evidence of growth.

Figure 9 graphically illustrates the effect of added lactic acid upon the generation time of *S. aureus* 243. By extrapolation, it can be estimated that the addition of approximately 0.23% or more lactic acid would result in a generation time greater than was observed in the control culture. The addition of 0.23% lactic acid would give an initial pH of about 5.5 to 5.6 (by interpolation from pH data in Table 9).

The diameters of inhibitory zones observed when several strains each of *S. lactis* and *P. cerevisiae* were used as effector organisms on agar diffusion cup assays, in which the plates were seeded with *S. aureus* 243, are given in Table 10. Figure 10 illustrates typical plates from this investigation. In general, the zones of inhibition produced by the strains of *S. lactis* were wider than those produced by the strains of *P. cerevisiae*. *S. lactis* 11454 produces nisin, an antibiotic substance effective against a variety of gram positive organisms. It is noteworthy that the zone of inhibition produced by *S. lactis* 11454 was substantially wider than that produced by any of the other strains examined.

Figure 11 represents data obtained from the growth of *S. aureus* 243 in spent APT broth produced by the growth of *S. lactis* A64. Growth in the spent medium alone as well as growth in spent broth containing added glucose, tryptone, yeast extract and catalase are illustrated. Figure 12 represents similar data obtained from the growth of *S.*

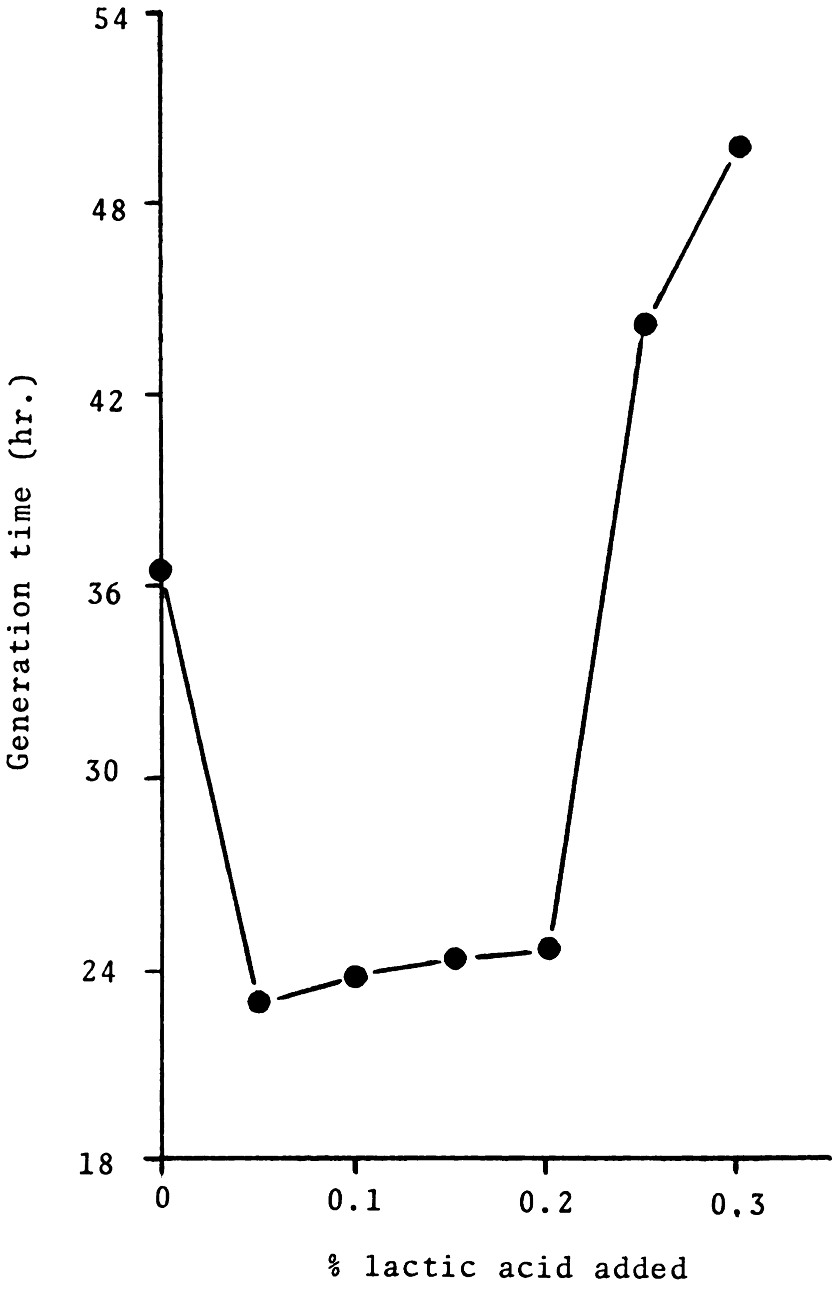

Figure 9.--Effect of the addition of lactic acid upon the generation time of S. aureus 243 grown in APT broth at 30 C under constant agitation.

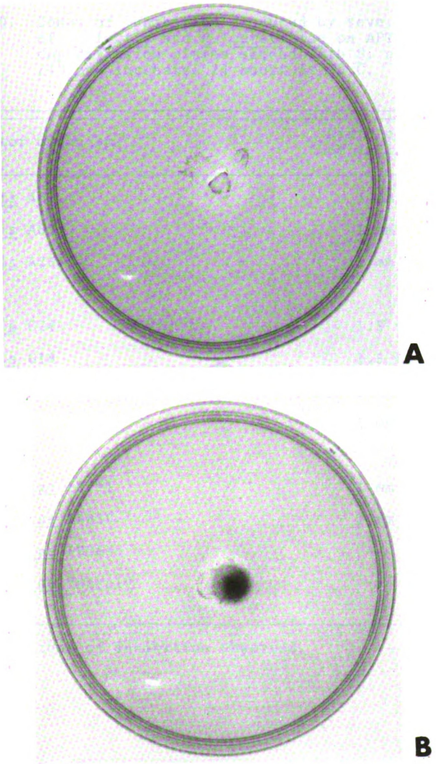

Figure 10.--Typical agar diffusion cup assay plates. (A) S. lactis 11454 (known Nisin producing strain) used as effector organism; (B) *S. lactis* G18 used as effector organism.

Table 10. Zones of inhibition produced by several strains of *S. lactis* and *P. cerevisiae* on APT agar cup diffusion plates seeded with *S. aureus* 243 and incubated 24 hours at 30 C.

Effector organism	Width of zone of inhibition
S. lactis A62	3 mm
S. lactis A64	1 mm
S. lactis A254	1 mm
S. lactis C24	1.5 mm
S. lactis G18	1.5 mm
S. lactis 11454	6 mm
S. lactis W	1 mm
P. cerevisiae 10791	1 mm
P. cerevisiae FBB39	1 mm
P. cerevisiae FBB63	--
P. cerevisiae "accel"	--

-- No zone of inhibition observed.

