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Staphylococcal Growth and Enterotoxin Production in the Presence of Meat Cultures (Non LAB)

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ABSTRACT

The effect of meat cultures (non lactic acid bacteria) on the growth and production of enterotoxins and thermonuclease by Staphylococcus aureus *was studied.* Micrococcus varians *did not affect growth nor the synthesis of metabolites. Levels of enterotoxins A, B and D produced by the respective* S. aureus *strains were reduced by* S. xylosus, S. saprophyticus *and* S. carnosus. *The two latter species prevented production of enterotoxin* C_1 *and* S. xylosus *markedly reduced the amount produced. The three coagulase-negative staphylococci showed little inhibitory effect on the growth of* S. aureus. Penicillium nalgiovense *did not show inhibitory activity against the four* S. aureus *strains.* Debaryomyces hansenii *slightly inhibited growth of the enterotoxin A-producing strain, but reduced enterotoxin synthesis at 30°C. Thermonuclease was detected whenever enterotoxins were detected though the influence of the effector organism was dependent on the test strain. Copyright © 1996 Elsevier Science Ltd*

INTRODUCTION

A great variety of fermented sausages can be produced using lactic acid bacteria (LAB), Micrococcaceae and moulds and yeasts. It has been well established that LAB are antagonistic to growth and survival of pathogenic microorganisms, as well as spoilage organisms (Salminen & von Wright, 1993).

Certain species of the genera *Staphylococcus* and *Micrococcus* are often used as starter cultures because of their ability to reduce nitrate and produce catalase. The possible contribution of these bacteria to the control of pathogens has also been suggested (Niskanen & Nurmi, 1976; Cantoni *et al.*, 1992).

Specific strains of yeasts, particularly of the *Debaryomyces* genus play a role in ripening, colour and flavour formation. Moulds also contribute to the characteristic flavour, appearance and texture of air-dried sausages, with *Penicillium* spp. being the most desirable. In Europe, *Debaryomyces hansenii* and *Penicillium nalgiovense* are used as meat starters (Bacus, 1986).

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A high percentage of raw meats and sausage ingredients are contaminated with *Staphylococcus aureus*. This bacterium is tolerant to salt and nitrite and capable of growing under anaerobic conditions. Therefore, staphylococcal food poisoning caused by incorrectly fermented sausages is a potentially significant problem (Bergdoll, 1989).

Although the beneficial effect of LAB in inhibiting staphylococcal growth and/or enterotoxin production is well known, little attention has been given to non LAB meat cultures as possible producers of antagonistic compounds against *S. aureus*.

This study was undertaken to investigate the effect of meat cultures other than LAB on *S. aureus* growth and enterotoxin synthesis.

MATERIALS AND METHODS

Organisms

The following organisms were used: *S. aureus* ATCC 13565 (-SEA- enterotoxin A producer), ATCC 14458 (-SEB-enterotoxin B producer), FRI 137 (-SEC_1- an enterotoxin C_1 producer strain from the Food Research Institute, University of Wisconsin-Madison, Madison, WI, USA), and ATCC 23235 (-SED- enterotoxin D producer), *Micrococcus varians* (DSM 20033); *Staphylococcus carnosus* (DSM 20501); *Staphylococcus saprophyticus* (DSM 20029); *Staphylococcus xylosus* (DSM 20266); *Debaryomyces hansenii* (ATCC 36239); and *Penicillium nalgiovense* (ATCC 10472). Stock cultures of bacteria were maintained on Brain Heart Infusion (BHI) agar (Oxoid) slants. Mould and yeast cultures were stored on Potato Dextrose agar (Difco) and Malt Extract agar (Oxoid), respectively. The strains, except *P. nalgiovense*, were subcultured three times in BHI broth immediately before use. Micrococcaceae were incubated at 37 °C for 18 hr and *D. hansenii* at 30 °C for 18 hr.

Inocula

The cultures (except *P. nalgiovense*) were washed twice with 0.1% peptone water by being centrifuged (10 min at 12 200 *g*) in a refrigerated RC-5B Sorvall centrifuge (Du Pont Co., Newtown, CT, USA) and then suspended in the same volume of 0.1% peptone water. The optical density at 620 nm was read, and the cell suspension was diluted in 0.1% peptone water for inoculation (*ca* 10^5 CFU/ml).

Enumeration of microorganisms

The CFU/ml of *S. aureus* and *M. varians* were determined by spreading 0.1 ml of the appropriate dilution on Mannitol Salt agar plates (Oxoid), followed by incubation at 37 °C for 48 hr. Colonial morphology was used to differentiate between *S. aureus* and *M. varians*. The replacement of mannitol with another sugar (maltose, mannose or xylose) allowed enumeration of *S. carnosus* (maltose−), *S. saprophyticus* (mannose−) and *S. xylosus* (xylose+). Yeast cells were enumerated on Oxytetracycline Glucose Yeast Extract agar (Oxoid) incubated at 25 °C for 3–5 days.

