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CONTROLLING THE GROWTH OF *STAPHYLOCOCCUS AUREUS* AND PRODUCTION OF ENTEROTOXIN-B

Nadia M. Awny, Mohey Z. El-Fouly*, Yehia A. El-Zawahry and Halla A. Hussin

Botany Department, Faculty of Science, Zagazig University, Egypt.
**National Center for Radiation Research and Technology, Cairo.*

ABSTRACT

Fifty nine samples of milk and dairy products, and forty samples from meat products were studied for their content of *S. aureus* . 32 Strains of *S. aureus* were isolated and identified, 8 Strains were found to be enterotoxin-B producers. The highly toxic one was used for further investigation. The effect of thermal treatment at 55, 60, 65 and 70°C for different periods on the viability of *S. aureus* and productivity of enterotoxin-B was studied. The sublethal thermal treatment stimulated the organism to produce toxin to 800% compared with non treated cells. Low dose (0.5 KGy) of γ-radiation increased the strain's productivity for enterotoxin-B. The strains lost the ability to produce toxin at 1.5 and 1.25 KGy in BHI and minced meat media, respectively. Also, 2% of clove was enough to inactivate the strain, while this concentration of garlic and mint inactivated the toxin production by 87.5% and was insufficient to kill the organism. Water activity (a_w) had a great effect on the viability of *S. aureus* . Decreasing a_w also inhibited the toxin production.

INTRODUCTION

Enterotoxin production was reported as an exclusive property of coagulase positive strains of *Staphylococcus areus*[1]. Also, enterotoxins were reported as metabolic products of *S. aureus* formed at the cell surface and secreted into the medium[2].

Enterotoxins are relatively heat resistant[3-5]. The thermal stability of enterotoxins was further reported and found to be complicated by variations in the initial concentration of each type in foods, and the medium in which the toxin is heated and the temperature of heating. Some observations suggested great thermal inactivation at low temperatures than high temperatures.

The effect of various individual spices (clove, pepper, ginger, garlic powder, fresh garlic extracts, nutmeg) on the enterotoxin production of *S. aureus* strains at 35°C were examined and reported that the spices inhibited the growth and production of enterotoxin-A[6,7].

Inactivation of enterotoxin-B by irradiation was studied[8], and showed that a dose of 5 or 20 M. rad was required to reduce an enterotoxin-B concentration of 31 μg/ml to less than 0.7 μg/ml and 30 μg/ml to less than 0.5 μg/ml respectively.

Furthermore the effect of low dose of irradiation (2KGy) on the growth and toxin production by *S. aureus* . was investigated[9].

The growth and metabolism of microorganisms depend on the presence of viability water. The most useful measurement of the viability of water is water activity (a_w)[10] . The total cell number and rate of growth of *S. aureus* C-243 were found to diminish at low levels and enterotoxin synthesis was extremely sensitive to reduction in a_w[11]. Enterotoxin production has not been reported in food below a_w 0.93[12].

The present work was undertaken to determine the total bacterial content of *S. aureus* in different Egyptian food, and screening assay for enterotoxin-B production by the isolated strains of *S. aureus* . Also, the Effect of thermal treatments, irradiation, water activity and different spices on both viable count and production of enterotoxin-B, were studied.

EXPERIMENTAL

Samples:

Fifty nine samples were collected from milk and dairy products; 40 samples from meat and meat products. These samples were taken from different food markets in Cairo, Egypt.

The samples were subjected to isolation of coagulase positive *S. aureus* which was identified according to Cwan and Steel[13].

Reference Enterotoxin and Anti-enterotoxins:

Lyophilized references were used after rehydration and dilution, according to the instructions delivered with those materials (Sigma).

Irradiation Source:

The source of irradiation was cobalt-60 gamma cell 220 located at National Center for Radiation Research and Technology (Nasr city, Cairo). This source gave dose of 5.2 Gy/min at the time to of experiments.

Spices:

Three kinds of spices were used; clove, garlic bulb and fresh mint leaves. They were sterilized by 0.1% mercuric chloride and washed twice with sterile distilled water to be ready for use.