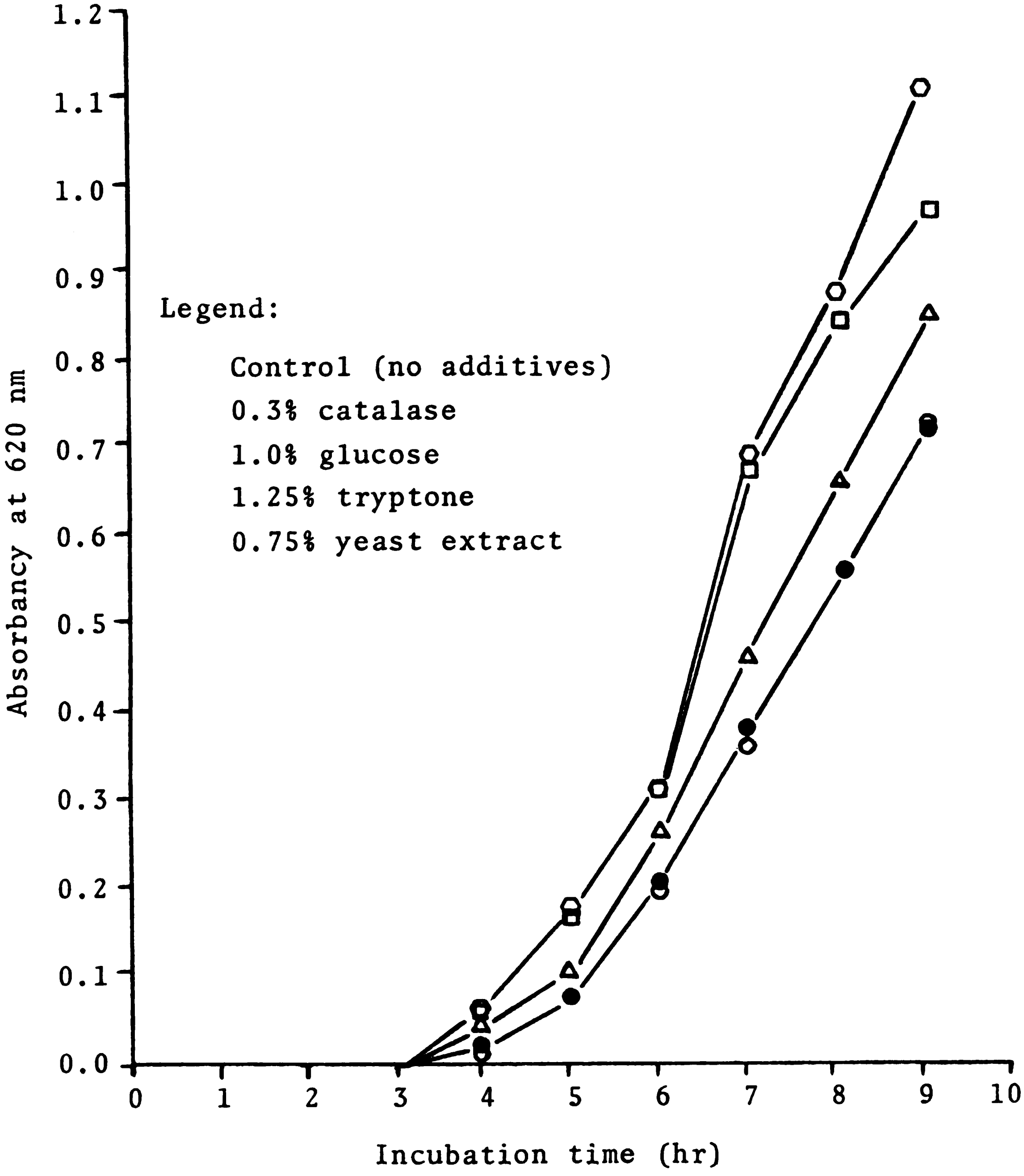

Figure 11.--Effect of various nutrient additions upon the growth of S. aureus 243 in spent APT broth produced by the growth of S. lactis A64. The temperature of incubation was 30 C and the initial pH was 6.5.

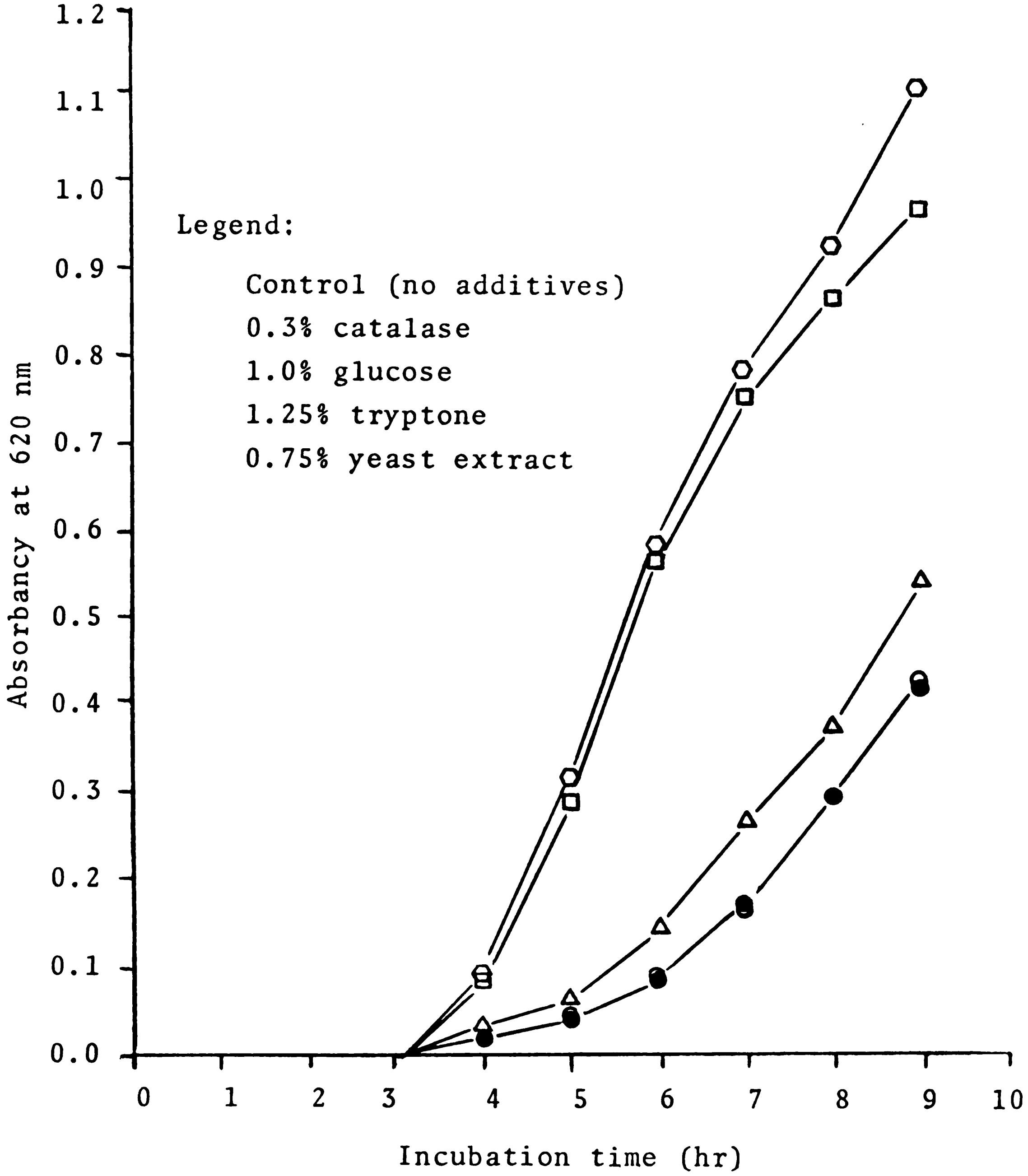

Figure 12.--Effect of various additions upon the growth of *S. aureus* 243 in spent APT broth produced by the growth of *P. cerevisiae* 10791. The temperature of incubation was 30 C and the initial pH was 6.5.

aureus 243 in spent APT broth produced by the growth of P. cerevisiae 10791. In both studies, it was observed that the growth of S. aureus 243 in the spent medium containing glucose was the same as the growth in the spent medium alone. The addition of catalase to the spent medium resulted in a slight increase in growth in both studies. Additions of either tryptone or yeast extract resulted in a greater increase in the growth of S. aureus.

