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Minimal Water Activity for Enterotoxin A Production and Growth of *Staphylococcus aureus*

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The minimal water activity (a_w) for growth was correlated with enterotoxin A formation by two strains of *Staphylococcus aureus* in a salt mixture broth. Within 7 days at 30°C both strains grew and formed enterotoxin A minimally between a_w 0.864 and 0.867, but at 25°C, the minimal a_w for both activities was increased to between 0.870 and 0.887 after a 2-week incubation.

Various intermediate moisture foods (IMF) with a water activity (a_w) in the range of 0.90 to 0.60 have been prepared, such as sweet and sour pork (2), ready-to-eat chicken (9), and hennican (1). Several IMF are adjusted to a_w 0.85; however, some of these foods share a higher value, as from 0.85 to 0.90, in order to avoid off flavors by excess use of humectants. Moreover, food laws of several countries forbid addition of such humectants as glycerol and propylene glycol (10).

The ability of *Staphylococcus aureus* to grow aerobically down to a_w 0.86 in laboratory media is well known (4, 12, 13, 17). However, this organism is inhibited anaerobically at an a_w below 0.91 (17).

S. aureus forms five enterotoxins: A, B, C, D, and E; enterotoxin A is the most commonly encountered type from food-borne strains. This toxin is associated with over 50% of the staphylococcal strains isolated from food poisoning outbreaks (3, 6) and from foods (15). Thus, from a practical standpoint, contamination of IMF by enterotoxin A-forming staphylococci could constitute a serious risk. According to Pawsey and Davies (14), slow growth or prolonged survival of *S. aureus* in IMF is possible.

The minimal a_w for staphylococcal enterotoxin A production in laboratory media has been previously reported as 0.90 (23). We have attempted in this present report to pinpoint the limiting a_w for growth and toxin A formation by *S. aureus* within the range of 0.90 to 0.85.

The laboratory medium for the effect of a_w on growth and enterotoxin A production by *S. aureus* contained the following ingredients: 2.5% Standard I Nutrient Broth (Merck); 1.0% yeast extract (Difco); and the salts sodium chloride, potassium chloride, and sodium sulfate in a ratio of 5:3:2 mol. Table 1 indicates the approximate amounts of the salt mixture required to calculate a particular level of a_w and the measured a_w of the uninoculated salt mixture broths. Water activity, symbolized by a_w, is commonly defined as either the ratio of vapor pressure of a solution to that of pure water (17) or the equilibrium relative humidity pertaining to the atmosphere above a material enclosed in a chamber divided by 100 (23). The a_w levels of control and salted broths were determined by SINA sensors (NOVA-SINA, Switzerland) containing lithium chloride and attached to an ISO apparatus (International Sales Organisation, Ottobrunn/Munich, West Germany), which can measure 22 samples at one time. With this instrument, a_w can be measured accurately to three decimal places (8). Each 15-ml sample in duplicate was equilibrated to 25°C for 2 to 3 h before measurement of a_w. Before use, sensors of the ISO instrument were calibrated against saturated $BaCl_2$, KBr, KCl, K_2SO_4, and NaCl solutions.

A striking discrepancy exists between calculated and measured a_w values, as the calculated a_w data average consistently about 0.02 higher than those measured. This discrepancy can be explained by the fact that the calculated a_w data apply to salt solutes in pure water, whereas measured a_w values concern salt mixture broths containing protein hydrolysates, sugars, and soluble salts, and these can depress water activity. For example, 1% of such materials as sodium chloride, glucose, and milk protein, all together in a pure water system, can depress a_w by about 0.01 (8).

The minimal a_w for growth and enterotoxin A production was determined for strains 100 and M 7/1 of *S. aureus* in salt mixture broths at 30°C (Table 2). Strain 100 was donated by C. A. Genigeorgis (Veterinary School, University of California, Davis), and M 7/1 was donated by H. J. Sinell (Institute of Food Hygiene, Free University of Berlin, West Berlin, Germany).