Separation of enterotoxin-B by modification of Sac culture method[14] :

Dialysis tubing sacs (spectrapore 48°A, 3.5 cm wide, 30 cm length) were washed with distilled water, knotted at one end and inflated to make a sac. The knotted end was placed in 250 ml conical flask and 50 ml double strength (we used single strength) of BHI broth was placed in the sac and the open end was knotted. The sac was positioned in the flask in a U shape and autoclaved at 121°C for 15 min. Fourteen ml of phosphate-buffer pH 7.0, was added to the flask

outside the sac. The buffer was inoculated by 1 ml of *S.aureus* suspension. After 24 hr. of incubation at 37°C in shaking incubator at 6 rpm, the growth surrounding the sac was removed from the flask and the culture fluid was separated from the cells by centrifugation in high speed centrifuge at 15000 rpm **(Beckman Model J. 218)** for 30 minutes. The supernatent fluid (crude enterotoxin) was preserved with 1:10000 merthiolate and stored at 4°C.

Extraction of enterotoxin from food:

The method recommended by Bennett[15] was used, for enterotoxin extraction.

Microslide method for enterotoxin detection:

The microslide double diffusion test for the detection and assay of staphylococcal enterotoxins developed by Casman[16] as described by Bennett[17]. The test was carried out as follows.

a. Rehydration of the Lyophilized reagents:

Lyophilized preparation of reference enterotoxin-B was dissolved in saline containing 10% BHI broth (Oxide) in portions of 3 ml per vial reference enterotoxin (working solution) and 0.6 ml saline solution was added to reference antienterotoxin-B (stock solution) as recommended in the instruction supplied with these materials. Working solutions (reference enterotoxin) and antienterotoxin (stock solution) were prepared by diluting 0.1 ml of each with 0.9 ml saline.

b. Microslide preparation:

Clean microscopic slides were prepared with plastic electrical insulating tape twice around each end of the slide, leaving 2 cm space in the center. This area was coated with 2 layers of melted cooled noble agar (55-66°C). First layer 0.2 ml of 0.2% agar, second layer 0.4 ml of the following 1.2 g noble agar, 0.90% sodium chloride, 0.80% sodium barbitol, 1:10.000 merthiolate plastic template coated by silicon grease was placed over the second agar layer carefully.

c. Addition of reagents and test material to the slide:

After incubation for 48 hr at 37°C, the template was removed and the slide was immersed into water for 10 minutes in each of the following baths:

a) 0.1% Thiazin Red-R in 1% acetic acid.

b) 1% acetic acid containing 1% glycerol.

The results were performed by precipitine lines observed between the central well and the outer well and exposing the slide to an indirect light. The positive test fluids exhibit a precipitine lines joined to the lines formed by the reference enterotoxins.

Enterotoxin Estimation:

The determination of the amount of enterotoxin quantitatively was carried out according to the effect of amount of enterotoxin test preparation on the development of the reference line of precipitation.

The highest dilution and the most distinct line of the preparation was detected . The toxin concentration from the microslide was multiplied by the reciprocal number of the dilution. The result was the concentration of enterotoxin per ml of the original test preparation. The amount of the toxin was given by multiplying the toxin concentration/ml of the original solution by the volume of solution[18].

RESULTS

Occurrance of *S. aureus* in some Egyptian foods:

The presence of *S. aureus* in both dairy and meat products was arranged according to the log viable count as follow: white cheese (3.97), Karish cheese (3.72), raw milk (3.5), Yoghurt (2.05) and ice-cream (1.4). With regard to meat products, sausage comes in the first position (4.4), followed by luncheon (2.3), hot dog (0.7) and bastirma (0.5).

- Enterotoxigenicity of *S. aureus* isolates:

The ability of staphylococcal isolates to produce enterotoxin-B was summerized in table (1). From thirty two *S. aureus* isolated from the previous examined food stuff, only 8 isolates could produce entertoxoin-B. They were isolated from white cheese (2 strains), Yoghurt three, Karish cheese (one) and also only one isolated from bastirma and sausage.