Figures 13 and 14 represent data from an investigation designed to determine the effect of the addition of certain vitamins to the spent media upon growth of S. aureus 243. Addition of thiamine resulted in no increase in growth of S. aureus 243 in either of the spent media. Addition of biotin stimulated growth of the Staphylococcus slightly in both media, while niacin addition stimulated growth even more.

Release of Intracellular Enterotoxin by Disintegration of the Cells

Data in Table 11 indicate that grinding, freezing and thawing, and sonic treatment of the washed cells of S. aureus 243 resulted in no release of detectable quantities of enterotoxin. Disintegration of the cells by either the acetone-dry powder technique, toluene treatment or lysozyme treatment did result in the release of a quantity of enterotoxin which could be detected. The quantity of toxin

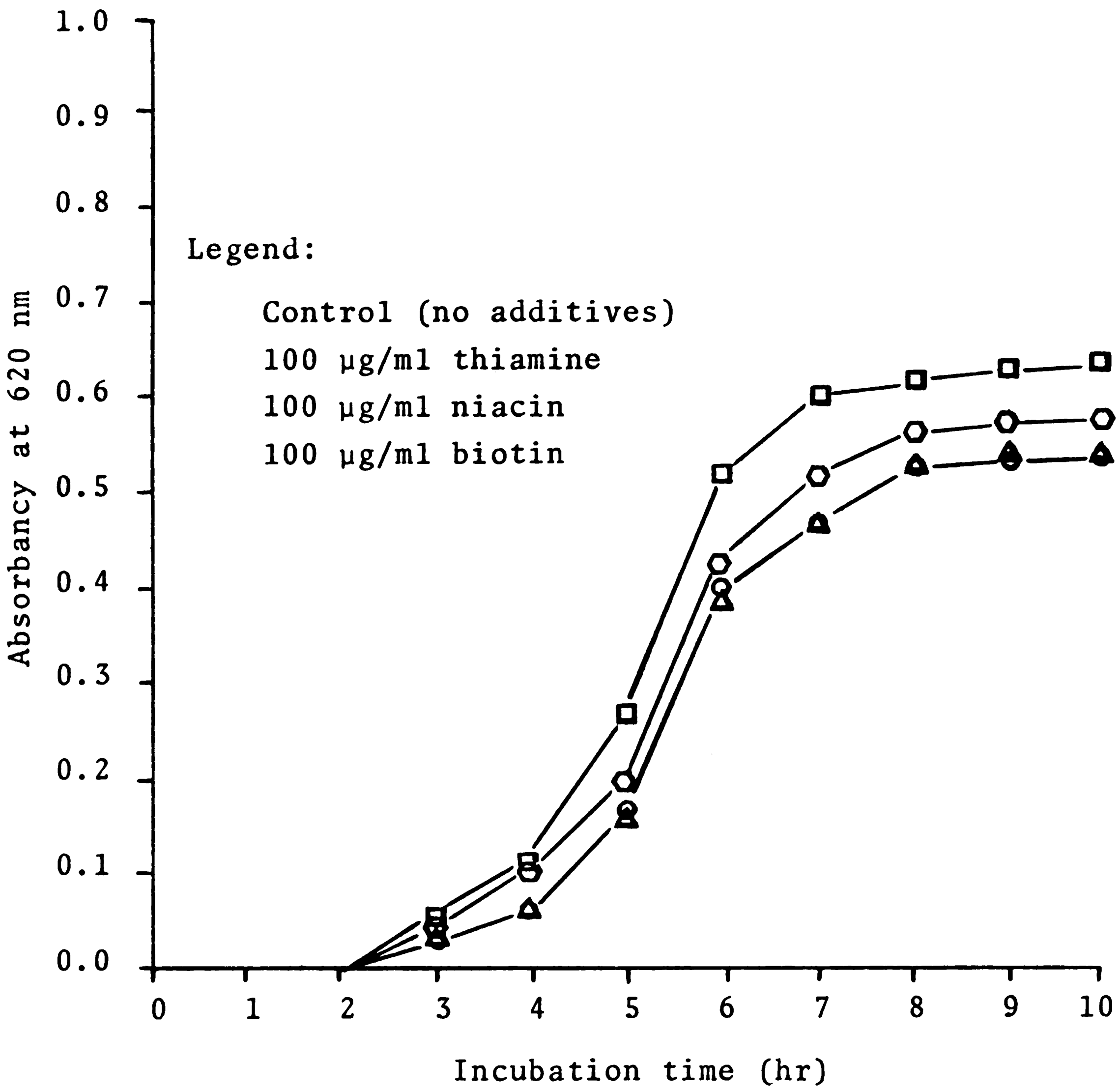

Figure 13.--Effect of certain vitamin additions upon the growth of *S. aureus* 243 in spent APT broth produced by the growth of *S. lactis* A64. The temperature of incubation was 30 C and the initial pH was 6.5.