Associative growth

Each *S. aureus* strain was inoculated (*ca* 10^5 CFU/ml) into BHI broth. The inoculated medium was thoroughly mixed and volumes of 200 ml aseptically dispensed into sufficient 500 ml Erlenmeyer flasks to provide controls and one or more flask for each effector organism to be tested (Micrococcaceae and *D. hansenii*). The flasks designated for the

associative growth were additionally inoculated with the desired organism. The four *S. aureus* strains were tested against three levels of *M. varians* (10^5, 10^6 and 10^7 CFU/ml). Only *S. aureus* ATCC 13565 was tested against three levels of each coagulase negative *Staphylococcus*. The remaining *S. aureus* strains were tested against 10^7 CFU/ml of coagulase negative staphylococci (see Results section). The inoculum of *D. hansenii* was 10^5 CFU/ml. Mixed cultures with Micrococcaceae were incubated (without shaking) for 3 days at 37 °C, while mixed cultures with *D. hansenii* were incubated at 25 °C for 7 days.

Samples were withdrawn at intervals and CFU/ml (using the appropriate selective media), enterotoxin and thermonuclease and pH measurements were determined. Samples (10 ml) for thermonuclease and enterotoxin detection were centrifuged for 10 min at 12200 ***g*** at 5 °C and the supernatant frozen at −30 °C for later analysis. Percentage growth inhibition was calculated using the following formula (Ibrahim, 1978):

$$\%\ Inhibition = \frac{\log_{10} a - \log_{10} b}{\log_{10} a}$$

with *a* being the cfu/ml of *S. aureus* in pure culture, and *b* the cfu/ml of *S. aureus* in mixed culture.

Each experiment was performed twice and each sample assayed in duplicate. Data reported correspond to mean values.

Antibacterial activity of *P. nalgiovense*

The assay of Geisen *et al.* (1988) was used to study the antibacterial activity of *P. nalgiovense*.

Working mould cultures were prepared by streaking spores from stock cultures on plates of Malt Extract agar (Oxoid) which were incubated at 25 °C for 7 days. Working bacterial cultures (the four strains of *S. aureus*) were grown in 5 ml of BHI broth at 37 °C for 18 hr before use. The turbidity was adjusted with sterile saline (*ca* 10^5 CFU/ml) and each *S. aureus* strain streaked on the entire surface of Standard I Nutrient agar (Merck) plates. Agar 'disks' (8 mm) were aseptically removed from the sporulated mould cultures using a sterile cork borer and placed on the plates inoculated with *S. aureus*. After 48 hr of incubation at 37 °C, plates were examined for the presence of inhibition zones.

Thermonuclease assay

Thermonuclease activity was determined by the quantitative procedure of Ibrahim (1981). Before testing, each supernatant was heated in a boiling water bath for 15 min and its pH adjusted to 10. A standard curve was prepared using Micrococcal Nuclease (100 μmolar units/mg, Sigma Chemical Co., St. Louis, MO, USA) and Salmon Sperm DNA (Difco).

Detection of enterotoxins

Enterotoxins were detected using the sandwich ELISA technique of Fey *et al.* (1984). The reagents for ELISA tests were obtained from W. Bommeli (Bern, Switzerland).

RESULTS

M. varians vs *S. aureus*

The results of growing the four *S. aureus* strains in the presence of *M. varians* are given in Tables 1–4. Growth of *S. aureus* and production of enterotoxins (SEA–SED) and

thermonuclease were not inhibited by *M. varians*. The level of inocula affected neither growth nor the synthesis of metabolites.

Growth, enterotoxin production and thermonuclease activity of *S. aureus* strain ATCC 13565 (SEA producer) in mixed culture were determined against three levels of the three coagulase negative staphylococci. On the basis of the results obtained (little influence of the inocula), 10^7 CFU/ml only of effector bacteria were used in experiments with *S. aureus* strains producing SEB, SEC_1 and SED.

