APPLIED MICROBIOLOGY, May, 1966
Vol. 14, No. 3
Printed in U.S.A.
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Influence of Food Microorganisms on Staphylococcal Growth and Enterotoxin Production in Meat

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Received for publication 2 December 1965

ABSTRACT

McCOY, D. W. (U.S. Department of Agriculture, Beltsville, Md.), AND J. E. FABER. Influence of food microorganisms on staphylococcal growth and enterotoxin production in meat. Appl. Microbiol. **14**:372–377. 1966.—Forty-four microorganisms were studied for their influence on staphylococcal growth and enterotoxin production. Inhibition was found to be more common than stimulation. Two types of inhibition were observed: inhibition of staphylococcal growth, and inhibition of enterotoxin formation with no apparent effect on growth. By use of a plate test, 12 of the 44 food microorganisms were found to inhibit staphylococcal growth at 35 C. Of the 12, 3 also inhibited growth at 25 C. No significant differences in inhibition were observed with the 15 strains of enterotoxigenic staphylococci. In meat slurries, inhibition of staphylococcal growth was found to be greater at 25 C than at 35 C. Results on inhibition obtained from the plate test could not be correlated with the effect of the organisms in slurries. Environmental conditions were found to affect markedly the influence of food microorganisms on staphylococci. Of the 44 food microorganisms studied, only *Bacillus cereus* was observed to stimulate significantly staphylococcal growth and enterotoxin formation. Stimulation was more pronounced with *Staphylococcus aureus* 196E than with other strains of enterotoxigenic staphylococci. *Bacillus megaterium* and *Brevibacterium linens* were inhibited by staphylococci. These organisms were completely inhibited when inoculated in mixed cultures with staphylococci. In pure cultures, good staphylococcal growth was found to be accompanied by enterotoxin production; however, in the presence of food microorganisms, good staphylococcal growth occurred without the formation of detectable levels of enterotoxin A.

Casman, McCoy, and Brandly (2) observed that when good staphylococcal growth occurred on meat, enterotoxin A was produced. However, to obtain good growth it was necessary to use either fresh meat taken aseptically from the internal portions of various cuts, or cooked meat, because of the inability of staphylococci to compete with other organisms found in meats. When contaminating organisms were excluded, staphylococci grew abundantly and produced detectable levels of enterotoxin without marked organoleptic changes in the meat.

Miller (7) reported that the natural flora of ground pork outgrew added staphylococci at temperatures above 18.3 C. Sufficient numbers of staphylococci to produce food poisoning were never attained. Dack and Lippitz (4) reported the failure of staphylococci to grow when inoculated into a food slurry which included a naturally occurring bacterial population. Peterson, Black, and Gunderson (10) found that appreciable numbers of staphylococci did not develop in chicken pies under any conditions of defrost, even though the pies spoiled under the test conditions. Staphylococci grew well at 37 C; however, rank spoilage occurred within 24 hr, rendering the product inedible. Thus, these workers were unable to obtain an edible product containing large numbers of staphylococci. They also found definite repressive effects on the growth of staphylococci when a mixture of saprophytic and psychrophilic bacteria was present (11). The repressive effect became more pronounced as the staphylococcal portion of the bacterial population decreased. These workers found that the competition be-

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tween staphylococci, saprophytic organisms, and psychrophilic organisms was extremely active up to room temperature. Even when large numbers of staphylococci were achieved, the tremendous numbers of saprophytes also obtained would render a food organoleptically unacceptable before sufficient staphylococci grew to be a potential hazard. Troller and Frazier (13) found that maximal inhibition occurred between 20 and 25 C and at a *p*H range of 6.2 to 7.4. These workers also observed that inhibition was markedly increased as the ratio of inhibitor to staphylococci increased.