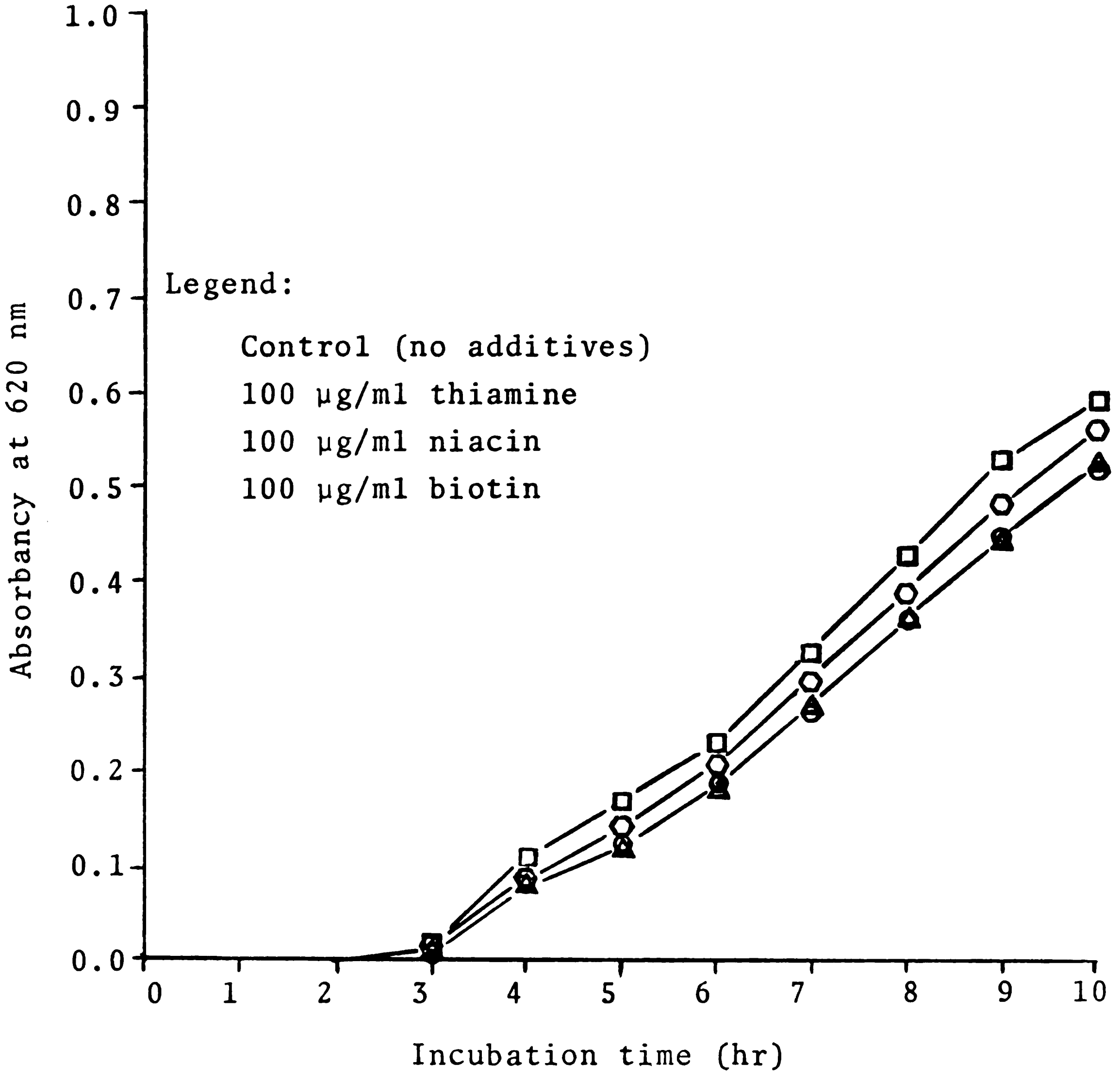

Figure 14.--Effect of certain vitamins upon the growth of *S. aureus* 243 in spent APT broth produced by the growth of *P. cerevisiae* 10791. The temperature of incubation was 30 C and the initial pH was 6.5.

Table 11. Comparison of the ability of several disintegration procedures to release enterotoxin B from washed cells of *S. aureus* 243.

Disintegration procedure	Toxin titer
Grinding	N.D.[a]
Freeze-thaw	N.D.
Sonic treatment	N.D.
Acetone-powder	1
Toluene	1
Lysozyme	1

[a] None detected.

released by the latter procedures was apparently between 1 and 2 µg/ml of cell extract solution.

The data in Table 12, which gives absorbancies at 280 nm for 1/20 dilutions of the cell extract solutions, represent a crude comparison of the abilities of the various procedures to release intracellular proteins. There was no apparent correlation between comparative ability of a technique to release proteins and ability to effect a release of enterotoxin.

An enterotoxin titer of 128 was obtained for the supernatant fluid from the growth medium. A minimal quantity of enterotoxin was detected in the saline solution used for the first washing of the cells. No toxin was detected in the saline used for the second and third washings.

Samples of the supernatant fluid from the growth medium were subjected to sonic treatment, freezing and thawing, and grinding, and subsequently re-assayed for enterotoxin. Titers of 128 were obtained in all such assays, indicating no detectable denaturation of enterotoxin had occurred as a result of these cell disruption procedures.

Table 12. Absorbancy at 280 nm of 1/20 dilutions of cell extract solutions obtained from disintegration of cells of *S. aureus* 243 by several procedures.

Disintegration procedure	Absorbancy at 280 nm
Grinding	0.48
Freeze-thaw	0.60
Sonic treatment	0.61
Acetone-powder	0.65
Toluene	0.88
Lysozyme	0.43

DISCUSSION

Associative Culture Studies

Data obtained during the experiments in which competitive lactic cultures were associated with *S. aureus* indicate great differences exist between lactic culture organisms with respect to the ability to affect the growth and production of enterotoxin by *S. aureus*. While only a limited number of representative lactic culture bacteria were studied, some general observations can be made.

The streptococci used as effectors were consistently more antagonistic to *S. aureus*, both as inhibitors of growth and as inhibitors of production of enterotoxin, than the other organisms studied. *P. cerevisiae* was only slightly less antagonistic than the streptococci. The lactobacilli studied and *L. citrovorum* were the least antagonistic. McCoy and Faber (1960) found that strains of *Streptococcus* were more consistently inhibitory to growth of *S. aureus* than were *L. lactis*, *P. cerevisiae* and three species of *Leuconstoc*. Similarly, Kao and Frazier (1965) obtained data which indicated that streptococci isolated from foods inhibited growth of *S. aureus* to a greater degree than lactobacilli and the *Leuconostoc* species. In a survey of the ability of several strains of *L. bulgaricus* and *L. lactis* to inhibit growth of *S. aureus*, Dahiya and Speck (1967) found only one strain of each which demonstrated any effect. Thus, the data obtained in this survey are consistent with reports in the literature.

Minor changes in the pH of the growth medium and in the temperature of incubation were found to have only slight effects upon the inhibition of *S. aureus* by *S. lactis* and *P. cerevisiae*. Inhibition of *S. aureus* was, however, slightly greater at a pH of 6 than at 7. Similarly, *P. cerevisiae* and *S. lactis* were more inhibitory at 25 C than at 30 C. Kao and Frazier (1965), DiGiancinto and Frazier (1965) and Donnelly *et al*. (1968) all reported somewhat greater inhibition of staphylococci in mixed culture with decreasing temperatures of incubation. Oberhofer and Frazier (1960) demonstrated greater inhibition of *S. aureus* by *Escherichia coli* at 15 C and 44 C than at 30 C. According to Breed *et al*. (1957), the optimum temperature for growth of *S. aureus* is 37 C. Thus, it appears that inhibition of *S. aureus* in associative culture is greatest when the conditions of incubation differ substantially from the optimum for growth of staphylococci.

The proportion of staphylococci in the inoculum was especially significant. It can be seen in Table 4 that inhibition of both growth of *S. aureus* 243 and production of enterotoxin by *S. lactis* A64 and *P. cerevisiae* 10791 was greatest when 10% of the inoculum was *S. aureus*. Several workers, including Peterson *et al.* (1962b), Troller and Frazier (1962), Kao and Frazier (1965) and DiGiancinto and Frazier (1965), have made reports indicating the importance of the relative proportion of staphylococci upon growth in association with other microorganisms.

Differences between strains of *S. lactis* and *P. cerevisiae* with regards to the ability to inhibit the growth of *S. aureus* and the production of enterotoxin were not great. Kao and Frazier (1965) also found only slight differences between strains of certain other species of lactic acid bacteria in ability to inhibit growth of *S. aureus*. It would appear that differences between strains of a given species of lactic acid bacterium are probably not, in general, significant.

