EFFECT OF SODIUM CHLORIDE ON

*Staphylococcus aureus* GROWTH

By:

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Dedication

To my family

To my supervisor

To my friends

With my deep love
Acknowledgements

First I thank Allah, who gave me health and ability to complete this work.

Great thanks go to my supervisor for his guidance, advice and help.

Special thanks are due to the technicians in the Department of Botany and Agricultural Biotechnology, Faculty of Agriculture, University of Khartoum, especially A. Aziz, Mona, Altage and A. Alwahed.
# List of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedication</td>
<td>i</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>ii</td>
</tr>
<tr>
<td>List of contents</td>
<td>iii</td>
</tr>
<tr>
<td>List of tables</td>
<td>vi</td>
</tr>
<tr>
<td>List of figures</td>
<td>vii</td>
</tr>
<tr>
<td>List of plates</td>
<td>viii</td>
</tr>
<tr>
<td>Abstract Arabic</td>
<td>ix</td>
</tr>
<tr>
<td>abstract</td>
<td>x</td>
</tr>
</tbody>
</table>

## INTRODUCTION

## CHAPTER TWO: REVIEW OF THE LITERATURE

- History of staphylococci food poisoning                                | 2    |
- *Staphylococcus aureus* description                                    | 2    |
- Morphology                                                            | 2    |
- Colonies                                                              | 2    |
- Colony pigmentation                                                   | 3    |
- Coagulase                                                             | 3    |
- Habitat and distribution                                              | 3    |
- Incidence in foods                                                    | 4    |
- Nutritional requirement for growth                                     | 5    |
- Temperature growth range                                              | 5    |
- Effect of pH                                                           | 6    |
- Effect of water activity (a<sub>w</sub>)                               | 6    |
- Effect of pH, a<sub>w</sub> and temperature                           | 6    |
- Effect of NaNO<sub>2</sub>, pH and temperature of growth              | 6    |
- Toxins                                                                | 7    |
- Exotoxins                                                             | 7    |
- Enterotoxins                                                          | 7    |
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,1</td>
<td>Toxic shock syndrome toxin (TSST)</td>
<td>8</td>
</tr>
<tr>
<td>3,3</td>
<td>Resistance</td>
<td>10</td>
</tr>
<tr>
<td>3,4</td>
<td>Ecology of <em>S. aureus</em> growth</td>
<td>10</td>
</tr>
<tr>
<td>3,5</td>
<td>Clinical infections</td>
<td>10</td>
</tr>
<tr>
<td>3,6</td>
<td>The Gastroenteritis syndrome</td>
<td>11</td>
</tr>
<tr>
<td>3,7</td>
<td>Source of infection</td>
<td>12</td>
</tr>
<tr>
<td>3,8</td>
<td>Mechanisms of transmission in cross infection</td>
<td>13</td>
</tr>
<tr>
<td>3,9</td>
<td>Prevention of staphylococcal and other food poisoning syndromes</td>
<td>14</td>
</tr>
<tr>
<td>3,10</td>
<td>The factors leading to food poisoning</td>
<td>14</td>
</tr>
<tr>
<td>3,11</td>
<td>Sodium chloride concentrations</td>
<td>14</td>
</tr>
<tr>
<td>3,12</td>
<td>Effect of salt on foods</td>
<td>14</td>
</tr>
<tr>
<td>3,13</td>
<td>Effect of NaCl on growth of <em>S. aureus</em> and enterotoxin production</td>
<td>15</td>
</tr>
</tbody>
</table>

CHAPTER THREE: MATERIALS AND METHODS

3,1  Sterilization  
3,1,1  Hot air oven (160°C for one hour)  
3,1,2  Autoclaving at 121°C (15 lb/ in²)  
3,1,3  Disinfection  
3,2  Collection of samples  
3,3  Identification of isolated bacteria  
3,4  Preparation of media  
3,4,1  Liquid media  
3,4,1,1  Nutrient broth agar  
3,4,2  Solid media  
3,4,2,1  Nutrient agar  
3,4,2,2  Urea agar  
3,4,2,3  Blood agar  
3,5  Semi solid media  
3,5,1  Hugh and Leifson's (O/F) medium
CHAPTER FOUR: RESULTS AND DISCUSSION

The effect of NaCl on S. aureus growth

Growth on solid media

Growth at 0% NaCl

Growth at 0.5% NaCl

Growth at 1% NaCl

Growth at 1.5% NaCl

Growth at 2% NaCl

Growth in liquid media

Changes in pH values of growth medium of S. aureus

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

Conclusions

Recommendations
LIST OF TABLES

Table 1: Virulence factors, including toxins of *S. aureus* and their pathogenic effects (Quinn, 2002)

Table 2: The biochemical properties of *S. aureus* isolated from cheese and milk

Table 3: The relationship between growth (OD) of *S. aureus* isolated from milk and NaCl concentration.