Table (1) revealed that strain No. 2 which was isolated from white cheese had the highest productivity of enterotoxin-B; so it was chosen for further studies in the following experiments, while strain No. 8 showed the lowest productivity.

Factors affecting the viability of *S. aureus* And production of its Enterotoxin-b:

Effect of thermal treatments:

The results of the effect of different temperatures (55,60,65,70°C) at different periods of time (2.5 up to 30 min.) on the viability of the isolated strain of *S. aureus* . were presented graphically in Fig. 1. It has been found to be ideal logarithmic curve. The death of vegetative cells was decreased by increasing temperature and exposure time for each suspending media (nutrient broth or minced meat). The results revealed a complete inhibition after exposure period 7.5 minutes at 70°C for both suspending media. These curves were useful to determine the D_t values (exposure time in minutes required to reduce the surviving fraction by one log cycle) (Table 2).

Effect of thermal treatments on toxin productivity :

Table 3 shows the effect of different thermal treatments at the sublethal time on the productivity rate of *S. aureus* to 800% in all examined treatments.

Effect of certain spices on viable count:

The results represented in Fig. 2 indicated that increasing concentration of clove; garlic or mint inhibited the growth of the tested strain.

The data revealed that the cells were more sensitive to clove than garlic and mint in the same concentrations.

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Thus 2.0% of clove was sufficient to inhibit the bacterial growth completely. The same concentration of garlic and mint induced 6.4 and 6.3 log cycle reduction for the strain, respectively.

The examined stains showed simple exponential relationship (Fig.3), these curves were useful to determine the D_e values (the spice concentration required to kill 90% of the population) (Table 2).

Effect of certain spices on toxin productivity :

Table 4 showed a remarkable decrease in the productivity rate of enterotoxin-B by isolated strain with increase of spices concentrations. Clove had the strongest effect on the productivity rate followed by garlic and mint. Clove at concentration 1% reduced 75% of toxin productivity while 2% completely inactivated the organism. With regard to garlic, 1.0% and 2% reduced toxin productivity 74% and 87.5%, respectively. Meanwhile mint at concentration 1.0% and 2% recorded 50% and 87.5% reduction of toxin productivity, respectively.

Response of *S. Aureus* to the increasing doses of gamma radiation :

Effect on viable count :

The effect of increasing doses of gamma radiation (0.0 up to 3.0 KGY) on the viability of the tested strain was detected. Sterile minced meat and nutrient broth were used as suspending media Fig. (3). Data in the curves showed simple exponential relation and used for calculating the D_{10} values (dose required to reduce number of microorganism by one log cycle) (Table 2). It was clear that radiosterile meat as a suspending medium was more protective for *S. aureus* cells than nutrient broth.

Effect of radiation on toxin productivity:

The productivity of unirradiated and irradiated *S. aureus* strain to enterotoxin-B was examined in both artificial medium and minced meat.

The data in table 5 show that the productivity of enterotoxin-B in artificial medium (0.375 µg) was higher than in minced meat (0.25 µg) i.e. the value decreased by 12.5%.

From the table, it is clear that exposing *S. aureus* to low doses of gamma radiation (0.25 and 0.5 KGy) resulted in a sharp increase in the productivity of enterotoxin-B which reached 400% and 600% in both suspending media, respectively.

Thereafter, the rate of the productivity started to decrease and the amounts of enterotoxin were almost as the control in artificial medium at dose level 1.0 KGy. At the dose levels 1.25 KGy in minced meat and 1.5 KGy in BHI the organism lost its ability to produce enterotoxin-B, although it was still alive until 2.0 KGY and 2.5 KGy for the same suspending media, respectively.

Effect of water activity (a_w) on *S. aureus*:

Effect on viable count:

Sodium chloride and potassium chloride were used to adjust the a_w in BHI media. The concentrations of solutes employed the different levels of a_w according to **Troller**[19].

The effect of various levels of a_w on the growth rate of *S. aureus* was represented in Fig. 4. It was clear that a slight decrease in the viable counts occurred by decreasing the a_w from 0.97 to 0.91. It was also clear that KCl as solute was relatively more effective than NaCl at low levels of a_w.