Similarly, very little difference in susceptibility to antagonism by the lactic acid bacteria was found between the four enterotoxigenic strains of *S. aureus* studied. McCoy and Faber (1965) found 15 strains of *S. aureus* to be approximately equal in susceptibility to inhibition of growth by various food microorganisms. Two enterotoxigenic strains of *S. aureus* were found by Oberhofer and Frazier (1960) to be somewhat more sensitive to antagonism by various microorganisms than were two non-enterotoxigenic strains.

A noteworthy observation which can be made from data obtained during the associative culture studies is that in no case was enterotoxin detected if the population of *S. aureus* was prevented from reaching approximately 50 million cells per ml. Donnelly *et al.* (1968) similarly observed that production of detectable quantities of enterotoxin A occurred in milk only after a population of 50 million cells of *S. aureus* per ml was reached. Hood (1968) reported production of enterotoxin A in BHI broth could be detected only after a population of at least 270 million organisms per ml was reached, while a population of at least 40 million organisms per ml was required for production of a detectable quantity of enterotoxin B. Hood found, however, that a detectable quantity of enterotoxin A was produced in a colby cheese sample in which a maximum population of only 6.5 million staphylococci per gram was reached. Thus, some discretion should probably be exercised in predicting maximum populations of *S. aureus* necessary to produce detectable quantities of enterotoxin in semi-solid foods on the basis of broth culture studies. Furthermore, it is possible that at low populations quantities of enterotoxin may be produced, which can be toxic, but which cannot be detected, due to the limit of sensitivity of the assay procedure used.

Mechanisms of Antagonism of S. aureus by P. cerevisiae and S. lactis

The Role of Lactic Acid

Studies conducted by Nunheimer and Fabian (1940) indicated that lactic acid has inhibitory and germicidal properties which are disproportionate to effects caused by the hydronium ion concentration and which can affect the growth of staphylococci. However, it was necessary to decrease the pH to 4.27 to cause inhibition, and 3.8 to cause destruction of S. aureus. Nunheimer and Fabian concluded that the inhibitory and germicidal actions of lactic acid are properties of the undissociated acid.

The results obtained from the investigation to determine the growth of S. aureus 243 in APT broth to which increasing quantities of lactic acid were added were somewhat surprising. Growth of the Staphylococcus was apparently stimulated by additions of lactic acid in quantities of up to 0.20%. These additions of lactic acid to APT broth caused a gradual decrease of the pH of the medium from approximately 6.5 to 5.7. Genigeorgis and Sadler (1966) reported a steady decrease in growth of S. aureus when the pH of the growth medium was decreased from 6.9 to 5.1. Data reported by Hood (1968) indicated that growth of staphylococci in BHI broth was greater at a pH of 7.4 than at 6.0. Scheusner (1972) indicated that a decrease in pH from approximately 7 to 5.5 effected a decrease in the growth of S. aureus 243 in BHI broth. Apparently the stimulation of growth of

S. aureus by addition of lactic acid was not a result of a change in pH, but was due to some other property of the acid.

Additions of lactic acid in quantities of 0.25% (pH 5.45) and 0.30% (pH 5.29) did result in a decreased growth rate of S. aureus compared to growth in non-acidified APT broth. No growth was detected in APT broth acidified with 0.40% and 0.50% lactic acid. The pH resulting from addition of the latter quantities of lactic acid was 4.71 and 4.46, respectively. Lechowich et al. (1956) and Scheusner (1972) have indicated that growth of S. aureus does not occur in media in which the pH is lower than 4.8. It is probable that the inhibition of growth of S. aureus 243 by quantities of lactic acid of 0.25% or greater was a result of a lowering of the pH of the medium, and not due to any inhibitory properties of lactic acid.

The data in Tables 7 and 8 offer further support for the contention that the inhibition of growth of S. aureus in the presence of lactic acid was not a result of any inhibitory or germicidal properties of the acid apart from a lowering of the pH. A comparison of lactic acid production by S. lactis A64 and P. cerevisiae 10791 with the data in Table 10 indicates that both organisms can produce sufficient acid to inhibit the growth of S. aureus 243. In the case of both organisms production of acid resulted in a pH of the medium sufficiently low to be inhibitory to growth of staphylococci. However, in no case was a pH of 4.27 or lower observed, where according to the previously mentioned data of Nunheimer and Fabian, it would be expected that the lactic acid would exhibit antagonistic properties apart from a lowering of the pH. Therefore, no information was obtained which gave any indication that production of lactic acid by the organisms studied affected growth of *S. aureus* by any means other than lowering the pH of the growth medium.

The role of Antibiotics and Antibiotic-Like Substances

The agar diffusion cup assay and spot plate techniques have been used by a number of researchers to demonstrate the production of metabolic by-products by one organism which inhibits the growth of another. Sabine (1963) demonstrated that the acid produced by lactobacilli was not responsible for zones of inhibition observed on agar diffusion cup assay plates seeded with *S. aureus*, since the pH of the cups, the zones, and areas where the staphylococci grew was the same.

S. lactis 11454, a known nisin producing strain, produced a zone of inhibition significantly greater than that of any of the other strains examined. That *S. aureus* is sensitive to nisin has been reported by Hirsch and Wheater (1951) and Oberhofer and Frazier (1960). The other strains of *S. lactis* and *P. cerevisiae* produced only very small zones of inhibition. Furthermore, there is no apparent correlation between the width of zones of inhibition on agar diffusion cup assays and the results obtained with corresponding strains in the associative culture studies. It is therefore believed that production of antibiotics and antibiotic-like substances was not the primary mechanism of inhibition of the growth of *S. aureus* 243 by the majority of *S. lactis* and *P. cerevisiae* strains examined.

The Role of Hydrogen Peroxide

The lactic acid bacteria cannot synthesize heme compounds. As a result, these organisms cannot synthesize the enzyme catalase, which catalyzes the breakdown of hydrogen peroxide. Therefore, the hydrogen peroxide produced as a by-product of the growth of the organisms tends to accumulate in the growth medium.

Thompson and Johnson (1951) demonstrated that hydrogen peroxide was the principal inhibitory agent produced by certain salivary streptococci which were demonstrated to be antagonistic to growth of various microorganisms including staphylococci. Dahiya and Speck (1967) observed that inhibition of staphylococci by certain strains of *L. bulgaricus* and *L. lactis* was a result of hydrogen peroxide production.

Data obtained from the spent broth studies (Figures 11 and 12) indicate that the addition of catalase to the spent media produced by growth of *S. lactis* A64 and *P. cerevisiae* 10791 stimulated growth of *S. aureus* 243. It would appear that the spent broths contained sufficient hydrogen peroxide to be somewhat inhibitory to the growth of *S. aureus*. The addition of catalase apparently caused the breakdown of at least some of the hydrogen peroxide, resulting in better growth of the *Staphylococcus* than in the control media. There is some evidence, then, that production of hydrogen peroxide may have been a factor in the inhibition of the growth of *S. aureus* 243 by at least some of the organisms studied.

The Role of Competition for Vital Nutrients

Various studies have indicated that *S. aureus* strains are not particularly nutritionally fastidious. The work of Surgalla (1947) demonstrated that staphylococci can grow and produce enterotoxin in a medium consisting of certain salts, glucose, two amino acids and the vitamins niacin (nicotinic acid) and thiamine. Earlier studies by Knight (1937) and Porter and Pelczar (1940) indicated that thiamine, niacin and biotin were especially important nutrients for growth of staphylococci.