Table 4: The relationship between growth (OD) of *S. aureus* isolated from white cheese and NaCl concentration.

Table 5: The relationship between concentration of NaCl and pH of medium in which grew *S. aureus* isolated from white cheese.

Table 6: The relationship between concentration of NaCl and pH of medium in which grew *S. aureus* isolate from milk.
LIST OF FIGURES

Fig. 1: Level of growth of *S. aureus* isolated from milk as affected by different concentration of NaCl

Fig. 2: Level of growth of *S. aureus* isolated from cheese as affected by different concentrations of NaCl

Figure 3: The relationship between concentration of NaCl and pH of medium in which grew *S. aureus* isolated from white cheese

Figure 4: The relationship between concentration of NaCl and pH of medium in which grew *S. aureus* isolated from milk
LIST OF PLATES

Plate ١: Growth of *S. aureus* isolated from milk at ٪٠ NaCl ٢٧
Plate ٢: Growth of *S. aureus* isolated from white cheese at ٪٠ NaCl ٢٧
Plate ٣: Growth of *S. aureus* isolated from milk at ٪٠ NaCl ٢٨
Plate ٤: Growth of *S. aureus* isolated from white cheese at ٪٠ NaCl ٢٨
Plate ٥: Growth of *S. aureus* isolated from milk at ٪٠ NaCl ٢٩
Plate ٦: Growth of *S. aureus* isolated from white cheese at ٪٠ NaCl ٢٩
Plate ٧: Growth of *S. aureus* isolated from milk at ٪٠ NaCl ٣٠
Plate ٨: Growth of *S. aureus* isolated from white cheese at ٪٠ NaCl ٣٠
Plate ٩: Growth of *S. aureus* isolated from milk at ٪٠ NaCl ٣١
Plate ١٠: Growth of *S. aureus* isolated from white cheese at ٪٠ NaCl ٣١
البحث

أجريت التجربة لمعرفة أثر تركيز ملح كلوريد الصوديوم على نمو بكتريا

Staphylococcus aureus

ومعرفة كيفية منعه لتلوث الغذاء وأثره في تثبيط نمو هذه البكتريا

الخطيرة الممرضة للإنسان والحيوان.

في هذه الدراسة تم عزل Staphylococcus aureus من عينتين، عينة جبنه وعينة لبن

وزنعت على وسط غذائي Nutrient agar و Nutrient broth تحتويان على تركيزات مختلفة % من الملح تشمل 5%, 10%, 15%, 20% من تركيز الملح وحضنت في 37 درجة مئوية وpH 7.00. أخذت النتائج بعد كل 12, 24, 48, 72 ساعة في شكل الكثافة الضوئية للنمو.

من هذه التجربة خلصنا إلى أن زيادة تركيز الملح يؤدي إلى انخفاض نمو بكتريا Staphylococcus aureus بالإضافة إلى أن هذه البكتريا يمكن أن تنمو في تركيز ملح بسيطة وكبيرة قد تصل إلى 20% من تركيز الملح لمدة تمتد إلى 48 ساعة الذي وصلت إليه بيئة النمو تراوح بين 6 و 7,7 في عينة pH تتحلل. مدى المعزولة من الجبن و 7, 9.9 في العينة المعزولة من اللبن. أما الكثافة الضوئية فتراوحت بين 0,0 و 0,0 في العينة المعزولة من الجبن و 0,0000، 0,0000 في العينة المعزولة من اللبن.
ABSTRACT

This study was done to see the effect of different concentrations of salt (NaCl) on the growth of *Staphylococcus aureus*, to find out how to prevent contamination of food by using this chemical, and to see its effect on the inhibition of *S. aureus* growth which is a dangerous pathogenic species that causes many diseases of humans and animals.