Oberhofer and Frazier (9) examined 66 cultures of food bacteria, and found that *Escherichia coli*, fecal streptococci, a nisin-producing strain of *Streptococcus lactis*, and meat lactobacilli inhibited staphylococci. The inhibitory effect varied with the temperature and the test medium. Troller and Frazier (14) studied the nature of inhibition produced by seven different food bacteria. The inhibition of staphylococci by *Serratia marcescens* and *Pseudomonas* sp. was caused by a competition for nutrients. However, five other organisms (*Bacillus cereus*, *Proteus vulgaris*, *E. coli*, *Aerobacter aerogenes*, and *Achromobacter* sp.) were found to produce an inhibitory substance relatively specific for staphylococci and micrococci. Graves and Frazier (5) reported that, of 14 cultures influencing the growth of staphylococci, over one-half were inhibitory, whereas some were stimulatory. The lactic acid bacteria were mostly inhibitory, but the other groups of bacteria contained more stimulatory cultures than inhibitory ones. It should also be noted that some cultures were stimulatory according to one test, but were inhibitory in other tests.

In the present study, the influence of food microorganisms on growth and enterotoxin A production by strains of staphylococci in meats was studied.

MATERIALS AND METHODS

Cultures. Fifteen strains of *Staphylococcus aureus* that produce enterotoxin A, as demonstrated by gel-diffusion techniques, were used in this study. Preliminary studies revealed that all 15 strains reacted similarly when grown with various food microorganisms. Therefore, only the results obtained with strain 265-1 (Casman) will be given in detail.

The influence of bacteria commonly found in meats was studied, by use of 44 organisms representing 17 different genera. These included 12 strains recently isolated from meats.

Preparation and inoculation of meat samples. Five types of meat were used in this study: raw beef, raw pork, cooked beef (internal temperature of 62 C), cooked pork (internal temperature of 75 C), and canned perishable ham. Slurries of meat were prepared by blending 1 part meat with 2 parts sterile distilled water in a Waring Blendor for 3 min at high speed. Quantities of 60 ml of the slurries were placed in 300-ml Erlenmeyer flasks, and were inoculated with appropriate dilutions of the test organisms, based on optical density of saline suspensions prepared from overnight Trypticase Soy Agar (TSA; BBL) slants. Ratios of staphylococci to food microorganisms of 1:1, 10:1, and 100:1 were employed. Slurries were incubated in a shaker-bath at 25 and 35 C.

Cultural methods. Staphylococcal counts were performed by spreading 0.1 ml of 10-fold dilutions of slurries on the surface of Vogel and Johnson Agar (VJA; BBL) plates. Typical black colonies surrounded by yellow zones were counted after incubation at 35 C for 48 hr. In mixed culture studies, the staphylococcal counts recorded were those of the highest dilution of slurry containing staphylococci as demonstrated by growth in Trypticase Soy Broth (TSB; BBL) containing 10% NaCl.

Serological methods. Antiserum used in this study was produced by intramuscular injection of rabbits with a partially purified (20%) enterotoxin A mixed with an equal volume of Freund's complete adjuvant. Each rabbit received approximately 1.3 mg of enterotoxin A over an 11-week period. Two gel-diffusion techniques were used for the detection of enterotoxin A. These were the micro-Ouchterlony slide test of Crowle (3), and the double diffusion tube test of Oakley and Fulthrope (8), as described by Hall, Angelotti, and Lewis (6).

Antiserum giving a single band of precipitation in gel-diffusion tests was obtained by absorbing the antiserum with cells of enterotoxin A-nonproducing strains of *S. aureus* and soluble staphylococcal antigens adsorbed onto aluminum hydroxide (1).

Preparation of enterotoxin A. In selecting a suitable method for detecting the presence of enterotoxin A in meat slurries, a comparison was made of the sensitivities of the slide and tube gel-diffusion tests. The enterotoxin A present in 250 ml of culture filtrate from a 72-hr semisolid Brain Heart Infusion Agar (BHIA) culture was concentrated approximately 50 times. This was accomplished by first precipitating the protein with 85% saturated ammonium sulfate followed by dialysis against 0.85% saline until a negative barium chloride reaction was obtained. The dialyzed protein fraction was concentrated further by lyophilization. The dried preparation was then reconstituted to 5 ml with distilled water. Dilutions of the concentrate from 1:10 to 1:200 were tested by both gel-diffusion techniques. Precipitin bands were formed by a 1:90 dilution of concentrated toxin in the micro-Ouchterlony side test, and by a 1:110 dilution in the double-diffusion tube test. The apparent difference in the sensitivities of the two tests thus afforded a semiquantitative method for assaying enterotoxin A production by staphylococci under various cultural conditions. Therefore, in subsequent studies, culture filtrates were tested for the presence of enterotoxin A, first by the slide test and, if negative, then by the tube test. If a filtrate was still negative, it was concentrated approximately 10 times by lyophilization and retested by the tube test. Any filtrate

not producing a precipitin band after 10 times concentration was considered negative for the presence of enterotoxin A.