TABLE 1
Effect of *M. varians* and Coagulase-Negative Staphylococci on Growth of *S. aureus* ATCC 13565 in BHI Broth

Strain	*Time of incubation (hr)*					
	8	*16*	*24*	*36*	*48*	*72*
Control	7.32*	9.20	9.31	9.15	9.32	9.23
M. varians[a]	2.14†	ND	0.63	NT	4.25	1.59
M. varians[b]	0.63	ND	1.59	NT	ND	3.83
M. varians[c]	3.02	3.78	0.42	NT	3.93	2.13
S. xylosus[a]	ND	ND	1.40	ND	2.68	0.54
S. xylosus[b]	1.64	ND	ND	ND	0.54	1.08
S. xylosus[c]	ND	3.04	0.97	ND	2.68	0.76
S. saprophyticus[a]	ND	ND	0.11	0.55	3.97	0.98
S. saprophyticus[b]	ND	ND	2.79	ND	3.43	4.66
S. saprophyticus[c]	ND	ND	ND	ND	1.82	1.30
S. carnosus[a]	ND	ND	ND	3.06	0.54	4.77
S. carnosus[b]	ND	7.50	3.44	7.65	9.23	8.88
S. carnosus[c]	ND	17.39	24.81	23.50	21.57	25.68

*Levels of *S. aureus* in pure culture (log_{10} CFU/ml).
†Percentage of inhibition of *S. aureus* growth.
ND: Inhibition not detected. NT: Not investigated.
[a]Inocula of 10^5 CFU/ml, [b]10^6 CFU/ml, [c]10^7 CFU/ml.

TABLE 2
Effect of *M. varians* and Coagulase-Negative Staphylococci on Growth of Three *S. aureus* Strains in BHI Broth

Strain	*Time of incubation (hr)*				
	8	*16*	*24*	*48*	*72*
Control	7.02–7.14*	8.91–9.41	9.16–9.42	9.18–9.84	9.08–9.19
M. varians[a]	0.80–7.21†	2.36–3.46	ND–2.15	ND–0.63	ND–1.61
S. xylosus[a]	4.76–10.26	5.31 8.53	0.66–5.10	0.87–5.08	ND–2.20
S. saprophyticus[a]	0.42–3.42	0.44–7.07	0.11–5.74	ND–4.88	1.87–3.26
S. carnosus[a]	3.42–9.24	22.50–33.33	20.31–25.69	20.22–29.41	23.21–24.56

*Minimum and maximum levels of *S. aureus* in pure culture (log_{10} cfu/ml).
†Minimum and maximum percentage of inhibition observed (all three strains).
ND, Inhibition not detected.
[a]Inocula of 10^7 CFU/ml.

TABLE 3
Effect of *M. varians* and Coagulase-Negative Staphylococci on Staphylococcal Enterotoxins (A-D) Synthesis in BHI Broth

Strain	*Type strain of* S. aureus																				
	ATCC 13565 (Toxin A)						*ATCC 14458 (Toxin B)*					*FRI 137 (Toxin C_1)*					*ATCC 23235 (Toxin D)*				
	8†	*16*	*24*	*36*	*48*	*72*	*8*	*16*	*24*	*48*	*72*	*8*	*16*	*24*	*48*	*72*	*8*	*16*	*24*	*48*	*72*
Control	ND	19*	31	25	32	31	ND	71	81	88	91	ND	2	11	11	13	ND	21	19	38	41
M. varians[a]	ND	15	15	NT	16	44															
M. varians[b]	ND	14	15	NT	11	47															
M. varians[c]	ND	18	12	NT	17	49	ND	49	72	72	106	ND	3	4	6	5	ND	24	22	29	46
S. xylosus[a]	ND	13	32	22	16	13															
S. xylosus[b]	ND	16	31	27	14	9															
S. xylosus[c]	ND	3	23	11	7	24	ND	41	60	45	61	ND	1	3	4	4	ND	16	27	5	17
S. saprophyticus[a]	ND	20	27	20	8	18															
S. saprophyticus[b]	ND	12	22	31	6	11															
S. saprophyticus[c]	ND	11	10	26	7	10	ND	47	42	54	39	ND	ND	ND	ND	ND	ND	12	10	13	11
S. carnosus[a]	ND	9	26	19	4	8															
S. carnosus[b]	ND	4	3	2	1	1															
S. carnosus[c]	ND	ND	1	1	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1	1	2

*ng/ml. †Incubation time (hr).
ND, Not detected. NT, Not investigated.
[a]Inocula of 10^5 CFU/ml, [b]10^6 CFU/ml, [c]10^7 CFU/ml.