In this study *S. aureus* was isolated from cheese and milk and its growth at 73°C and pH 0.7 on nutrient agar and in nutrient broth which contained different concentrations of salt (5%, 10%, 15% and 20% NaCl) was studied. The results were taken after 21, 42, 63 and 84 hour, as optical density.

From these results it was concluded that increasing NaCl concentration led to decrease in *S. aureus* growth. Moreover, *S. aureus* grew not only at low concentrations of salt but was also able to grow at high concentrations of salt like 10-20% NaCl at 63 hours but after 84 hour *S. aureus* lysed.

The range of pH of the medium in which *S. aureus* grew in these experiments was 0.6-7.7 for isolate from cheese and pH 9.5-10.8 for the isolate from milk. The optical density ranged between 0.0 and 1.0 for the isolate from cheese and 0.0-0.8 isolate from milk.
CHAPTER ONE

INTRODUCTION

Staphylococci are Gram - positive cocci usually arranged in clusters, are facultatively anaerobic, catalase - positive and non - motile. The word Staphylococcus derives from the Greek words ‘staphyle’ and ‘kokkos’ for a bunch of grapes and berry, respectively (Quinn, et al., ٢٠٠٢).

The genus *Staphylococcus* belongs to family micrococcaceae; at least ٠٣ species of it occur as commensals on skin and mucus (Quinn, et al., ٢٠٠٢). This genus is very important because it includes the common and versatile pathogenic species *S. aureus* (Duguid, et al., ١٩٨١).

*Staphylococcus aureus* rapidly became resistant to most antibiotics and is a great source of danger in hospital infection (Barker and Breach, ١٩٨٠). The different agents that prevent growth and toxin production by *S. aureus* have been studied by many investigators especially the effect of NaCl and pH on the growth of *S. aureus* and food poisoning.

In this research we have tried to study the effect of NaCl on the growth of *Staphylococcus aureus*. 
CHAPTER TWO
LITERATURE REVIEW

١.١ History of staphylococcal food poisoning:

This syndrome was first studied in ٤١٩١ by Denys and later in ٤١٩١ by Barber who produced in himself the signs and symptoms of the disease by consuming milk which has been contaminated with a culture of S. aureus (Duguid, et al., ٣٧٩١).

١.٢ S. aureus description:

١.٢.١ Morphology:

These are Gram - positive cocci about ١٠м in diameter, mainly joined in grape - like clusters, but some cocci are single and some in pairs, non - sporing and non - motile. (Duguid, et al., ٣٧٩١).

١.٢.٢ Colonies:

Colonies are smooth, raised, glistening circular, entire and translucent. With increasing age, colonies become nearly transparent. Under conditions inhibitory to normal growth, rough or dwarf colony variants may be produced (Sneath, et al., ٣٧٩١).
Colony pigmentation:

Colonial pigment is variable; however, most strains demonstrate some degree of color or cell pigmentation ranging from gray or gray-white with yellow tint.

The production of pigment may be influenced by growth conditions; growth on agar slants is abundant, slightly sticky in consistency, and translucent to nearly opaque. In broth, growth changes from a uniform turbidity to a fine, easily suspended deposit (Sneath, et al., 1981).

Coagulase:

This is an enzyme-like product of S. aureus that converts the fibrinogen in citrated human or rabbit plasma into fibrin aided by an activator in plasma. Staphylocoagulase occurs in two forms, soluble coagulase and bound coagulase (Duguid, et al., 1975).

Habitat and distribution:

In man, the main reservoir of S. aureus is the nose; these organisms find their way to the skin and into wounds. The nasal carriage rate is about 5-7% for adults and somewhat higher among children. The most common skin sources are arms, hands and face, where the carriage rate is between 5-7% (Elek, 1959). In addition to skin and nasal cavities, S. aureus may be found in eyes, throat and in the intestinal tract. From these sources, the organism
finds its way into air and dust and onto clothing from which it may contaminate food. Most domesticated animals harbor *S. aureus* (Jay, 1994), but transfer of *S. aureus* strains between animal species and between animal and man is limited (Quinn, 2004).

Staphylococcal mastitis is not unknown among dairy herds and if milk from infected cows is consumed or used for cheese making it may cause poisoning (Jay, 1987).

**Incidence in foods:**

In general, staphylococci may be expected to exist in any or all food products that are of animal origin or that are handled directly by humans (Jay, 1994).