- Effect on toxin productivity:

Although a_w at range of 0.99 to 90 had a slight effect on the viable count of *S. aureus* as mentioned above. It had a remarkable effect on its productivity of enterotoxin-B as clearly shown in (table 6).

The production of toxin was 4.0 µg when a_w was 0.99. By decreasing a_w to 0.97, the toxin production decreased to 2.5 ug (i.e. 73.5% reduction) either by adjusting the a_w by NaCl or KCl. The productivity of enterotoxin was completely inhibited at a_w 0.90 in case of NaCl and 0.91 in case of Kcl.

DISCUSSION

The enterotoxigenicity of *S. aureus* is considered one of the main problems of the food poisoning all over the world, especially in hot countries such as Egypt. The organism grows well in the presence or absence of oxygen and in the presence of salt[20]. In the present study, 32 strains of *S. aureus* were isolated from dairy and meat products. Eight strains of those isolates were enterotoxin-B producer in this connection. Previously, **Sharaf**[21] represented the enterotoxigenicity (A and B types) of *S. aureus* strains isolated from diary products by 16.1% isolated from yoghurt, 9.8% isolates from ice-cream and 15% isolates from soft cheese. On the other hand **Reabi**[22] isolated 7 strains from 30 sausage samples; only one produced enterotoxin-B, while **Yessa**[23] found that 87% of 140 strains of *S. aureus* isolated from bastirma were non enterotoxigenic and only 2% of the toxigenic strains produced enterotoxin-B.

Thermal treatment was used as traditional method for food preservation and elemination of pathogenic microorganisms from several types of food. In the present work, intensive reduction was observed in *S.aureus* counts (Fig. 1) when suspended in nutrient broth compared with cells suspended in radiosterile ground beef after application of thermal treatments. these results were in agreement with previous results of **N.Awny**[24] and **Abd El-ALI**[25].

Table (1) : Productivity of enterotoxin-B of *S. aureus* strains isolated from various food and grown on aritifical medium (B.H.I.).

Strain No.	Source of food Stuff.	Enterotoxin-B (ug/ml)
1	White cheese	1.75
2	White cheese	2.25
3	Youghurt	1.00
4	Youghurt	1.50
5	Youghurt	0.75
6	Karish cheese	2.00
7	Bastirma	1.00
8	Sausage	0.5

Table (2) : Calculated D_X values of *S. aureus* isolated from the Egyptian food products with thermal, spices and radiation treatment.

Temperature	Spices	Value of D_X	Suspending medium	
			Broth	Meat
55°C		D_t	5.037	7.542
60°C			2.917	5.459
65°C			1.640	2.093
70°C			0.805	0.777
	Clove	D_C	0.225	*
	Garlic		0.306	*
	Mint		0.316	*
		D_{10} Gy	439	512

* Not examined.

Table (3) : Effect of thermal treatment on the productivity rate of *S. aureus* strain for enterotoxin-B.

Temperature	Time of exposure in min	Toxin conc. (µg /ml)	Productivity %
0	0	0.375	100
55°C	30	3.00	800
65°C	10	3.00	800
70°C	2.5	3.00	800

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Table (4) : Effect of different concentrations of clove, garlic, and mint on the production of enterotoxin-B by *S. aureus* .

% of Spice conc.	Clove		Garlic		Mint	
	Toxin conc. (µg)	P.R. %	Toxin conc. (µg)	P.R. %	Toxin conc. (µg)	P.R. %
0.0	2.00	100	2.00	100	2.00	100
0.5	0.75	37.5	1.00	5.0	1.50	75.0
1.0	0.50	25.0	0.52	26.0	1.00	50.0
1.5	0.25	12.5	0.25	12.5	0.50	25.0
2.0	0.00	0.00	0.25	12.5	0.25	12.5

