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Growth and enterotoxin production of *Staphylococcus aureus* in shrimp

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SUMMARY

Strains of *Staphylococcus aureus* isolated from shrimp were examined for phage pattern and enterotoxin production; 63% of the strains isolated from North Sea shrimp were typable with the International and additional set of phages, as were 38% of the strains isolated from South-East Asian shrimp. Staphylococcal enterotoxin(s) (SE) were produced by 48% and 35% of strains isolated from North Sea and South-East Asian shrimp respectively. Growth and enterotoxin production by *S. aureus* in shrimp was examined in storage experiments at 22 °C. *S. aureus* increased by 1–2 log units in 24 h when the organism was only a minor part of the total microflora of shrimp. When *S. aureus* was an equivalent part of the total flora its numbers increased by 3–4 log units in 24 h. Enterotoxins A and B became detectable when the number of *S. aureus* exceeded 10^7 per g in aseptically peeled shrimp. Results indicate that *S. aureus* is able to produce enterotoxin in shrimp, but its production depends upon a number of factors, including the relationship between *S. aureus* and competitive micro-organisms. It is concluded that the presence of *S. aureus* on commercially produced shrimp represents a potential hazard to health.

INTRODUCTION

Since peeling of shrimp is mostly carried out by hand, it may be assumed that it will be contaminated with *Staphylococcus aureus* of human origin. Enterotoxin production is more often observed in staphylococci of human origin than in those from other sources. It has become clear from a number of studies that about 50% of *S. aureus* isolated from humans produce enterotoxin (Wieneke, 1974; Olsvik *et al.* 1981; De Nooy, van Leeuwen & Notermans, 1982). However, only a few food-poisoning outbreaks have been ascribed to staphylococcal enterotoxins in cooked peeled shrimp (Gilbert & Wieneke, 1973; Turnbull & Gilbert, 1982); no recent outbreaks have been reported in the annual reports of Centers for Disease Control, USA, the Health Protection Branch, Canada, or elsewhere. There is no information available on the ability of *S. aureus* to produce enterotoxin in shrimp. To gain insight into the potential health hazard of *S. aureus* in shrimp the phage-pattern and enterotoxigenicity of strains isolated from shrimp was determined. Storage experiments were also carried out to determine whether *S. aureus* could multiply and produce enterotoxins in shrimp.

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MATERIALS AND METHODS

Source of shrimp

Study on enterotoxigenicity of S. aureus. Cooked peeled shrimp were obtained from a wholesale dealer. Samples of North Sea shrimp were from 50 different batches and those from South-East Asia from 27 different batches. The shrimp from the North Sea were peeled in the Netherlands while those from South-East Asia were peeled at source.

Storage experiments. These experiments were carried out with shrimp originating from the North Sea. Cooked but unpeeled shrimp were obtained from the fish auction where the shrimp was brought ashore. These shrimp already contained 0·4% (w/w) benzoic acid. Cooked, peeled shrimp were obtained from a wholesale dealer. These differed from the unpeeled shrimp only in that they had been peeled in the normal manner. They also contained 0·4% benzoic acid.

All the shrimp were transported under cooled conditions and stored at −20 °C.

Bacteriological examinations

Samples of 10 g were macerated in a blender with 90 ml of peptone saline and decimally diluted in peptone saline as described in ISO 6887 for enumeration of the different groups of bacteria (Anon, 1983).

The aerobic plate count was determined according to ISO 4833 (Anon, 1978) with the pour-plate method using Plate Count Agar (Oxoid CM 325) incubated at 30 °C for 3 days. The Enterobacteriaceae count was determined according to ISO 5552 (Anon, 1979) with the pour-plate method with overlayer using Violet Red Bile Agar (Difco 0012) supplemented with glucose (10 g/l) incubated at 37 °C for 24 h.

The *S. aureus* count was determined according to the method of Beckers *et al.* (1984) with the pour-plate method using rabbit plasma bovine fibrinogen agar incubated at 37 °C for 24–48 h. Typical *S. aureus* colonies, black and surrounded by a turbid halo, were isolated and stored for phage typing and enterotoxin testing.