Donnelly *et al.* (1984) found 2% of 343 market cheddar cheese samples contained these organisms. It should be noted also that most domesticated animals harbor *S. aureus* (Morrison, *et al.*, 1974). If milk from such cows is consumed or used for cheese making, the chances of contracting food poisoning are high. The organism will not grow in mayonnaise because the pH of mayonnaise is never above 0.4 but when it is added to other materials such as potatoes, chicken or even ham, the pH of the mixture is much higher than 0.4. The mixture becomes suitable medium for growth of *Staphylococcus* (Martins, 1964).
Nutritional requirements for growth:

Staphylococci are typical of other Gram-positive bacteria in having a requirement for certain organic compounds in their nutrition, like amino acids as nitrogen sources and thiamine and nicotinic acid are required among the B vitamins. When grown anaerobically, they appear to require uracil. In one minimal media for aerobic growth and enterotoxin production, monosodium glutamate served as C, N and energy source. This medium contained only three amino acids (arginine, cystine and phenylalanine) and four vitamins (pantothenate, biotin, niacin and thiamine), in addition to inorganic salts (Jay, 2020).

Temperature growth range:

Angelotti, et al. (1991) reported that some strains of S. aureus can grow at temperatures as low as 80°C. In general, growth occurs over the range of 8°C to 35°C and enterotoxins are produced between 41°C to 64°C (Jay, 1997). These organisms have an optimum growth temperature around 37°C.

Abd Alsamed, (2002) found S. aureus was able to survive at 80°C for five minutes but did not survive at 8°C for the same period.
Effect of pH:

*S. aureus* can grow over the range of pH 4-8.9 but its optimum is in the range of pH, 6-7 (Jay, 1991). Abd Alsamed (2002) reported that *S. aureus* failed to grow at pH 5 and pH 9-11.

Effect of water activity (aw):

With respect to aw, staphylococci are unique in being able to grow at values lower than for any other non halophilic bacteria. Growth has been demonstrated at as low as 38.0 under otherwise ideal conditions, although 68.0 is the generally recognized minimum aw (Jay, 1991).

Effect of pH, aw and temperature:

No growth of mixture of *S. aureus* strains occurred in brain heart infusion (BHI) broth containing NaCl and sucrose as humectants either at pH 4.3, aw of 0.86, or 20°C. No growth occurred with a combination of pH <5.0, 12°C and aw of 0.9 or 0.93 and no growth occurred at pH <4.8 and 14°C (Jay, 2002).

Effect of NaNO2, pH and temperature of growth:

*S. aureus* strains grew and produced enterotoxin B in cured ham under anaerobic conditions with brine content up to 9%, but not below pH 5.7 and 9°C, or below 0.8 at 10°C. Under aerobic conditions, enterotoxin production occurred sooner than under anaerobic conditions. As the
concentration of HNO$_2$ increased, enterotoxin production decreased (Jay, 2002). NaNO$_2$ and NaNO$_3$ at concentrations allowable in cured meats have been reported to have no effect on production of this enterotoxin (Mclean, et al., 1968).

**Toxins:**

Toxin is formed by the organism in food before it is eaten and not after it had entered the body (Jay, 2002).

**Exotoxins:**

Exotoxins are secreted by growing S. aureus cells (Duguid, et al., 1973).

**Enterotoxin:**

This is an intestinal toxin, formed by 0.5-2% of strain of S. aureus. It is heat-stable and withstands exposure at 100 °C for few minutes (Duguid et al, 1973) to 30 minutes (Colle et al, 1985). By use of serologic methods, seven different enterotoxins are recognized and designated A, B, C$_1$, C$_r$, C$_r$, D and E. In general, toxin A is recovered from food poisoning outbreaks more than any other, with toxin D being second most frequent. The fewest number of outbreaks are associated with toxin E (Jay, 1994).

Casman and Bennett, (1965) found that large numbers of S. aureus must be present in foods to cause poisoning, it was estimated that 1-2 µg of
toxin A would be required to cause symptoms of food staphylococcal poisoning. *S. aureus* may grow (count of $2 \times 10^2 - 2 \times 10^3$ per gram) in foods without causing changes in odors, taste or physical appearance (Bergdoll, 1981).