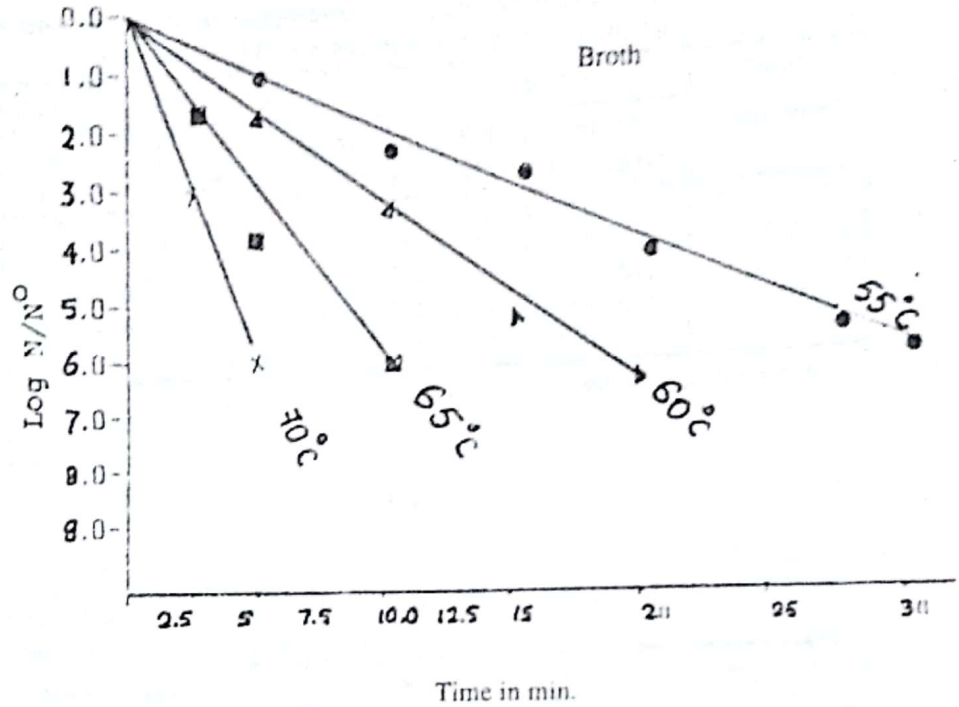
* P.R = Productivity rate.

Table (5) : Effect of gamma radiation on the productivity of *S. aureus* for Enterotoxin-B in artificial medium (BHI) and minced meat.

Dose KGY	Artificial medium		Minced meat	
	Toxin conc. (µg /ml)	Productivity %	Toxin conc. (µg /ml)	Productivity %
0.11	0.375	100	0.250	100
0.25	1.500	400	1.000	400
0.50	2.250	600	1.500	600
0.75	1.400	373.3	0.750	300
1.00	0.375	100	0.250	100
1.25	0.125	33.3	0.000	0.00
1.50	0.000	0.00	0.000	0.00

Table (6) : Effect of water activity on the production of entorotoxin-B by *S. aureus* in aritifical medium (BHI).

NaCl				KCl			
a_w	Wieght % (g)	Enterotoxin conc. (µg)	Productivity	a_w	Wieght % (g)	Enterotoxin conc. (µg)	Productivity
0.99	0.0	4.0	100	0.99	0.0	4.0	100
0.97	3.0	2.5	62.5	0.98	4.0	3.5	87.5
0.94	5.0	1.0	25	0.97	8.0	2.5	62.5
0.92	7.5	0.25	6.2	0.95	15.0	1.0	25
0.90	10.0	0.0	0.0	0.91	20	0.0	0.0