Results and Discussion

Effect of food microorganisms on staphylococcal growth and enterotoxin production. Forty-four food microorganisms were tested for the production of antistaphylococcal substances. Sterile filter paper discs were saturated with saline suspensions of the various organisms and placed on TSA plates seeded with the 18 strains of *S. aureus*. Of the 44 organisms tested, only 12 inhibited staphylococcal growth at 35 C, and only 3 of the 12 also inhibited staphylococcal growth at 25 C. The three organisms inhibiting growth at both 25 and 35 C (*Pseudomonas aeruginosa*, *P. fluorescens*, and *Pseudomonas* sp. MID-1) produced zones of complete inhibition approximately 20 mm in diameter. *Micrococcus flavus*, *M. freudenreichii*, *Proteus vulgaris*, *Salmonella enteritidis*, *S. plymuthica*, three strains of *Streptococcus faecalis*, and *S. faecium* produced zones of diminished staphylococcal growth in the area surrounding the discs. The lactic acid organisms failed to grow under the above conditions, and experiments to be discussed later revealed that only *Lactobacillus casei*, *L. plantarum*, *Leuconostoc mesenteroides*, and *S. lactis* were able to grow under the conditions necessary for good staphylococcal growth and enterotoxin formation. No difference in the susceptibility of the 15 staphylococcal strains to inhibitory organisms was noted. Graves and Frazier (5) also found that strains of *S. aureus* used in their studies reacted similarly when grown with an inhibiting organism. Therefore, only the results obtained with strain 265-1 are reported in detail.

When mixed shake-cultures of staphylococci and food organisms were grown in meat slurries at 35 C, some of the food microorganisms failed to grow or showed only slight growth. Only *Bacillus megaterium* and *Brevibacterium linens* appeared to be inhibited by the staphylococci, since these organisms grew well in the meat slurries without staphylococci. The conditions of the shake-cultures were unfavorable for the lactic acid bacteria, and only *L. casei*. *L. plantarum*, *L.*

TABLE 1. *Influence of food microorganisms on staphylococcal growth and enterotoxin formation when grown in association with Staphylococcus aureus 265-1 in cooked beef slurries at 35 C*

Organism	Staphylococcal counts*			Enterotoxin A formation†			
	12 hr	24 hr	36 hr	24 hr	36 hr	48 hr	72 hr
Aerobacter aerogenes	10^8	10^8	10^8	—	—	—	—
Alcaligenes faecalis	10^9	10^9	10^9	ST	ST	ST	TT
A. viscolactis	10^8	10^9	10^9	ST	ST	ST	TT
Bacillus cereus	10^7	10^8	10^9	ST	ST	ST	TT
Brevibacterium acetylicum	10^9	10^9	10^9	—	TT	TT	TT
Escherichia coli P-1	10^7	10^8	10^9	—	—	—	—
E. coli 9637	10^8	10^9	10^9	TTC	TTC	TT	TT
E. coli 10536	10^8	10^9	10^9	—	—	—	—
Klebsiella pneumoniae	10^8	10^8	10^8	—	TT	TT	TT
Leuconostoc mesenteroides	10^8	10^8	10^8	ST	ST	ST	TT
Micrococcus flavus	10^9	10^9	10^{10}	ST	ST	ST	TT
M. freudenreichii	10^8	10^9	10^{10}	ST	ST	ST	TT
Proteus mirabilis	10^9	10^8	10^8	—	—	—	—
P. vulgaris	10^8	10^8	10^8	TT	TT	TT	TT
Pseudomonas aeruginosa	10^8	10^8	10^8	—	—	TT	TT
P. fluorescens	10^8	10^8	10^8	—	—	TT	TT
Pseudomonas sp. MID-1	10^7	10^7	10^7	—	—	—	—
Salmonella enteritidis	10^7	10^7	10^7	—	—	—	—
Serratia marcescens	10^8	10^8	10^9	—	—	—	—
S. plymuthica	10^6	10^6	10^7	—	—	—	—
Streptococcus faecalis 11	10^8	10^8	10^8	ST	ST	ST	ST
S. faecium	10^8	10^8	10^9	TT	TT	TT	ST
S. lactis	10^8	10^8	10^9	TTC	TT	TT	TT
S. aureus 265-1 control	10^8	10^9	10^9	ST	ST	ST	TT

* Highest dilution of slurry containing staphylococci.