Iandolo *et al*. (1965) were able to stimulate growth of *S. aureus* in spent Trypticase Soy broth, produced by the growth of *S. diacetilactis*, by adding Yeast Nitrogen Base to the spent medium. They further demonstrated that nicotinamide was a limiting nutrient in the spent medium.

Data in Figures 11 and 12 indicate that growth of *S. aureus* 243 in spent APT broth produced by the growth of *S.*

lactis A64 or P. cerevisiae 10791 could be stimulated by the addition of either Yeast Extract or Tryptone. Both of these nutrients supply B vitamins to the medium. Results from the studies to determine the effects of vitamin additions to the spent media (Figures 12 and 13) indicate that biotin and niacin were apparently limiting nutrients. The addition of thiamine had no apparent effect. Evidence was obtained, therefore, that competition for certain vital nutrients, especially niacin, and to a certain extent biotin, may be a factor in the antagonism of S. aureus by S. lactis and P. cerevisiae.

Release of Enterotoxin from Cells Treated by Certain Disintegration Procedures

Attempts to release enterotoxin from washed cells of S. aureus have generally been unsuccessful. Friedman (1966) and Markus and Silverman (1968) reported no success in releasing enterotoxin by sonic treatment. Baird-Parker (1971) has reported the release of small amounts of enterotoxin B from S. aureus S-6 by treatment of washed cells with lysostaphin, a lysozyme-like enzyme with a specificity for the cell wall of S. aureus.

Grinding, freezing and thawing, and sonic treatment were unsuccessful in releasing detectable quantities of enterotoxin B from washed cells of S. aureus 243. Treatment with toluene or lysozyme, as well as disintegration by the acetone-powder technique, did result in the release of detectable quantities of enterotoxin B. There was no apparent correlation between the abilities of the various disintegration procedures to effect a release of enterotoxin and the comparative ability to release proteins in general. Thus, it may be concluded that the procedures which were successful in effecting a release of enterotoxin demonstrated some specificity of action for the sites within the cell where the enterotoxin is at its highest concentration.

Lysozyme catalyzes the degradation of the cell wall mucopeptide. The ability of lysozyme in particular to effect a release of enterotoxin from the washed cells gives evidence that enterotoxin is associated with the cell wall. The work of Friedman and White (1965), in which enterotoxin was detected on the cell surface by means of a fluorescent-antibody technique also pointed to enterotoxin being associated with the cell wall. Studies by Friedman (1966, 1968) using inhibitors of cell wall synthesis gave indication that enterotoxin is synthesized at or near the site of the cell wall. It is likely, then, that release of toxin from washed cells was accomplished by the procedures which did in some manner affect the integrity of the cell wall or other surface layers.

SUMMARY AND CONCLUSIONS

Great differences were found between the species of lactic acid culture bacteria studied with respect to their ability to inhibit the growth and production of enterotoxin by *Staphylococcus aureus*. In general, the streptococci and pediococci exhibited greater inhibition of *S. aureus* than did the lactobacilli. It was found that production of a detectable quantity of enterotoxin was prevented when the maximum population of *S. aureus* was not allowed to reach approximately 50 million cells per ml. On the other hand, toxin was detected in all cases in which a population of 50 million staphylococci per ml or greater was attained. Thus, none of the organisms examined demonstrated a specific ability to inhibit production of enterotoxin apart from the ability to inhibit growth of *S. aureus*.

Minor changes in environmental conditions, including pH of the growth medium and temperature of incubation, resulted in small changes in the inhibition of growth of *S. aureus* and production of enterotoxin, when *Streptococcus lactis* and *Pediococcus cerevisiae* were used as effector organisms. Similar observations probably would have been made in studies using other lactic acid culture bacteria as effectors.

Little difference was found between strains of *S. lactis* and *P. cerevisiae* in the ability to inhibit growth of *S. aureus* and production of toxin. Similarly, all four enterotoxigenic strains of *S. aureus* appeared to be approximately equal in sensitivity to the inhibitory effects of *S. lactis* and *P. cerevisiae*. It is not likely, therefore, that variations between strains of either staphylococci or effector organisms are of major significance in determining the outcomes of interactions of the organisms in associative culture.

The relative proportion of *S. aureus* in the initial population was very significant. When either *S. lactis* or *P. cerevisiae* was used as the effector organism, both growth of *S. aureus* and production of enterotoxin were inhibited to the greatest extent when the relative proportion of staphylococci in the inoculum was least.

The mechanism by which *S. lactis* and *P. cerevisiae* exert their inhibitory characteristics appears to be complex. Studies on the growth of *S. aureus* in APT broth acidified with lactic acid indicated that acid production cannot be considered as the sole means of inhibition, since low concentrations of lactic acid were stimulatory. Agar diffusion cup assays gave evidence that production of antibiotic-like substances may be of significance in the inhibition of the growth of *S. aureus* by some strains of lactic acid bacteria, but is probably not the major means of inhibition by most strains. Since the addition of catalase stimulated the growth of *S. aureus* in spent broth produced by growth of *S. lactis* on *P. cerevisiae*, it is probable that production of hydrogen peroxide is of some importance in the overall mechanism of inhibition by the two organisms. Additional information from the experiments with spent broth indicated that competition for vital nutrients, especially niacin and biotin, probably plays a major role in the mechanism of inhibition of *S. aureus* by *S. lactis* and *P. cerevisiae*.

Treatment of washed cells of *S. aureus* 243 with lysozyme or toluene, or subjection of the washed cells to disintegration by the acetone-dry powder procedure effected a release of small quantities of enterotoxin. Disintegration of the washed cells by sonic treatment, grinding and freeze-thawing did not result in the release of detectable quantities of enterotoxin. It was felt that the procedures which were successful in releasing enterotoxin from the washed cells were those which especially affected the integrity of the cell surface. Thus, evidence was found indicating the association of enterotoxin with the cell surface, in support of earlier references.

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APPENDIX

Table A1. Growth of *S. aureus* 243 in APT broth incubated at 30 C under constant agitation.

Hours	Population (cells/ml)	pH
0	$1.6x10^5$	6.42
3	$3.4x10^5$	6.43
6	$2.2x10^6$	6.43
9	$2.1x10^7$	6.39
14	$1.6x10^9$	6.27
24	$7.3x10^9$	5.92
30	$7.6x10^9$	5.85
36	$1.0x10^{10}$	5.88
48	$1.1x10^{10}$	6.54

Table A2. Growth of *S. aureus* 243 in APT broth incubated at 41 C under constant agitation.

Hours	Population (cells/ml)	pH
0	2.0×10^{5}	6.64
3	9.0×10^{4}	6.60
6	2.0×10^{7}	6.44
9	2.4×10^{9}	5.84
12	3.7×10^{9}	5.24
18	3.8×10^{9}	4.64
24	3.8×10^{9}	4.58
30	3.8×10^{9}	4.86
36	3.5×10^{9}	5.11
48	3.3×10^{8}	4.88

Table A3. Growth of *S. lactis* A64 in APT broth incubated at 30 C under constant agitation.