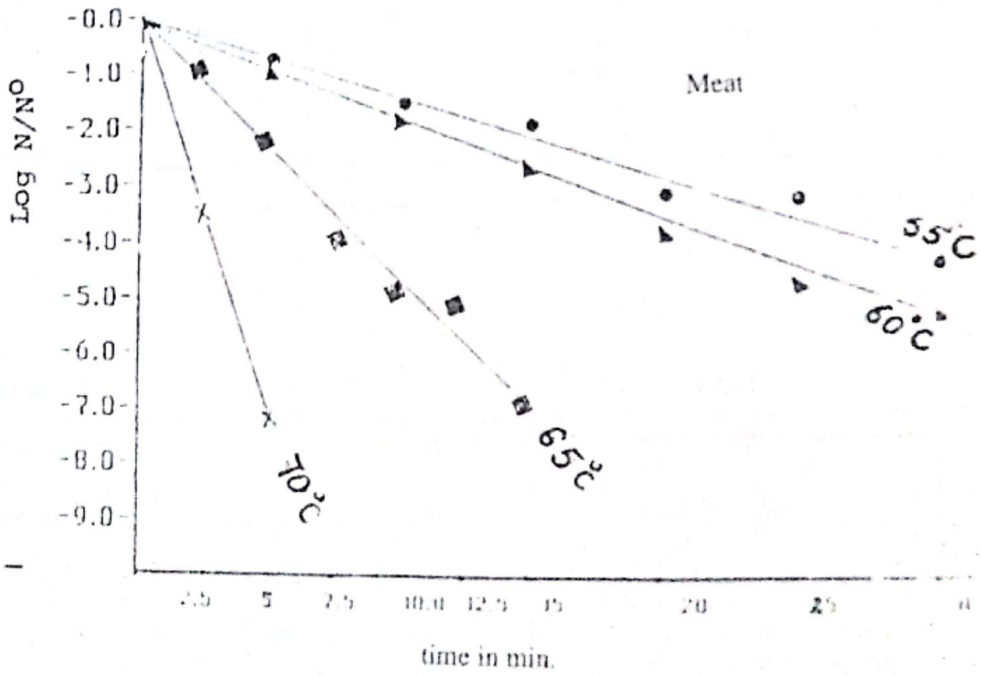


Figure (1) Effect of different suspending media (broth or meat) and different thermal treatments for different exposure time on the viable count of *S. aureus*.

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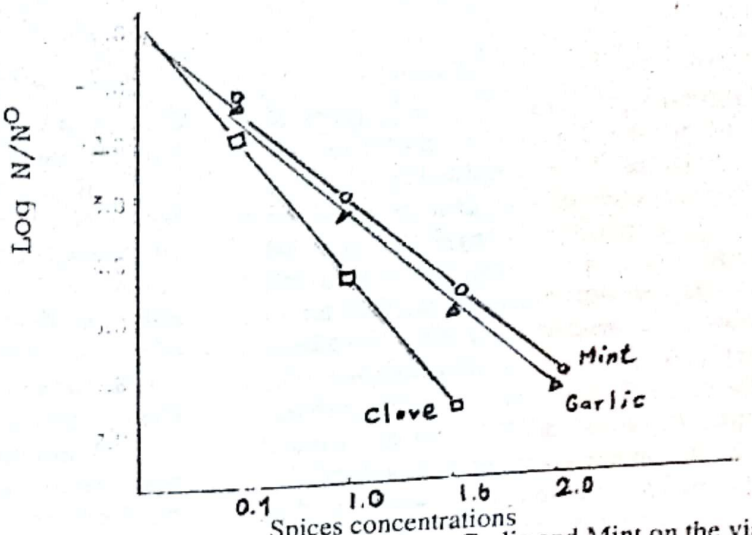

Figure (2) Effect of different conentrations of clove, Garlic and Mint on the viable count of *S.aureus*.

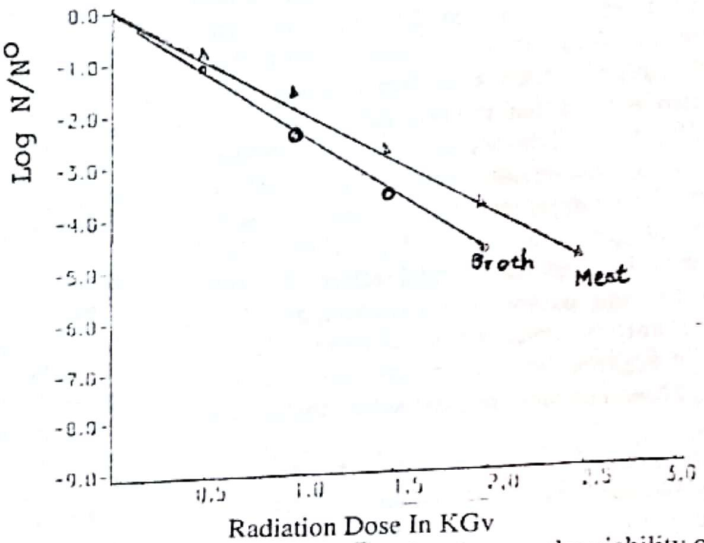

Figure (3) Effect of increasing radiation dose on the viability of *S.aureus* suspended in nutrient broth and minced meat.