† ST = positive test by the micro-Ouchterlony slide method; TT = negative slide test, positive test by the double-diffusion tube method; TTC = positive test after 10 times concentration with the tube method; — = no enterotoxin A detected after concentrating 10 times.

TABLE 2. *Comparison of the growth and enterotoxin formation of Staphyococcus aureus 265-1 grown in association with food microorganisms in ham slurries at 25 and 35 C*

Organism	Staphylococcal growth*				Enterotoxin formation†			
	25 C		35 C		25 C		35 C	
	36 hr	72 hr	36 hr	72 hr	36 hr	72 hr	36 hr	72 hr
Achromobacter sp. 104	10^{6}	10^{8}	10^{8}	10^{9}	—	—	ST	TT
Aerobacter aerogenes	10^{5}	10^{6}	10^{8}	10^{8}	—	—	—	—
Alcaligenes faecalis	10^{6}	10^{10}	10^{9}	10^{9}	—	TTC	ST	ST
Bacillus cereus	10^{8}	10^{6}	10^{9}	10^{11}	TT	TT	ST	ST
Brevibacterium acetylicum	10^{7}	10^{8}	10^{8}	10^{9}	—	TTC	ST	ST
Escherichia coli 9637	10^{6}	10^{6}	10^{8}	10^{9}	—	—	TTC	TT
E. coli 10536	10^{7}	10^{8}	10^{8}	10^{8}	—	TTC	—	—
Flavobacterium suaveolens‡	10^{8}	10^{9}	10^{8}	10^{9}	—	TTC	ST	TT
Klebsiella pneumoniae	10^{7}	10^{6}	10^{9}	10^{9}	—	—	ST	TT
Micrococcus flavus	10^{8}	10^{8}	10^{9}	10^{10}	—	TTC	ST	ST
Proteus mirabilis	10^{7}	10^{7}	10^{8}	10^{9}	—	—	—	—
P. vulgaris	10^{7}	10^{7}	10^{9}	10^{9}	—	—	—	—
Pseudomonas aeruginosa	10^{6}	10^{8}	10^{8}	10^{10}	—	TTC	ST	TT
Pseudomonas sp. MID-1	10^{7}	10^{6}	10^{8}	10^{8}	—	—	—	—
Pseudomonas sp. 93‡	10^{8}	10^{8}	10^{8}	10^{9}	—	—	ST	TT
Pseudomonas sp. 97‡	10^{8}	10^{8}	10^{8}	10^{9}	—	TTC	ST	TT
Salmonella enteritidis	10^{6}	10^{8}	10^{8}	10^{8}	—	—	ST	ST
Serratia marcescens	10^{6}	10^{7}	10^{6}	10^{7}	—	—	—	TTC
S. plymuthica	10^{5}	10^{6}	10^{7}	10^{8}	—	—	—	—
Streptococcus faecalis 11	10^{8}	10^{7}	10^{8}	10^{9}	—	—	—	TT
S. faecium	10^{7}	10^{7}	10^{9}	10^{9}	—	—	—	TTC
S. lactis	10^{6}	10^{8}	10^{8}	10^{8}	—	—	—	TT
S. aureus 265-1 control	10^{7}	10^{8}	10^{8}	10^{10}	—	TTC	ST	ST

* Highest dilution containing staphylococci.

† ST = positive test with the micro-Ouchterlony slide technique; TT = positive test with the double-diffusion tube test; TTC = positive tube test after 10 times concentration; — = no enterotoxin A detected after concentrating 10 times.