The count of Gram-positive cocci was determined with the surface plate method using laboratory-made blood agar containing 75 ml/l defibrinated sheep blood and 40 mg/l nalidixic acid incubated at 37 °C for 24 h. After incubation the colonies were examined macroscopically and microscopically and the number of Gram-positive cocci was calculated.

Phage typing and enterotoxin production of strains

Strains were phage-typed using the International basic set of typing phages (report Subcommittee on Staphylococcal Phage-typing, 1975). If necessary they were further tested with an additional set of phages (Van Leeuwen & Rost, 1976).

For enterotoxin production culture supernatants were prepared using the dialysis-sac culture method of Donnelly *et al.* (1967) and examined for enterotoxins A–F by the Optimal Sensitivity Plate (OSP) method of Robbins, Gould & Bergdoll (1974). All of the reference enterotoxins (A–F) and specific antisera used were provided by Professor M. S. Bergdoll of the Food Research Institute, Madison, Wisconsin, USA.

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Artificial contamination of shrimp with S. aureus

The following *S. aureus* strains were used (laboratory code numbers): 196E, producing enterotoxin A; 14458, producing enterotoxin B; 137, producing enterotoxin C; and Set 13, producing enterotoxin F.

The strains were grown in Brain Heart Infusion (Difco 0037) at 37 °C for 18 h on a gyratory shaker. The cultures were diluted in peptone saline to give different levels of *S. aureus* (see Table 3); 500 g of cooked peeled shrimp was mixed with 100 ml of a dilution in a Turbula mixer (W. A. Bachofer, Basel, Switzerland) for 20 min and subsequently put into a sterile strainer for about 20 min to remove the remaining liquid.

Shrimp storage experiments

Cooked and normally peeled shrimp, as well as cooked and aseptically peeled shrimp were used for storage experiments. In the latter case shrimp were peeled in the laboratory by staff wearing sterile gloves. Some portions of cooked and normally peeled shrimp were previously pasteurized to eliminate competitive micro-organisms.

Portions of shrimp prepared in different ways were artificially contaminated with *S. aureus* as described in the previous paragraph. Artificially and naturally contaminated shrimp were stored at 22 °C and samples were taken after 0, 1, 2 and 3 days for bacteriological examination and for the detection of *S. aureus* enterotoxins.

Detection of S. aureus *enterotoxins in shrimp*

The extraction and concentration method described by Notermans *et al.* (1983) was used for detecting *S. aureus* enterotoxins. The sandwich ELISA technique was used for the determination of enterotoxins as described by Bühning-Pfaue, Timmermans & Notermans (1981) and Notermans *et al.* (1983).

RESULTS

Enumeration of S. aureus *in shrimp*

The frequency distribution of *S. aureus* counts on shrimp from the North Sea and from South-East Asia are presented in Fig. 1; 22% of the North Sea shrimp and 29% of the South-East Asian shrimp had *S. aureus* counts exceeding 5×10^3 per g, this being the value beyond which a lot is rejected according to draft FAO/WHO specifications (1977).

Phage types and enterotoxin production of strains isolated from shrimp

The results of phage typing are presented in Table 1. Using the International set of phages, 39% of the 224 strains isolated from 50 samples of North Sea shrimp were typable.

A further 34% were typable using the additional phages. Phage groups I, II, I/III, XI and XVI were predominant (11–17%). Twenty-seven per cent of the strains remained untypable. Only 38% of the 37 strains isolated from 27 samples of shrimp originating from South-East Asia were typable using both the Inter-