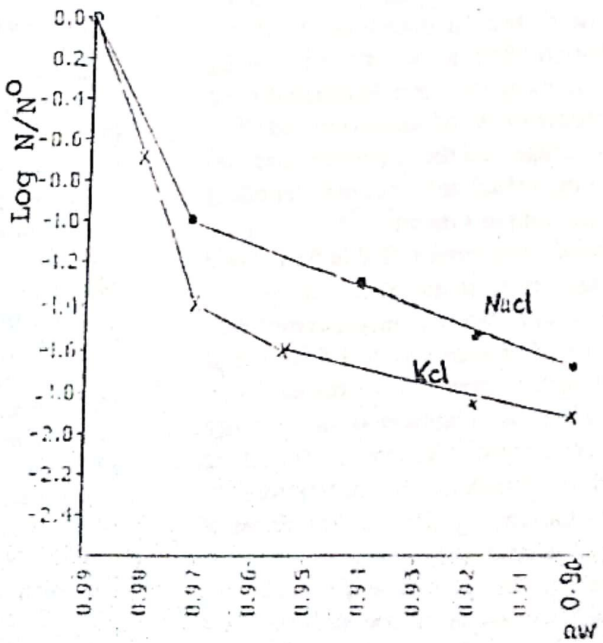

Figure (4) Effect of water activity on the growth of *S. aureus*.

An explanation to this logarithmic order of reduction can be attributed to the loss of reproductive power of a bacterial cell when subjected to moist heat was due to denaturization of one gene essential for reproduction[26]. It is possible however that a lethal agent, as moist heat, can damage other vital cell functions such as an enzyme activity. This might render cell production even though the reproductive genes has not undergone irrepairable damage. The sensitivity to thermal treatment in the prensent investigation was expressed as D_t values of the tested isolates (table 2), this in agreement with **Angelotti**[27].

On the other hand thermal treatment induced an activation for enterotoxin-B production by *S. aureus*. The rate of productivity increased by 800% when the organism thermally treated at 55°C for 30 min. or at 65°C for 10 min. or at 70°C for 2.5 min. in liquid medium (Table 3). This increase could be due to the occurrence of mutants which may have the ability to produce enterotoxin more than the original strain.

Generally, the inhibitory effect of spices differs with the kind of spices as well as the kind of microorganism. The results revealed that the cells were more sensitive to garlic and mint (Fig. 2). **Phillip *et al.*,**[28] reported that the bactericidal effect of some spices is due to their essential oils content, and they added that the antimicrobial action of clove is due to the presence of eugenol, while onion and garlic inhibit the bacterial growth because of their sulfur containing compound.

The results (Table 4) revealed that the enterotoxin-B productivity was decreased by increasing the spices concentration. These results were in the agreement with that of **Ebeid** and **Rizk**[29], **Nes *et al.*,**[7] and **Kumar and Gupta**[6].

It was reported in this investigation that the growth of *S. aureus* cells was greatly affected by exposing to gamma rays and the dose level 2.5 & 3.0 KGy completely destroyed the *S. aureus* cells suspended in broth medium or radiosterile minced meat respectively. A logarithmic order of death occured (Fig. 3). Several investigators reported that complex medium relatively protected the microbial cells against the effect of gamma radiation than simple system[25,30].

On the otherhand, the results (Table 5) showed that low doses of γ-radiation stimulated *S. aureus* to produce enterotoxin-B., and the organism completely lost its toxin productivity after expsoure to 1.5 KGy and 1.25 KGy in (B.H.I) and minced meat, respectively. Since gamma radiation is considered as energy elaboration and toxin excretion is an energy, requiring process as mentioned by **Markus** and **Silverman**[31], this could explain the enhancing effect of low doses of radiation for enterotoxin production.