**Toxic shock syndrome toxin (TSST):**

Some strains of *S. aureus* cause TSS (Cowan and Steel, 1981), but TSST is not an enterotoxin. Some enterotoxin producing strains also produce TSST, and some of the symptoms of TSS appear to be caused by toxins A, B and C (Jay, 1991). TSST (enterotoxin F, pyrogenic exotoxin C) is implicated in toxin shock syndrome, which particularly affects menstruating women using tampons. Some staphylococcal toxins are phage or plasmid - encoded (Hirsh and Zee, 1999). Table (1) lists the various virulence factors of *S. aureus*. 
Table 1: Virulence factors, including toxins of *S. aureus* and their pathogenic effects (Quinn, *et al.*, 2002).

<table>
<thead>
<tr>
<th>Virulence factor</th>
<th>Pathogenic effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulase</td>
<td>Conversion of fibrinogen to fibrin.</td>
</tr>
<tr>
<td>Lipase, esterases, elastase, staphylokinase, deoxyribonuclease, hyaluronidase phospholipase*</td>
<td>Enzymes which contribute to virulence.</td>
</tr>
<tr>
<td>Protein A</td>
<td>Surface component which binds Fe</td>
</tr>
<tr>
<td>Leukocidin</td>
<td>Cytolytic destruction of phagocytes of some animal species</td>
</tr>
<tr>
<td>Alpha - toxin</td>
<td>The major toxin in gangrenous mastitis. Causes spasm of smooth muscle</td>
</tr>
<tr>
<td>Beta - toxin</td>
<td>Asphingomyelinase which damages cell membranes</td>
</tr>
<tr>
<td>Exfoliative toxins</td>
<td>Responsible for desquamation staphylococcal scalded skin syndrome in man</td>
</tr>
<tr>
<td>Enterotoxins</td>
<td>Heat-stable toxins associated with staphylococcal food poisoning.</td>
</tr>
<tr>
<td>Toxic shock syndrome toxins (TSST)</td>
<td>Include excessive lymphokine production, resulting in tissue damage. Bovine and human strains of <em>S. aureus</em> produce TSST.</td>
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</table>

*Note: Also *S. aureus* produces urease, protease (Sneath, *et al.*, 1987) and penicillinase (Duguid, *et al.*, 1973).
**Resistance:**

Staphylococci withstand drying for weeks, heating up to \( \text{\textdegree} C \) for \( \text{\textdegree} \) minutes, pH fluctuations from \( \text{\textdegree} \) to \( \text{\textdegree} \), and salt concentration of \( \text{\textdegree} \% \).

Staphylococci are inhibited by bacteriostatic dyes (e.g. crystal violet), bile salts, disinfectants like chlorhexidine, and many antimicrobial drugs, (Hirsh and Zee, \( \text{\textdegree} \)).

**Ecology of \( S. \text{ aureus} \) growth:**

In general, the staphylococci do not compete with the normal flora of most foods, especially those containing large numbers of lactic acid bacteria where conditions permit the growth of the latter organisms; also \( S. \text{ aureus} \) have the inability to compete in both fresh and frozen foods, (Jay, \( \text{\textdegree} \)).

**Clinical infections:**

Duguid \textit{et al} (\( \text{\textdegree} \)) reported that \( S. \text{ aureus} \) causes boils and infect wounds but these may also be caused by other bacteria.

\textbf{\( \textbf{1}. \) Superficial infections:}

\( S. \text{ aureus} \) Skin pustules, boils, carbuncles, impetigo, pemphigus neonatorum, sycosis barbae, surgical wounds and burns.

\textbf{\( \textbf{2}. \) Subcutaneous and sub-mucous abscesses:}

e.g. whitlow of finger or palm of hand and breast abscesses.

\textbf{\( \textbf{3}. \) Osteomyelitis, bronchopneumonia, particularly post - influenzal and pyelonephritis.}
٣. Lymphangitis, lymphadenitis, bacteremia, septicemia and acute bacterial endocarditis.

٤. Staphylococcal food - poisoning, a common cause of vomiting and diarrhea. The staphylococcus is capable of initiating an infection in apparently intact skin but infection is facilitated if the skin is breached or damaged.

*S. aureus* is a very common cause of infection in hospitals, and is most liable to infect newborn babies, surgical patients, old and malnourished persons, and patients with diabetes and other chronic diseases.