Hours	Population (cells/ml)	pH
0	$8.3x10^4$	6.72
3	$1.0x10^6$	6.69
6	$2.4x10^7$	6.66
9	$2.6x10^9$	6.28
14	$4.7x10^9$	4.78
24	$3.0x10^9$	4.49
30	$3.9x10^9$	4.48
38	$4.6x10^9$	4.54
48	$1.0x10^4$	4.53

Table A4. Growth of *S. cremoris* E8 in APT broth incubated at 30 C under constant agitation.

Hours	Population (cells/ml)	pH
0	2.9×10^5	6.72
3	1.1×10^6	6.70
6	9.2×10^7	6.65
9	5.9×10^8	6.51
14	7.2×10^8	4.56
24	2.1×10^8	4.34
30	$<10^4$	4.34
38	$<10^4$	4.35
48	$<10^4$	4.37

Table A5. Growth of *S. thermophilus* W in APT broth incubated at 41 C under constant agitation.

Hours	Population (cells/ml)	pH
0	$9.3x10^4$	6.56
3	$8.1x10^5$	6.58
6	$1.0x10^7$	6.49
9	$1.0x10^8$	5.54
12	$9.4x10^7$	4.86
18	$9.8x10^7$	4.62
24	$1.1x10^8$	4.58
30	$7.2x10^7$	4.56
36	$3.0x10^6$	4.55
48	$<10^5$	4.56

Table A6. Growth of *P. cerevisiae* 10791 in APT broth incubated at 30 C under constant agitation.

Hours	Population (cells/ml)	pH
0	$1.0\text{x}10^5$	6.43
3	$1.7\text{x}10^5$	6.49
6	$5.2\text{x}10^5$	6.46
9	$2.2\text{x}10^6$	6.47
14	$1.1\text{x}10^9$	5.37
24	$2.9\text{x}10^9$	4.36
30	$3.9\text{x}10^9$	4.52
38	$2.3\text{x}10^8$	4.57
48	$<10^5$	4.58

Table A7. Growth of *L. bulgaricus* W in APT broth incubated at 41 C under constant agitation.

Hours	Population (cells/ml)	pH
0	$1.1x10^5$	6.58
3	$7.0x10^4$	6.56
6	$2.5x10^5$	6.52
9	$3.8x10^6$	6.48
12	$5.2x10^7$	6.36
24	$4.0x10^9$	6.24
30	$3.8x10^9$	5.56
36	$3.0x10^8$	4.78
48	$<10^5$	4.52

Table A8. Growth of L. *casei* 54 in APT broth incubated at 30 C under constant agitation.

Hours	Population (cells/ml)	pH
0	1.6×10^{5}	6.71
3	4.4×10^{5}	6.69
6	1.8×10^{6}	6.69
9	8.1×10^{6}	6.64
14	7.8×10^{7}	6.55
24	2.7×10^{9}	4.38
30	2.5×10^{9}	4.40
38	3.0×10^{9}	4.35
48	3.2×10^{9}	4.34

Table A9. Growth of *L. plantarum* FBB 12 in APT broth incubated at 30 C under constant agitation.

Hours	Population (cells/ml)	pH
0	$9.7\text{x}10^4$	6.43
3	$2.7\text{x}10^5$	6.42
6	$1.5\text{x}10^6$	6.41
9	$1.6\text{x}10^7$	6.28
14	$1.9\text{x}10^8$	6.13
24	$2.7\text{x}10^9$	4.36
30	$2.7\text{x}10^9$	4.44
38	$7.4\text{x}10^8$	4.48
48	$<10^5$	4.49

Table A10. Growth of L. *citrovorum* W in APT broth incubated at 30 C under constant agitation.

Hours	Population (cells/ml)	pH
0	1.8×10^5	6.49
3	1.5×10^5	6.41
6	2.5×10^5	6.45
9	9.4×10^5	6.42
12	3.0×10^6	6.42
18	4.2×10^7	6.36
24	4.6×10^8	6.09
30	4.3×10^8	5.56
36	1.0×10^8	4.89
48	$<10^5$	4.80

Table A11. Growth of *S. aureus* 243 and *S. lactis* A64 in association in APT broth incubated at 30 C under constant agitation.

Hours	*S. aureus* population (cells/ml)	*S. lactis* population (cells/ml)	pH
0	$2.3x10^5$	$2.5x10^5$	6.67
3	$2.4x10^5$	$2.1x10^6$	6.65
6	$1.0x10^6$	$2.0x10^8$	6.59
9	$2.3x10^6$	$1.6x10^9$	5.54
12	$3.7x10^6$	$3.0x10^9$	4.84
24	$2.0x10^6$	$2.9x10^9$	4.47
36	$1.8x10^4$	$3.0x10^8$	4.52
48	$4.4x10^2$	$3.0x10^5$	4.58

Table A12. Growth of *S. cremoris* E8 and *S. aureus* 243 in association in APT broth incubated at 30 C under constant agitation.

Hours	*S. aureus* population (cells/ml)	*S. cremoris* population (cells/ml)	pH
0	$8.2x10^4$	$1.2x10^5$	6.59
3	$1.1x10^5$	$6.7x10^5$	6.58
6	$5.0x10^5$	$9.4x10^5$	6.49
9	$5.2x10^6$	$1.2x10^8$	5.68
14	$5.0x10^7$	$6.6x10^8$	4.47
24	$3.9x10^7$	$8.9x10^8$	4.31
30	$4.0x10^7$	$3.1x10^8$	4.34
38	$2.8x10^7$	$1.6x10^7$	4.37
48	$2.5x10^7$	$4.0x10^6$	4.37

Table A13. Growth of *S. aureus* 243 and *S. thermophilus* W in association in APT broth incubated at 41 C under constant agitation.

Hours	*S. aureus* population (cells/ml)	*S. thermophilus* population (cells/ml)	pH
0	1.0×10^5	2.5×10^5	6.46
3	7.0×10^4	1.2×10^7	6.44
6	5.1×10^4	1.7×10^8	6.02
9	1.1×10^5	3.2×10^8	5.08
12	6.8×10^4	3.5×10^8	4.81
18	3.6×10^4	3.3×10^8	4.59
24	1.2×10^3	1.7×10^8	4.57
30	$<10^3$	8.8×10^7	4.61
36	$<10^3$	1.4×10^6	4.63
48	8.0×10^1	6.0×10^1	4.59

Table A14. Growth of *P. cerevisiae* 10791 and *S. aureus* 243 in association in APT broth incubated at 30 C under constant agitation.

Hours	*S. aureus* population (cells/ml)	*P. cerevisiae* population (cells/ml)	pH
0	$8.1x10^4$	$9.5x10^4$	6.46
3	$1.5x10^5$	$8.4x10^5$	6.49
6	$3.7x10^5$	$3.0x10^6$	6.49
9	$1.9x10^6$	$9.7x10^7$	6.10
14	$5.2x10^7$	$1.3x10^9$	5.20
24	$1.7x10^8$	$4.2x10^9$	4.34
30	$1.1x10^8$	$3.5x10^9$	4.42
38	$3.8x10^7$	$4.0x10^9$	4.49
48	$3.6x10^6$	$3.3x10^9$	4.65

Table A15. Growth of *L. casei* 54 and *S. aureus* 243 in association in APT broth incubated at 30 C under constant agitation.