‡ Failed to grow at 35 C.

mesenteroides, and *S. lactis* showed an increase in numbers. The incubation temperature appeared to inhibit the *Achromobacter*, *Flavobacterium*, and six of the pseudomonad strains, since these organisms failed to grow in pure culture at 35 C. *B. megaterium* and *B. linens* also failed to grow with staphylococci when incubated at 25 C, and no improvement in the growth of the lactic acid bacteria was observed. The remaining organisms, however, grew well in association with staphylococci at 25 C.

The growth and enterotoxin production of strain 265-1 grown in association with food organisms at 35 C is presented in Table 1. The staphylococcal growth given is the highest 10-fold dilution of beef slurry containing staphylococci. Equal numbers of staphylococci and food microorganisms were inoculated into beef slurries. In all cases, the growth of the food microorganism appeared to be equal to or greater than that of the staphylococcus, as demonstrated by periodic Gram stains over the 72-hr incubation period. The relative amounts of enterotoxin A formed were determined by the use of the slide and double-diffusion tube tests. The data presented in Table 1 show that only *Pseudomonas* sp. MID-1, *S. enteritidis*, and *S. plymuthica* markedly inhibited staphylococcal growth. The strong inhibition by *P. aeruginosa* and *P. fluorescens*, as demonstrated by the plate test, did not occur in the meat cultures. It can also be seen that staphylococcal numbers cannot be used as an index of enterotoxin formation. For example, *Serratia marcescens* and *Escherichia coli* strains P-1 and 10536 showed no effect on staphylococcal growth while preventing the formation of detectable levels of enterotoxin. Based on the reported sensitivities of the two gel-diffusion tests (2, 12), it can be assumed that cultures forming a precipitin band with the slide test contain over 1 μg/ml of enterotoxin A, and those giving a negative slide test and a positive double-diffusion tube test would contain between 0.25 and 1 μg/ml of enterotoxin A. It can be seen from Table 1 that

16 of the 23 food microorganisms tested had an adverse effect on enterotoxin formation. Their effect ranged from a slight decrease in enterotoxin concentration to the complete prevention of the formation of detectable levels of enterotoxin. For example, there was no significant difference in staphylococcal growth with *A. aerogenes*, *Klebsiella pneumoniae*, *P. vulgaris*, *P. aeruginosa*, and *S. faecalis*; however, there were marked differences in the amounts of enterotoxin formed with these organisms.

When strain 265-1 was grown in cooked beef slurries in association with the 23 food microorganisms at 25 C, enterotoxin A could be demonstrated in only 1 culture (*B. cereus* after 36 hr) by the double-diffusion tube test. *B. cereus* also stimulated enterotoxin production by strain 196E when grown at 25 and 35 C in ham slurry.

A comparison of the growth and enterotoxin formation of *S. aureus* 265-1 grown in ham slurry with various food microorganisms is presented in Table 2. Of the 22 organisms tested, only *B. cereus* was found to stimulate enterotoxin production. Of the 22 food microorganisms, 14 inhibited enterotoxin formation at 25 C. However, of the 14 organisms inhibiting enterotoxin formation, only 10 also inhibited staphylococcal growth. Enterotoxin formation at 35 C was inhibited by 11 of the 22 organisms, but only 2 cultures (*S. marcescens* and *S. plymuthica*) markedly inhibited staphylococal growth. It is also apparent from the results presented in Table 2 that good staphylococcal growth is necessary for enterotoxin production. However, enterotoxin formation does not always accompany good staphylococcal growth. Both strains of *E. coli* inhibited enterotoxin formation at 35 C; however, only strain 9637 appeared to inhibit enterotoxin formation at 25 C. Four of the 22 food microorganisms failed to grow at 35 C. These four organisms grew well at 25 C, and two of the four (*Achromobacter* sp. 104 and *Pseudomonas* sp. 93) inhibited enterotoxin formation. It can also be concluded from the data presented in Table 2 that inhibition of staphylococcal growth and enterotoxin formation is more pronounced at 25 C than at 35 C.