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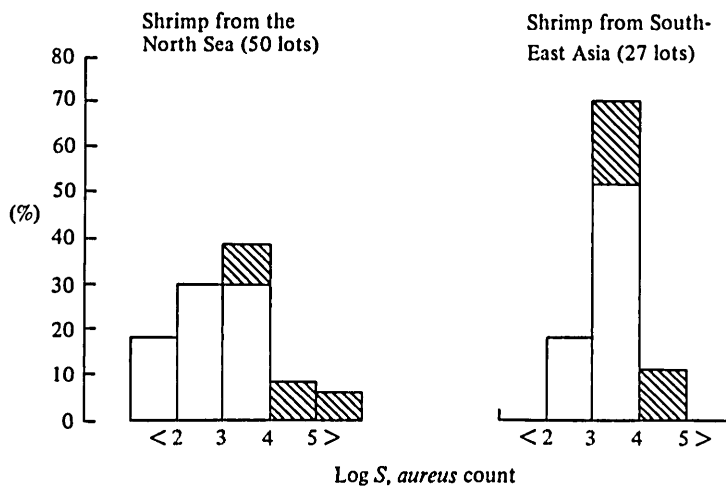

Fig. 1. Frequency distribution of *S. aureus* counts in cooked peeled shrimp originating from the North Sea and from South East Asia. □, Percentage of samples with *S. aureus* counts $<5\times10^3$/g; ▨, percentage of samples with *S. aureus* counts $>5\times10^3$/g.

Table 1. *Phage groups of strains of* Staphylococcus aureus *isolated from cooked peeled shrimp originating from the North Sea and from South-East Asia*

		Number of strains	
	Phage group*	Shrimp from the North Sea (50 lots)	Shrimp from South-East Asia (27 lots)
International set	I	41 (17%)	3 (8%)
	II	26 (11%)	4 (11%)
	III	14 (6%)	1 (3%)
	I/III	11 (5%)	4 (11%)
Additional set*	XI	31 (13%)	2 (5%)
	XII	11 (5%)	—
	XIII	1 (<1%)	—
	XV	12 (5%)	—
	XVI	28 (11%)	—
	Non-typable†	69 (27%)	23 (62%)
	Total	244	37

* Described by Van Leeuwen & Rost (1976). † With both sets of phages.

national and the additional set of phages; phage groups II and I/III were predominant (both 11%). Enterotoxin (SE) production by strains isolated from shrimp is presented in Table 2. Forty-eight per cent of strains isolated from North Sea shrimp produced SE. Thirty-five per cent of strains isolated from shrimp originating from South-East Asia produced SE. Some strains produced more than one type of toxin.

Growth of S. aureus *and competitive micro-organisms in shrimp during storage*

Growth of *S. aureus* and competitive micro-organisms in shrimp during storage is illustrated in Fig. 2. In normally peeled shrimp which contained the naturally

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Table 2. *Enterotoxin production by strains of* Staphylococcus aureus *isolated from cooked peeled shrimp originating from the North Sea and from South-East Asia*

Origin of shrimp	No. of strains tested	No. of strains which produce enterotoxin A–F	Frequency distribution of type of enterotoxine produced (no. of strains)‡					
			A	B	C	D	E	F
North Sea	50*	24 (48%)	3	3	8	—	—	13
South-East Asia	37†	13 (35%)	1	4	4	3	—	6

* Strains were isolated from 50 different lots. From each phage type one strain was tested.
† Strains were isolated from 27 different lots.
‡ Some strains produced more than one type of toxin.