٥. Bovine staphylococcal mastitis:

Staphylococcal mastitis, usually caused by *S. aureus*, is a common form of bovine mastitis worldwide. It may be sub - clinical, acute or chronic but the majority of infections are sub - clinical, (Quinn, *et al*, ٢٠٠٢).

٦. The Gastroenteritis syndrome:

The symptoms of staphylococcal poisoning are nausea, vomiting, abdominal cramps, prostration and diarrhea (Nickerson and Sinskey, ١٩٩٧) also sweating, headache and sometimes a fall in body temperature (Jay, ١٩٩٧). Generally the last symptoms from ٤٤ to ٤٨ hours and the mortality rate is very low or nil. The usual treatment for healthy persons consists of bed rest and maintenance of fluid balance (Jay, ٢٠٠٢).
While the symptoms last, suffering may be acute but this usually involves a period of 1-7 hr, usually 3-6 hr after ingesting toxin (Nickerson and Sinskey, 1971).

Upon cessation of symptoms, the victim possesses no demonstrable immunity to recurring attack, although animals become resistant to enterotoxin after repeated oral doses. The minimum quality of enterotoxin needed to cause illness in humans is about 0.1 ng. This value is derived from an outbreak of staphylococcal gastroenteritis traced to % chocolate milk (Jay, 1981).

% Sources of infection:

1. Patients with lesions discharging staphylococci into the environment.

2. Healthy carriers: S. aureus grows on the moist invaginated skin in 0.1-3.0% of healthy persons. The cocci are spread from these sites to the environment by hands, handkerchief, clothing and dust.

3. Domesticated animals and some wild species may disseminate S. aureus from infected lesions or carriage site and so cause infections in man (Duguid et al, 1971).
Mechanisms of transmission in cross infection:

These include:

1. Contact:

By direct contact with the contaminated hand or clothing of an infected person e.g. hand of nasal carrier who has picked his nose. Hundreds of staphylococci may pass in a drop of sweat that exudes from the hand of a carrier surgeon through puncture in rubber glove.

Moderate numbers of the staphylococci may be transmitted indirectly on hand of non-carrier nurse who has contaminated his hands by touching infected patients or babies. Small numbers of staphylococci are spread by contact with objects, such as furniture, clothing and bedding which were contaminated by a patient or carrier of S. aureus.

2. Air-born dust:

Staphylococci carried on fragments of desquamated keratin, fibers of cloth and particles of powdered dried pus or sputum are readily shed into the air from the skin, clothing and bedding; 95% of these particles will fall out of the air within 15-20 minutes but a few will remain air-borne for up to 4 hours or more.

3. Droplet-spray and air-borne droplet-nuclei:

Disseminated in speaking, coughing and sneezing by healthy nasal carriers.
Prevention of staphylococcal and other food poisoning syndromes:

When susceptible foods are produced with low numbers of staphylococci, they will remain free of enterotoxins and other food-poisoning hazards, if kept either below \(0^\circ\text{C}\) or above \(14^\circ\text{C}\) until consumed.

The factors leading to food poisoning:

1. Inadequate refrigeration.
2. Preparing foods far in advance of planned service.
3. Infected persons practicing poor personal hygiene.
4. Inadequate cooking or heat processing.
5. Holding food in warming devices at bacterial growth temperature.

Inadequate refrigeration alone comprised 80% of the contribution factors (Jay, 2002).

Sodium chloride concentrations:

NaCl is widely used as food preservative. Its effect on inhibition of microbial growth and toxin synthesis has been extensively studied (Hughes and Hurst, 1989).

Effect of salt on foods:

Salt dehydrates food by drawing out and tying up moisture, and it dehydrates microbial cells.
1. It ionizes to yield the chloride ion which is harmful to organisms.

2. It sensitizes the microbial cell against carbon dioxide.

3. It reduces the solubility of oxygen in water.

4. It interferes with the action of proteolytic enzymes (Frazier and Westhoff, 1978).

Effect of NaCl on growth of S. aureus and enterotoxin production:

The staphylococci are capable of growing in the presence of fairly high levels of salt and this property is utilized in the preparation of media selective for the organisms. Most strains grow well in 1% NaCl, some being able to grow in up to 2% NaCl (Jay, 1979). Generally growth is good at NaCl concentration up to 1% and is relatively poor at 5%, most strains of S. aureus grow between 0.6-35°C and at pH values between 4.0 and 5.0 (Sneath, et al., 1979). It has been well documented that S. aureus is highly resistant to NaCl surviving even in conditions of 5% NaCl (Stewart et al, 1980).