Water requirement for growth and metabolism of the organism depends on the a_w of the medium. This effect was clear in (Fig. 4). Decreasing a_w from 0.99 to 0.97 decreased the viability by1.0 and 1.4 log cycle in case of adjustement with NaCl and KCl respectively. Meanwhile the productivity of enterotoxin decreased by 37.5% and 12.5% by using NaCl or KCl respectively (Table 6). The results revelaed that a_w had a slight effect on the viable count of *S. aureus* and remarkable effect on its productivity on enterotoxin-B. These results were in agreement with that of **Mclean *et al.*,**[32] **Genigeorgis** and **Salder**[33].

From the previous results it is recomended that the *S. aureus* cells must be completely eleminated from the foods treated by temperature or radiation since the sublethal doses of these 2 factors increase the productivity of enterotoxin-B, hence it may induce food poisning to the customers.

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التحكم فى نمو الاستافيلوكوكس اوريوس وانتاج التوكسين المعوى (ب) بواسطة عوامل مختلفة

نادية عونى - محيى الفولى* - يحى الظواهرى - هاله احمد حسين

قسم النبات - كلية العلوم - جامعة الزقازيق- مصر

*المركز القومى لأبحاث وتكنولوجيا الاشعاع- القاهره

استخدم فى البحث ٥٩ عينه من منتجات الالبان (الجبن الابيض ، الجبن الرومى ، الزبادى ، المثلجات اللبنية ، اللبن الحليب ، الجبن القريش) ، و٤٠ عينه من منتجات اللحوم (اللانشون بسطرمه ، السجق الطرى الرفيع ، السجق الناشف الغليظ)

ثم عزل ٣٢ سلاله للميكروب ودرست قدرتها على افراز الانتيروتوكسين ووجد أن ٨ سلالات فقط لها القدرة على افراز التوكسين وكانت اكثر السلالات افرازا له هى سلاله معزوله من الجبن الابيض ، وقد استخدمت هذه السلاله فى اجراء جميع التجارب الخاصة بالبحث .

بدراسة تأثير درجات الحراة ٥٥ ، ٦٠ ، ٦٥ ، ٧٠°م وجد انه يمكن القضاء على الميكروب عند درجة ٦٠°م لمدة ١٢,٥ دقيقة وعند ٧٠°م لمدة ٧,٥ دقيقة كما لوحظ ان افراز الميكروب للتوكسين المعوى (ب) تزيد بدرجة كبيرة وصلت الى نسبة ٨٠٠٪ عن الميكروب الغير معامل حراريا وذلك عند الدرجة القبل قاتله .

تمت دراسة تأثير القرنفل والثوم والنعناع على نمو وافراز التوكسين للكائن وجد ان تركيز ٢٪ من القرنفل كان كافيا لقتل الكائن ، اما عند استخدام الثوم والنعناع كان هذا التركيز مثبطا لافراز السم بنسبة ٨٧,٥٪ الا انه لم يكن كافيا للقضاء عليه.

تمت دراسة حساسية الكائن للاشعاع فكانت الجرعة القاتله فى العينة السائلة ٢,٥ كيلوجراى وفى اللحم المفروم ٣ كيلوجراى . كما تم تحديد د١٠ فى الوسطين (الجرعة الاشعاعية التى تقتل ٩٠٪ من الخلايا) .

وجد ان للاشعاع تأثير منشط لافراز الميكروب للتوكسين المعوى عند الجرعات المنخفضة (٥ كيلوجراى) ثم بدأ معدل الافراز فى الانخفاض حتى يفقد قدراته عن الجرعه ١,٢٥ كيلوجراى وعند ١,٥٠ كيلوجراى فى بيئة اللحم المفروم والبيئة السائلة على التوالى .

كان للنشاط المائى (aw) تأثيرا ملحوظا على تثبيط افراز السم وفقدت السلاله المعزوله قدرتها على الافراز تماما عند النشاط المائى ٠,٩١ حيث ان معدل النمو انخفض بمقدار ١,٥ دورة لوغاريتمية.