TABLE 4
Effect of *M. varians* and Coagulase-Negative Staphylococci on Staphylococcal Thermonuclease Synthesis in BHI Broth

Strain	*Type strain of* S. aureus			
	ATCC 13565	*ATCC 14458*	*FRI 137*	*ATCC 23235*
Control	3.86*	3.86	3.21	3.43
M. varians[a]	3.21	NT	NT	NT
M. varians[b]	3.39	NT	NT	NT
M. varians[c]	3.00	3.29	3.65	2.81
S. xylosus[a]	4.15	NT	NT	NT
S. xylosus[b]	3.50	NT	NT	NT
S. xylosus[c]	3.21	3.21	1.41	2.64
S. saprophyticus[a]	3.43	NT	NT	NT
S. saprophyticus[b]	3.14	NT	NT	NT
S. saprophyticus[c]	2.78	2.78	2.13	2.42
S. carnosus[a]	3.29	NT	NT	NT
S. carnosus[b]	3.07	NT	NT	NT
S. carnosus[c]	2.35	1.26	0.40	1.56

*Log ng/ml after 72 hr incubation at 37 °C without shaking.
NT, Not tested.

S. xylosus vs *S. aureus*

S. xylosus had little or no apparent effect on the growth of *S. aureus* strains ATCC 13565, 14458 and 23353, though enterotoxin synthesis was partially inhibited (Tables 1–3). In the presence of *S. xylosus*, strain FRI 137 at first declined in numbers but later grew, the final counts being in the range of the control values. A marked reduction in SEC_1 and thermonuclease production was noted (Table 4).

S. saprophyticus vs *S. aureus*

In mixed culture, SEC_1 production was completely inhibited even when the final counts of *S. aureus* strain FRI 137 were greater than 10^8 CFU/ml (Table 3). There was partial inhibition of SEA, SEB and SED synthesis but very little effect on the growth of the respective *S. aureus* strains (Tables 1–3).

S. carnosus vs *S. aureus*

SEA, SEB and SED synthesis was strongly inhibited by 10^7 CFU/ml of *S. carnosus*. SEC_1 was not detected. After 72 hr incubation, counts of *S. aureus* were always lower ($> 2 \log_{10}$ CFU/ml) than those in pure cultures (Tables 1–3). Inocula of 10^5 and 10^6 CFU/ml had significant inhibitory effect on SEA synthesis, but growth of *S. aureus* strain ATCC 13565 was only slightly inhibited (Tables 1 and 3). Decrease of thermonuclease activity was observed with all three levels of inoculum (Table 4).

D. hansenii vs S. aureus

Table 5 illustrates the effect of *D. hansenii* upon growth of *S. aureus* ATCC 13565 and production of SEA and thermonuclease. Depending on the incubation time, *D. hansenii*

TABLE 5
Growth and Synthesis of Thermonuclease and SEA by *S. aureus* ATCC 13565 in Pure Culture and in Mixed Culture with *D. hansenii*

Strain	*Hour*	log_{10} S. aureus/*ml*	*%inhib*	*SEA (ng/ml)*	*TNase log ng/ml*
S. aureus at 30 °C	0	4.94		ND	ND
	8	7.15		ND	1.61
	24	8.93		13.88	2.54
	48	9.05		16.93	3.33
	72	9.05		19.24	3.25
	168	8.80		29.89	3.64
S. aureus + *D. hansenii* at 30 °C	0	5.05		ND	ND
	8	6.97	2.50[a]	ND	0.67
	24	8.81	4.34	13.95	3.25
	48	9.15	ND	10.75	3.33
	72	8.56	5.41	5.84	3.33
	168	8.84	ND	18.11	13.95
S. aureus at 25 °C	0	4.96		ND	ND
	8	5.93		ND	0.08
	24	7.59		6.56	2.08
	48	8.33		4.49	3.25
	72	8.19		5.49	3.33
	168	8.09		14.82	2.43
S. aureus + *D. hansenii* at 25 °C	0	5.10		ND	ND
	8	5.95	ND	ND	−0.27
	24	8.80	ND	7.38	2.08
	48	8.81	ND	3.91	2.54
	72	8.12	0.86	5.49	2.93
	168	8.01	6.85	17.87	4.19

ND, Not detected.
[a]Percentage of inhibition of *S. aureus* growth.

had little or no apparent effect on the growth of *S. aureus*. SEA synthesis was partially inhibited at 30 °C and slightly stimulated after 7 days incubation at 25 °C.

P. nalgiovense vs *S. aureus*

Good growth was evident for both mould and bacteria. However, no zones of inhibition surrounding the mould plugs were observed.

DISCUSSION

In sausages, Andres (1977) observed that micrococci suppressed the growth of undesirable microorganisms and Daly *et al.* (1973) suggested that *Micrococcus* spp. were responsible for the inhibition of staphylococcal enterotoxin production. Using screening methods, other authors (Oberhofer & Frazier, 1961; Graves & Frazier, 1963; Brankova, 1976) have shown that the effect of micrococci on *S. aureus* depended upon the strains tested and the procedure used (plate assays or liquid media). Using agar plates, Oberhofer and Frazier (1961) reported that *M. varians* had little or no apparent effect on the growth of four strains of *S. aureus*, while 10 strains of micrococci, including two of *M. varians*, isolated

by Cantoni *et al.* (1992) from sausages were shown to produce bacteriocins active against five strains of *S. aureus*.