Tompkin et al. (1973) found that up to 1% NaCl did not essentially alter the ratio of enterotoxin A, although the quantity of enterotoxin decreased. Increased salt level of NaCl concentration higher than 1% inhibited bacterial growth.

Growth and enterotoxin C production occurred over the pH range 4.0-9.0 with no NaCl; with 1% NaCl, the pH range was restricted to 4.0-5.0. Toxin was produced at 1% NaCl with a pH of 5.0 or higher but none was
produced at ٪٢ NaCl.

Growth and enterotoxin B production by *S. aureus* occurred in ٪٠.٢ NaCl and pH ٩.٩, but not with ٪٤ NaCl at pH ٠.١. The general effect of increasing NaCl concentration is to raise the minimum pH of growth. At pH ٩.٩ and ٣°C enterotoxin B was inhibited by ٪١ or more NaCl (Jay, ١٩٩١).

Also growth occurred at pH ٩.٩ with ٪٠.٢ NaCl at pH ١.٢ and ٪٠.٣ at · and ٪٢ NaCl. Toxin was produced at ٩, ١.٠ and ٪٠.٢ NaCl for low (٩,٢×١.٠ cell/sac), medium (٩,٢×١.٠ cell/sac), and high (٩,٢×١.٠ cell/sac) inoculum levels (Tompkin *et al.*, ١٩٩٣).

Nunheimer and Fabian (١٩٩١) reported that ٪٠.٢-٪٠.٤ salt was inhibitory for growth, while ٪٠.٣-٪٠.٧ was found to be germicidal. Also Genigeorgis and Sadler (١٩٩٩) reported that *S. aureus* produced enterotoxin B at pH ٩.٩ with ٪٠.٢ salt, at pH ٠.٢ with ٪٠.٢ salt and at ٠.١ with up to ٪٢ salt.

The growth of *S. aureus* in Trypticase Soy Broth (TSB) containing ٪٠, ٪٠.١ or ٪١ NaCl after ٨٤ hr was not significantly different at all. Stern, *et al.*, (١٩٩٩) also stated that the effect of pH ١.٠ on the growth response of *S. aureus* in TSB containing ٪٠, ٪٠.١ or ٪١ NaCl was little, but at pH ٪٠.١ the lag phase of growth increased as the salt concentration increased from ٪٠ to ٪١. However, at ٨٤ hr incubation, the log number of cells was not significantly different at all levels of NaCl.
Stern, *et al.* (1991) reported that the addition of BHA to growth media at various pH values, allowed lower concentrations of NaCl to inhibit growth of *S. aureus* when compared to other investigations using NaCl and pH alone.

Tompkin, *et al.* (1993) found growth of *S. aureus* to occur at pH 4.9 with 7% NaCl but Stern, *et al.* (1991) found that growth was inhibited with 7% NaCl at pH 6.0 and pH 6.6 in the presence of either 50 ppm or 100 ppm BHA.

Genigeorgis and Sadler (1977) reported that cell numbers were found to decrease as NaCl levels were increased at any pH value tested.
CHAPTER THREE
MATERIALS AND METHODS

١٫٣ Sterilization:

١٫١٫٣ Hot air oven (٦١۱°С for two hours):

Glassware such as Petri dishes, pipettes, test tubes, flasks and glass rods were sterilized in hot air oven at ٦١۱°С for two hours.

١٫٢٫٣ Autoclaving at ١٢١°С (١٠٠ b/in²):

Used for sterilization of media and solution by autoclaving at ١٢١°С for ٥١٠ minutes.

١٫٣٫٣ Disinfection:

For aseptic work such as transfers, addition of supplements and pouring plates, absolute alcohol was used first for disinfecting the floors and the benches.

١٫٤٫٣ Collection of samples:

The isolates of S. aureus from white cheese and milk were obtained from the Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum, after isolation, purification and identification.

١٫٥٫٣ Identification of isolated bacteria:

This was done according to Barrow and Felthman (٣٩٩١) and scheme of Elsanosi (٦٩٩١), after the isolates were grown in selective media (Baird-Parker and mannitol salt agar); identification of isolated bacteria was done...
by biochemical tests to confirm the identity of the isolates (Table 2)

Table 2: The biochemical properties of *S. aureus* isolated from cheese and milk.