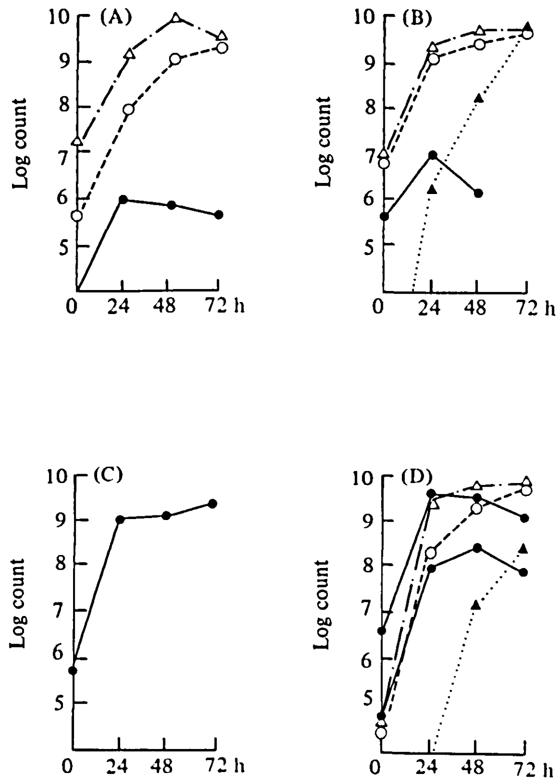

Fig. 2. Growth of *S. aureus* and competitive micro-organisms in cooked peeled shrimp during storage at 22 °C. (A) Normally peeled shrimp, naturally contaminated. (B) Normally peeled shrimp, artificially contaminated. (C) Pasteurized shrimp, artificially contaminated. (D) Aseptically peeled shrimp, artificially contaminated. △—·—△, Aerobes; ●—●, *S. aureus*; ▲.........▲, Enterobacteriaceae; ○-----○, Gram-positive cocci.

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occurring competitive micro-organisms the *S. aureus* present as a natural contaminant increased in 24 h from 6×10^3 to 1×10^6 per g, and decreased on prolonged storage (A). The competitive micro-organisms increased from 10^7 to 10^9 for aerobes and from 10^6 to 10^9 for Gram-positive cocci; Enterobacteriaceae did not appear in this experiment. In the same type of shrimp artificially inoculated, *S. aureus* increased from 5×10^5 to 1×10^7 per g in 24 h and then decreased on prolonged storage (B). At the same time the competitive aerobes and Gram-positive cocci increased from 10^7 to 10^9 per g. Levels of Enterobacteriaceae increased in this experiment to 10^9/g. When the shrimp was previously pasteurized the inoculated *S. aureus* grew as a pure culture from 10^6 to 10^9/g in 24 h (C). When the number of competitive micro-organisms was limited by peeling the shrimp aseptically in the laboratory the inoculated *S. aureus* increased in the order of 4 log-units in 24 h, regardless of the level of inoculation (D; two different inoculation levels are given). The competitive micro-organisms increased as in previous experiments.

Enterotoxin production during storage

The results of enterotoxin production during storage are presented in Table 3. The results after 2 and 3 days of storage did not differ from those after 24 h. Enterotoxin A production was only detected in aseptically peeled shrimp at a level of *S. aureus* exceeding 6×10^6/g. Enterotoxin B production was detected on one occasion in aseptically peeled shrimp at a *S. aureus* level of 4×10^7/g. Production of enterotoxins C and F was not observed. There was no relationship between increase of *S. aureus* and production of enterotoxin(s).

DISCUSSION

The contamination level of North Sea shrimp with *S. aureus* reported in this study is higher than that of Beckers *et al.* (1981). In the present study 22 % of the samples contained more than 5×10^3 *S. aureus*, while in the earlier one only 6 % contained more than 2×10^3. The contamination of South-East Asian shrimp with *S. aureus* is at a similar level in both studies: 29 % of samples with more than 5×10^3 *S. aureus* in the current study compared with 34 % of samples with more than 2×10^3 reported earlier. The high contamination levels with *S. aureus* indicate poor hygiene during peeling of shrimp.

The shrimp were contaminated with the strains of *S. aureus* most commonly found in humans (De Nooy, van Leeuwen & Notermans, 1982). This is explained by the fact that peeling is still done by hand. However, a relatively high percentage (27 %) of *S. aureus* present on North-Sea shrimp was untypable using the International and additional set of phages. Sixty-two per cent of the *S. aureus* isolated from South-East Asian shrimp were untypable, due mainly to the fact that only 5 % could be typed with the additional set. In the study reported by De Nooy, van Leeuwen & Notermans (1982) only 8 % of *S. aureus* strains isolated from Dutch human beings were untypable using both phage typing sets. The main reason for the high percentage of untypable strains from South-East Asian shrimp may be that the phages of the additional set originate from *S. aureus* isolated from humans in the Netherlands. The same may also be true for the North Sea shrimp as these

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Table 3. *Growth and enterotoxin production by* Staphylococcus aureus *in artificially contaminated cooked peeled shrimp during storage at 22 °C for 24 h*