Colony counts were carried out by first pregrowing either strain in standard I nutrient broth (Merck) and incubating the flask cultures on a 37°C water bath shaker for about 18 h. A 6-h subculture of each strain was prepared to seed the salt mixture broths and the unsalted control. Initial *S. aureus* totaled 10^5 viable cells per ml. The inoculated salt mixture broths containing *S. aureus* 100 or M 7/1 were incubated at 30°C for 7 days and were sampled for colony counts and enterotoxin assays. Cultures were diluted in sterile saline and plated on brain heart infusion agar plates by the drop-plating technique of Untermann (26). These plates were incubated at 37°C for about 24 h and then counted.

For toxin A isolation, 20-ml control and salt mixture broths containing cells of *S. aureus* were harvested by 4°C centrifugation at 40,000 × *g*. Supernatant fluid was concentrated in dialysis tubing (Kalle, 27-mm diameter, Wiesbaden, West Germany) overnight at room temperature against a 50% solution of Carbowax 20,000 (Serva Feinbiochemica, Heidelberg, West Germany). Material deposited in the tubing after concentration was suspended in 2.0 ml of distilled water and transferred to 5.0-ml serum vials for 25 h of lyophilization at −40°C (Christ, Delta II, Osterode/Harz, West Germany). Lyophilized samples were treated with 1.0% trypsin (2,000 Einheiten [units]/g, Merck) for 30 min at 37°C to remove foreign proteins interfering in the microslide for staphylococcal enterotoxin and reconstituted to 0.5 ml with distilled water by the method of Reiser et al. (16). Therefore, toxin material was concentrated by 40-fold. Enterotoxin A samples were assayed by microslide gel diffusion according to the method of Untermann (27) and quantitated by the microslide quantification procedure of Fung et al. (5) with a sensitivity of 0.20 μg of toxin per ml. M. S. Bergdoll, Food Research Institute, Madison, Wis., supplied reference enterotoxin A, and H.-J. Sinell, West Berlin, contributed the corresponding antiserum.

TABLE 1. *Approximate amounts of mixed solute needed to calculate the a_w of salt mixture broths and comparison of calculated and measured a_w values*

Solute (%, wt/wt)[a]			a_w calculated[b]	a_w measured[c]
NaCl	KCl	Na_2SO_4		
6.90[d]	5.40	6.75	0.900	0.890
7.55	5.90	7.35	0.890	0.870
7.75	6.10	7.55	0.888	0.867
7.95	6.25	7.80	0.886	0.864
8.15	6.40	7.95	0.884	0.860
8.35	6.55	8.20	0.882	0.855

[a] NaCl, 5 mol; KCl, 3 mol; Na_2SO_4, 2 mol; for convenience, the moles of each salt have been converted to percentages (wt/wt).

[b] a_w levels apply to percentage of salts in distilled water.

[c] Average of two samples of uninoculated salt mixture broth.

[d] Amounts of NaCl should be lowered by 1.0% if Standard I Nutrient Broth (Merck) supplemented with yeast extract is used as the basal medium.

In Table 2, the minimal a_w for growth of both *Staphylococcus* strains 100 and M 7/1 at 30°C was close to 0.864. This finding agrees with reports from previous investigators in which the minimal a_w is reported to be about 0.86 for aerobic growth in laboratory media (4, 12, 13, 17). However, in the present study neither strain grew appreciably within 7 days at 30°C in a salt mixture broth adjusted to an a_w of 0.860. Troller (23) observed that *S. aureus* 196 E, which forms enterotoxin A, extended its generation time of 30 min to 1,000 min at 37°C when the a_w of brain heart infusion broth was reduced from 0.98 to 0.885.