Hours	*S. aureus* population (cells/ml)	*L. casei* population (cells/ml)	pH
0	$1.1x10^5$	$1.2x10^5$	6.70
3	$1.0x10^5$	$4.0x10^5$	6.72
6	$6.7x10^5$	$1.2x10^6$	6.72
9	$1.8x10^7$	$1.0x10^7$	6.51
14	$1.5x10^9$	$3.9x10^9$	5.05
24	$1.6x10^9$	$4.6x10^9$	5.18
30	$1.6x10^9$	$4.7x10^9$	5.25
38	$1.3x10^9$	$4.7x10^9$	5.32
48	$1.4x10^9$	$6.6x10^9$	5.39

Table A16. Growth of *L. plantarum* FBB12 and *S. aureus* 243 in association in APT broth incubated at 30 C under constant agitation.

Hours	*S. aureus* population (cells/ml)	*L. plantarum* population (cells/ml)	pH
0	1.7×10^5	1.3×10^5	6.44
3	3.8×10^5	3.8×10^5	6.39
6	2.3×10^6	2.5×10^6	6.42
9	1.4×10^7	3.3×10^7	6.29
14	1.7×10^9	7.4×10^8	5.97
24	2.9×10^9	3.3×10^9	5.37
30	5.0×10^9	1.7×10^9	5.77
38	7.3×10^9	2.0×10^9	6.31
48	1.1×10^{10}	1.4×10^9	7.32

Table A17. Growth of *L. bulgaricus* W and *S. aureus* 243 in association in APT broth incubated at 41 C under constant agitation.

Hours	*S. aureus* population (cells/ml)	*L. bulgaricus* population (cells/ml)	pH
0	1.5×10^5	1.1×10^5	6.46
3	4.0×10^4	7.0×10^4	6.44
6	5.0×10^4	6.0×10^4	6.45
9	6.2×10^4	2.1×10^5	6.46
12	3.5×10^6	3.7×10^6	6.46
18	1.1×10^9	$<10^8$	5.97
24	4.2×10^9	4.5×10^9	4.77
30	3.9×10^9	4.4×10^9	5.01
36	1.8×10^9	4.6×10^9	5.02
48	7.9×10^8	3.8×10^8	5.01

Table A18. Growth of *L. citrovorum* W and *S. aureus* 243 in association in APT broth incubated at 30 C under constant agitation.

Hours	*S. aureus* population (cells/ml)	*L. citrovorum* population (cells/ml)	pH
0	$1.1x10^5$	$2.0x10^5$	6.51
3	$6.5x10^4$	$3.3x10^5$	6.56
6	$3.6x10^5$	$3.6x10^5$	6.58
9	$4.4x10^6$	$1.0x10^6$	6.56
12	$1.7x10^8$	$6.0x10^7$	5.59
18	$5.1x10^9$	$4.3x10^9$	6.19
24	$7.1x10^9$	$4.0x10^9$	5.32
30	$9.6x10^9$	$2.3x10^9$	5.72
36	$9.4x10^9$	$2.2x10^9$	6.01
48	$1.3x10^{10}$	$2.0x10^9$	6.15

Table A19. Absorbancy at 620 nm of cultures of S. aureus 243 grown in APT broth containing added lactic acid with incubation at 30 C under constant agitation. Values were read against an APT blank.

	% lactic acid								
Hours	0.00	0.05	0.10	0.15	0.20	0.25	0.30	0.40	0.50
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	0.010	0.010	0.015	0.015	0.010	0.000	0.000	0.000	0.000
4	0.032	0.032	0.035	0.039	0.035	0.011	0.000	0.000	0.000
5	0.100	0.169	0.221	0.218	0.210	0.147	0.023	0.000	0.000
6	0.277	0.490	0.450	0.455	0.450	0.377	0.053	0.000	0.000
7	0.520	0.650	0.700	0.700	0.700	0.620	0.135	0.000	0.000
8	0.740	0.860	0.920	0.930	0.930	0.840	0.289	0.000	0.000

Table A20. Absorbancy at 620 nm of cultures of *S. aureus* 243 grown in spent APT broth, produced by the growth of *S. lactis* A64, containing various additives. Incubation was at 30 C under constant agitation. Values were read against an APT broth blank.

	Nutrient Added				
Hours	None (Control)	0.03% Catalase	1.0% Glucose	1.25% Tryptone	0.75% Yeast Extract
0	0.000	0.000	0.000	0.000	0.000
1	0.000	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.000	0.000
3	0.000	0.000	0.000	0.000	0.000
4	0.020	0.041	0.010	0.035	0.060
5	0.075	0.102	0.073	0.161	0.179
6	0.208	0.261	0.188	0.402	0.404
7	0.380	0.460	0.350	0.670	0.690
8	0.560	0.660	0.560	0.840	0.870
9	0.720	0.850	0.730	0.970	1.100

Table A21. Absorbancy at 620 nm of *S. aureus* 243 cultures grown in spent APT broth, produced by the growth of *P. cerevisiae* 10791, containing various added nutrients. Incubation was at 30 C under constant agitation. Values were read against an APT broth blank.

	Nutrient Added				
Hours	None (Control)	0.03% Catalase	1.0% Glucose	1.25% Tryptone	0.75% Yeast Extract
0	0.000	0.000	0.000	0.000	0.000
1	0.000	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.000	0.000
3	0.000	0.000	0.000	0.000	0.000
4	0.020	0.031	0.025	0.089	0.091
5	0.040	0.067	0.045	0.289	0.319
6	0.085	0.142	0.090	0.560	0.580
7	0.169	0.269	0.168	0.750	0.780
8	0.291	0.373	0.291	0.860	0.920
9	0.420	0.540	0.450	0.960	1.100

Table A22. Absorbancy at 620 nm of *S. aureus* 243 cultures grown in spent APT broth, produced by the growth of *S. lactis* A64, containing added vitamins. Incubation was at 30 C under constant agitation. Values were read against an APT broth blank.

	Vitamin Added			
Hours	None (Control)	Thiamine*	Niacin*	Biotin*
0	0.000	0.000	0.000	0.000
1	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.000
3	0.028	0.028	0.052	0.049
4	0.062	0.062	0.107	0.102
5	0.172	0.165	0.268	0.500
6	0.399	0.370	0.520	0.580
7	0.450	0.450	0.600	0.610
8	0.530	0.530	0.620	0.620
9	0.530	0.540	0.630	0.620
10	0.540	0.540	0.640	0.620

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[*] Final concentration of 100 µg/ml.

Table A23. Absorbancy at 620 nm of S. *aureus* 243 cultures grown in spent APT broth, *produced* by the growth of P. *cerevisiae* 10791, containing added vitamins. Incubation was at 30 C under constant agitation. Values were read against an APT broth blank.

	Vitamin Added			
Hours	None (Control)	Thiamine*	Niacin*	Biotin*
0	0.000	0.000	0.000	0.000
1	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.000
3	0.005	0.010	0.010	0.005
4	0.086	0.085	0.102	0.085
5	0.121	0.115	0.169	0.137
6	0.178	0.177	0.229	0.209
7	0.263	0.268	0.326	0.289
8	0.362	0.360	0.430	0.385
9	0.450	0.450	0.530	0.480
10	0.520	0.530	0.590	0.560

*Final concentration of 100 μg/ml.