M. varians is particularly sensitive to the level of oxygen (Lücke & Hechelmann, 1987) and is therefore a poor competitor with facultative anaerobes in liquid media. Reduction in oxygen levels produced by metabolism of staphylococci could be partly responsible for the observed lack of effect of *M. varians* strain DSM 20033 against the *S. aureus* strains.

Dubos and Ducluzeau (1969*a*,*b*) studied the interaction between micrococci and *Staphylococcus pyogenes* in liquid media. One strain of *Micrococcus* was repressed when the Eh fell to values below 100 mV.

Staphylococci are known to produce a wide variety of substances including bacteriolytic enzymes, antibiotics and bacteriocins, which show an inhibitory effect on other staphylococci and related bacteria (Tagg *et al.*, 1976). Several workers have also shown the antagonistic action of some strains of coagulase-negative staphylococci (*S. epidermidis*, *S. xylosus* and unidentified isolates) against strains of *S. aureus* (Jetten & Vogels, 1972; Tagg *et al.*, 1976; Cantoni *et al.*, 1992). The strains of coagulase-negative staphylococci tested by us (*S. xylosus*, *S. saprophyticus* and *S. carnosus*) showed little inhibitory effect on growth of four strains of *S. aureus* although they reduced the amount of enterotoxin produced (SEA, SEB and SED), or prevented enterotoxin production (SEC_1) entirely. The most effective strain was *S. carnosus*. This contrasts with the results of Erol and Hildebrandt (1992) who reported that *S. carnosus* did not significantly affect *S. aureus* inoculated in dry sausages.

Noleto and Bergdoll (1980) studied the production of staphylococcal enterotoxins (SEA, SED and SEC_1) in the presence of different levels of a non-enterotoxigenic strain of *S. aureus* in laboratory medium, milk and ham. This strain inhibited to some extent the growth of the enterotoxigenic strains in milk, but not sufficiently to prevent enterotoxin production. Enterotoxin was detectable in milk (37 °C) when counts of the non-enterotoxigenic strain were 15–20 times greater than those of the enterotoxigenic ones, and in ham (37 °C) when the ratio was 60–77:1. They also found that incubation conditions (temperature and agitation) could influence both growth of and enterotoxin production by *S. aureus*.

Detection of thermostable deoxyribonuclease is used as a rapid and inexpensive procedure for screening foods such as sausages for indication of extensive staphylococcal growth and possible presence of enterotoxins (NRC, 1985). In this study, TNase was detected whenever enterotoxin was detected, though the influence of the effector microorganisms on TNase synthesis was dependent on the test strain.

One might conclude from the above information that results from research designed to study interactions between enterotoxin-producing strains of *S. aureus* and other Micrococcaceae is far from conclusive. Strain differences, inoculum size, incubation conditions (temperature and aeration), media and foods all influence survival, growth, TNAse synthesis and toxinogenesis of *S. aureus*.

Little attention has been given to the antimicrobial activity of yeasts against *S. aureus*. In sausages, Meisel *et al.* (1989) noted that addition of *D. hansenii* contributed markedly to the inhibition of *S. aureus*. This was because of the depletion of oxygen and there was no evidence for the involvement of any other mechanism. However, in pickled cheese brine, Nussinovitch *et al.* (1987) reported that acid-consuming yeasts increased the pH to a level which enhanced staphylococcal growth.

In this study the strain of *D. hansenii* showed little inhibitory effect on growth of the SEA-producing strain of *S. aureus*, but reduced enterotoxin synthesis at 30 °C. However, after 7 days incubation at 25 °C, levels of SEA and thermonuclease were higher in associative culture than in pure culture. Differences in SEA production in mixed cultures at 25 and 30 °C could not be related to the pH change resulting from yeast growth, since pH values were very similar at both temperatures.

Finally, our data indicate that the strain of *P. nalgiovense* examined was not capable of inhibiting the four strains of *S. aureus*. Duitschaever and Irvine (1971) found that growth of *Penicillium* spp. on the surface of cheese can cause a gradual increase in pH which enhances growth of enterotoxigenic staphylococci. On the other hand, Geisen *et al.* (1988) reported that six strains of *P. nalgiovense* had in-vitro inhibitory activity against *Listeria monocytogenes*.

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