The minimal number of staphylococci required to produce enough enterotoxin to cause food poisoning is believed to be about 10^7 cells per gram of food (12). Previously, the minimal

TABLE 2. *Effect of a_w on growth and enterotoxin A production by S. aureus in salt mixture broths at 30°C*

a_w[a]	Strain 100			Strain M 7/1		
	Colony count/ml		Enterotoxin (μg/ml)	Colony count/ml		Enterotoxin (μg/ml)
	Initial	7 days	7 days	Initial	7 days	7 days
0.980[b]	4.0×10^5	5.6×10^8	6.0	2.8×10^5	9.1×10^8	4.0
0.890	4.0×10^5	1.0×10^8	4.0	2.8×10^5	1.9×10^8	2.0
0.870	4.0×10^5	4.7×10^7	3.0	2.8×10^5	7.1×10^7	1.0
0.867	4.0×10^5	2.6×10^7	2.0	2.8×10^5	1.2×10^7	0.8
0.864	4.0×10^5	5.1×10^5	—[c]	2.8×10^5	8.1×10^5	—
0.860	4.0×10^5	2.9×10^5	—	2.8×10^5	6.2×10^4	—
0.855	4.0×10^5	8.0×10^3	—	2.8×10^5	3.0×10^3	—

[a] Uninoculated samples.

[b] Standard nutrient broth supplemented with yeast extract, without added salts.

[c] —, Undetectable enterotoxin A in samples.

a_w for enterotoxin A production by *S. aureus* has not been determined. Troller (23) studied enterotoxin A production only down to an a_w level of 0.90.

In the present study the effect of water activity on growth and enterotoxin A formation by two strains of *S. aureus* was also determined at 25°C by the same procedures as those described for 30°C (Table 3). The data indicate that minimal growth and enterotoxin A production occurred with an a_w 0.02 value higher at 25°C than at 30°C (Table 2). At 25°C and an a_w of 0.887, the colony count for strain M 7/1 after 7 days resembled that of strain 100 after 14 days (Table 3). This suggests that strain 100 may be more sensitive to lowered a_w than strain M 7/1. It has already been observed that strains of *S. aureus* vary in their tolerance to decreased a_w (18, 24).

During the present investigation, no growth had occurred when samples of salted broth were taken at 0 and 7 days (30°C incubation), for colony counts had not appreciably increased (Table 2). An experiment was undertaken to prove that some growth, followed by a decline to the reported values, was not the case. It was believed that formation of metabolic products by staphylococci during cultivation in salt mixture broths might lead to a change in a_w. Therefore, water activity of salt mixture broths inoculated with *S. aureus* 100 or M 7/1 were determined after 7 days of incubation at 30°C. Results demonstrated that regarding a_w levels of 0.890 and 0.870 measured before and after incubation, water activity had decreased slightly, and perhaps this was due to small amounts of lactic acid (as sodium lactate) produced from the 0.1% glucose present in standard nutrient broth. Leistner and Rödel (unpublished data) have found that 1% sodium lactate in pure water can depress a_w by 0.0041, whereas 1% glucose depresses a_w by 0.0024 (8). Below a_w 0.867 very little change in water activity was observed between samples measured before and after incubation. A slight variation in this a_w range may be attributable to experimental error. Growth of staphylococci at 30°C had ceased below a_w 0.864 (Table 2).

In an attempt to attain optimal growth and enterotoxin A production, it was decided that 1% yeast extract be added to the salt mixture broths. A lower minimal a_w for growth has already been noted when vitamins are contained in the medium (19). The addition of 2% brewer's yeast to whole milk elevated enterotoxin A and D production by *S. aureus* Z 88 almost 100-fold (20). Hill, as cited by Tatini (19), observed that the limiting a_w for growth in nutrient broth was decreased when vitamins (yeast extract) were added to the system, thus suggesting the interplay of nutrients and a_w on growth and enterotoxin A production by *S. aureus*. Lotter (unpublished data) found that the addition of 1% yeast extract (Difco) to the growth medium permitted *S. aureus* 196 E to increase enterotoxin A production by about fourfold.