The data presented in Tables 1 and 2 are similar to results obtained with raw beef and raw pork (not tabulated). Slurries prepared from cooked pork did not appear to support the growth of staphylococci as well as the other meats tested. Reduced growth was more obvious at 25 C than at 35 C. Enterotoxin production was also reduced in the cooked pork slurries. Studies reported by Casman, McCoy, and Brandly (2), with enterotoxin formation on meat slices, also showed that cooked pork yielded the lowest level of enterotoxin of meats tested. These workers did not find, however, that staphylococcal growth was also decreased on slices of cooked pork. Determinations of the *p*H of various slurries revealed that cooked pork slurry had a *p*H of 6.6, and the other slurries ranged in *p*H from 6.1 to 6.4. When staphylococcal strain 265-1 was inoculated into slurries of ham (*p*H 6.3) and pork (*p*H 6.6) and incubated at 25 C for 7 days, the final *p*H of the two slurries was 6.5 and 7.3, respectively. Enterotoxin assays by means of the double-diffusion tube test were made daily over the 7-day period. Precipitin bands were formed from the ham filtrate after 3 days, and from the cooked pork slurry after 5 days. These results suggested that the *p*H of the two slurries may be responsible for the difference in growth-supporting properties of the two media. However, when the *p*H of the pork slurry was adjusted to 6.2, no significant increase in staphylococcal growth or enterotoxin production was observed. It was concluded, therefore, that decreased staphylococcal growth and enterotoxin formation in slurries of cooked pork was due to factors other than *p*H. This does not, however, rule out the possibility that *p*H may be one of the factors involved.

Results of this investigation showed that the micro-Ouchterlony slide test and the double-diffusion tube test are well suited for direct detection of staphylococcal enterotoxin A in meat filtrates. Although the formation of nonspecific precipitates in the gels could mask precipitin bands formed by low levels of enterotoxin, concentration, heating, filtration, or a combination of procedures can eliminate interference by nonspecific precipitates. Sufficient levels of enterotoxin were produced so that in most cases no treatment of the culture filtrates was necessary. It is probable that enterotoxin could have been detected in some filtrates if they had been concentrated more than 10 times. A 10 times concentration appeared to be sufficient to show the influence of various food microorganisms on enterotoxin formation. Because of the lack of purified enterotoxin A, the relative amounts of enterotoxin were estimated from the reported sensitivities of the gel-diffusion tests. As techniques are developed for the purification of enterotoxin A, and enterotoxin A thus becomes more readily available, it will be possible to carry out more definitive studies on enterotoxin formation.

Studies of the influence of food microorganisms on staphylococcal growth and enterotoxin production revealed that inhibition, as indicated by the plate test, was not always duplicated in mixed cultures. It was also observed that inhibition was

more common than stimulation. Graves and Frazier (5) reported that of seven cultures found to be stimulatory according to a plate test, two were stimulatory in broth, two were slightly inhibitory, and three were definitely inhibitory. Thus, it was apparent that the influence of a given culture on staphylococcal growth may vary from stimulation to inhibition, depending on environmental conditions.

In general, two types of staphylococcal inhibition by food microorganisms were observed; that is, marked inhibition of staphylococcal growth, and inhibition of enterotoxin formation with no apparent effect on growth. The first type may be due to the formation of an antibiotic substance. Reduced levels of enterotoxin may be the result of competition for an essential nutrient for enterotoxin formation, or a breakdown of the enterotoxin by food microorganisms. It is also possible that an antibiotic substance was produced in quantities sufficient for the inhibition of enterotoxin formation, but not for significantly reducing staphylococcal growth. The formation of antibiotic substances by food bacteria has been reported by Troller and Frazier (14). These workers isolated an antibiotic substance from *E. coli* that was found to be especially effective against staphylococci and micrococci. An antibiotic substance effective against *S. aureus* 196E was also found by these investigators in culture filtrates of *B. cereus*. Of the 44 organisms used in this study, however, only *B. cereus* was found to cause a significant increase in enterotoxin formation by strains of enterotoxigenic staphylococci, including strain 196E. This difference could have been due to environmental factors influencing antibiotic formation or a difference in the strain of *B. cereus* used.

It was obvious from the results that organisms commonly occurring on meats may influence staphylococcal growth or enterotoxin production, or both. Indications were that inhibition occurs more often than stimulation in meats. However, it was apparent that the effect of food microorganisms on strains of *S. aureus* was markedly influenced by environmental conditions. Evidence was also found indicating that the presence of relatively large numbers of staphylococci in meats was not sufficient proof of the presence of enterotoxin.

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