S. aureus strain(s)	Type of enterotoxin(s)	Log *S. aureus* count: After contamination	Log *S. aureus* count: After storage	Enterotoxin production*
		Aseptically peeled shrimp		
196E	A	1·9	5·2	−
		1·8	6·1	−
		2·2	6·8	+
		2·7	6·8	−
		3·2	7·2	+
		4·5	8·0	−
		4·5	8·0	+ +
		6·5	9·7	+ + +
14458	B	3·5	7·6	+
		3·5	8·9	−
14458, 137, Set 13	B, C, F mixture	5·0	8·0	−
		6·7	9·5	−
		Normally peeled shrimp		
196E	A	5·3	7·0	−
14458	B	5·5	6·9	−
137	C	5·5	6·7	−
Set 13	F	6·0	7·4	−
14458, 137	B, C mixture	5·9	7·2	−

* −, < 0·1 μg of SE/100 g of shrimp; +, 0·1 − 1 μg of SE/100 g of shrimp; + +, 1–10 μg of SE/100 g of shrimp; + + +, > 10 μg of SE/100 g of shrimp.

were frequently peeled by workers from other countries. The frequency of enterotoxin production by *S. aureus* isolated from shrimp is in agreement with the frequency reported for strains isolated from humans (De Nooy, van Leeuwen & Notermans, 1982; Wieneke, 1974; Petrás & Maskova, 1982). It is of interest that 22 % of the strains tested produced SEF. There are no clear indications whether SEF is a real enterotoxin (Bergdoll *et al.* 1981) and up till now there are no reports of food-borne disease caused by this toxin. Storage experiments demonstrated a limited increase of *S. aureus* by 1–2 log units in 24 h when *S. aureus* was only a minor part of the total microflora of shrimp as would be found in normally peeled shrimp. When the competitive flora, measured as aerobes and micrococci, reached 10^8/g the number of *S. aureus* decreased on prolonged storage. When *S. aureus* was an equivalent part of the total flora as on aseptically peeled shrimp, the numbers of this organism increased by 3–4 log units in 24 h. These results clearly indicate that *S. aureus* is inhibited by the activities of competitive micro-organisms, but that these have only a minor effect on *S. aureus* when the organism is an equivalent part of the total micro-flora.

Enterotoxins may become detectable when the number of *S. aureus* is aseptically peeled shrimp reaches or exceeds 10^7 per g. However, even under these conditions enterotoxin production did not occur in all cases.

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The results indicate that *S. aureus* is able to produce enterotoxin in shrimp, but whether production actually occurs seems to depend on a number of, as yet, unknown factors. Moreover, the results also indicate that enterotoxin production also depends on the relationship between *S. aureus* and competitive micro-organisms. If competitors predominate, outgrowth of *S. aureus* is limited even at 22 °C; under such conditions production of enterotoxin is unlikely. When *S. aureus* is equal to or exceeds the level of competitive micro-organisms production of enterotoxins can be expected.

The absence of recent reports on staphylococcal food poisoning from cooked peeled shrimp is explained by our findings, since *S. aureus* normally only constitutes a minor part of the micro-flora of cooked peeled shrimp (Beckers *et al.* 1981; Gilbert, 1982). Our results also give evidence to support the suspected staphylococcal food poisoning from shrimp described by Todd *et al.* (1973), the outbreak being caused by recontamination with *S. aureus* of previously sterilized shrimp. The same was true for a similar outbreak in the UK (Gilbert & Wieneke, 1973). In reports of other outbreaks such as those in the UK (Gilbert & Wieneke, 1973; Turnbull & Gilbert, 1982) data are lacking on the number of competitive micro-organisms, so that it was not possible to evaluate these outbreaks in relation to this study. Nevertheless, the presence of *S. aureus* on commercially produced shrimp still represents a potential health hazard. A possible shift in the relationship between *S. aureus* and competitive micro-organisms cannot be excluded. Furthermore shrimp are often used in other foods, such as prepared salads and in these foods the protection from competitive micro-organisms against *S. aureus* may be quite different.

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