The effect of a_w on the degree of enterotoxin A production may be influenced by the type of humectant. For example, twice as much enterotoxin B may be formed in media with mixed solutes consisting of NaCl, KCl, and Na_2SO_4 in a 5:3:3 molar ratio than with NaCl as the sole solute (24). Thus, in the present study the use of mixed solutes instead of NaCl to adjust a_w seems justified to achieve optimal enterotoxin A production. Furthermore, mixed solutes would reduce the inhibitory effect of NaCl on growth of enterotoxin A-forming staphylococci (11, 21).

The findings of the present investigation contain implications for IMF. Troller and Stinson (25) used food slurries with glycerol as humectant and reported that strain 196 E formed enterotoxin A down to a_w 0.95 and the C 243 strain produced toxin B at a reduced a_w of 0.93. Their results conflicted with those reported by Troller in laboratory media (22, 23). The difference between both sets of findings may be due to the fact that glycerol was the humectant employed

TABLE 3. *Effect of a_w on growth and enterotoxin A production by S. aureus in salt mixture broths at 25°C*

a_w[a]	Strain 100				Strain M 7/1			
	Colony count/ml			Enterotoxin (μg/ml)	Colony count/ml			Enterotoxin (μg/ml)
	Initial	7 days	14 days	14 days	Initial	7 days	14 days	14 days
0.980[b]	3.7×10^5	2.4×10^9	5.6×10^8	6.0	6.5×10^5	3.3×10^8	1.8×10^8	4.0
0.887	3.7×10^5	7.6×10^6	2.7×10^8	4.0	6.5×10^5	9.2×10^7	1.2×10^8	2.0
0.870	3.7×10^5	4.8×10^5	2.0×10^5	—[c]	6.5×10^5	5.0×10^5	1.5×10^4	—
0.868	3.7×10^5	6.3×10^5	9.4×10^4	—	6.5×10^5	3.3×10^5	1.0×10^4	—
0.867	3.7×10^5	1.1×10^5	2.0×10^5	—	6.5×10^5	5.6×10^5	1.2×10^4	—
0.860	3.7×10^5	3.4×10^5	3.9×10^4	—	6.5×10^5	5.6×10^5	1.5×10^4	—
0.855	3.7×10^5	3.0×10^5	5.3×10^4	—	6.5×10^5	3.7×10^5	1.0×10^4	—

[a] Uninoculated samples.
[b] Standard I Nutrient Broth (Merck) supplemented with yeast extract, without added salts.
[c] —, Undetectable enterotoxin A in samples.

in the food studies, in contrast to laboratory media where various salts served as humectants. It has already been noted that at a_w levels between 0.96 and 0.90, glycerol can inhibit the growth rate of *S. aureus* by 10% more than can NaCl (12). Staphylococcal enterotoxins A, B, and C may also be produced by nonreplicating cells which may serve as a source of contamination in IMF (7, 14).

The results of the present study have clearly correlated the minimal a_w for growth with that for enterotoxin A synthesis by two different strains of *S. aureus*. Under optimal conditions, i.e., in a medium rich in nutrients and vitamins, with a water activity adjusted by a salt mixture and incubated at 30°C, growth and enterotoxin A formation was inhibited at an a_w between 0.864 and 0.867. Therefore, IMF should contain an a_w not higher than 0.86. All staphylococcal growth in IMF must be inhibited, as growth implies the risk of enterotoxin production. To suppress growth of *S. aureus* in IMF, legally permissible food additives should be preferably employed. The inhibition of growth of *S. aureus* by selected food additives will be described in a separate communication.

However, it should be kept in mind that inhibition of staphylococcal growth in IMF should not solely depend on food additives, but could be supported by other factors. As was observed in this study, lowering the storage temperature from 30 to 25°C already increased the minimal a_w for enterotoxin A production from 0.867 to 0.887. Furthermore, the employment of starter cultures in IMF which lower the pH of the products might improve the margin of safety.

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