<table>
<thead>
<tr>
<th>Test</th>
<th>S. aureus from milk</th>
<th>S. aureus from cheese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidase</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Coagulase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pigment</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Haemolysis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Preparation of media:

Liquid media:

Nutrient broth:

<table>
<thead>
<tr>
<th>Contents</th>
<th>(g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal tissue</td>
<td>5</td>
</tr>
<tr>
<td>Beef extract</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Thirteen grams of dehydrated nutrient broth (Difco) were dissolved in one litre of distilled water, mixed well and pH adjusted to 4.7. The medium was sterilized by autoclaving at 121°C for 15 minutes and poured in tubes; every tube contained 5 ml.

Carbohydrate fermentation:

Hundred ml of peptone water were prepared by dissolving 0.5 g of dehydrate peptone and pH adjusted to 2.7. One ml of Andrades indicator was added. Exactly 36 ml of this mixture were distributed into test tubes each containing 9 ml and sterilized by autoclaving at 115°C for 20 minutes. Sugar solution was prepared by dissolving 0.1 g of appropriate sugar in 9.9 ml of distilled water and sterilized by autoclaving at 115°C (15 lb/in²) for 10
minutes. The sugar solution was added aseptically to the peptone water to make 0.1 ml total value, then half quantity was transferred to a sterile test tube so that now there were two test tubes of each sugar solution each contain 0 ml. The carbohydrate used were a mannose, sucrose, mannitol, trehalose, fructose, lactose and maltose.

3. Solid media:

3.3.4.3 Nutrient agar:

<table>
<thead>
<tr>
<th>Contents</th>
<th>(g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>1.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.0</td>
</tr>
<tr>
<td>Animal tissue</td>
<td>0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0</td>
</tr>
<tr>
<td>Agar</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Twenty-eight grams of dehydrated nutrient agar (Difco) were dissolved in a liter of distilled water by steaming, the pH was adjusted to 7.4 and the medium autoclaved at 121°C for 15 minutes before pouring onto plates.

3.4.5.4 Urea agar:

The basal dehydrated medium (Oxoid) was composed of dextrose,
disodium phosphate, peptone, sodium chloride, potassium dihydrogen phosphate, phenol red and agar. An amount of ٤٫٤ grams was dissolved in ٥٩ ml distilled water by boiling. After sterilization by autoclaving at ١١٥٠ْC for ٠٢ minutes and cooling to ٥٠ْC, ٠ ml of sterile ٪٤ urea solution were aseptically added. The pH was adjusted to ٨٫٦ and the medium distributed as ١٠ ml aliquots into sterile screw - capped bottles which were allowed to solidify in slope position.

٣٫٤ ٤ Blood agar:

Ten ml of fresh defibrinated sheep blood were added aseptically to ٠٩ ml of melted sterile nutrient agar (cooled to ٥٥ْC), mixed and distributed into sterile Petri dishes, ٢ ml in each dish.

٣٫٥ ٥ Semi solid media:

٣٫٥ ٤ Hugh and Leifson's (O/F) medium)

Peptone, ٢٫٠ gram; NaCl, ٥٫٠ grams, agar, ٣٫٠ grams and K٢HPO٤, ٣٠٫٠ grams were dissolved in one hundred distilled water, heated, then the pH was adjusted to ١٫٧, ٥١ ml of bromthymol blue, aqueous solution (٪٢٫٠) were added then the medium was sterilized by autoclaving at ١١٥٠ْC for ٠٢ minutes. Then ml of sterile glucose solution were added aseptically to ٣٫٠ ml of the medium to give the final concentration of ٪٢ and the medium distributed into test tubes, each tube containing ١٠ ml.
Modified liquid media:
Modified nutrient broth:

It was prepared by manipulation of NaCl concentration of nutrient broth to give concentrations of 0%, 1%, 5% and 10% NaCl. pH was adjusted to 4.7.

Modified solid media:
Modified Nutrient agar:

It is prepared by adding NaCl to the other ingredients of nutrient agar to give media with several salt concentrations (Benson, 1991). The salt concentrations were 0%, 1%, 5% and 10%. pH was adjusted to 4.7.

Physiological methods:

Growth method:

A stock culture was prepared by incubation of a tube of nutrient broth and plate of nutrient agar containing 0, 5, 10 and 20% NaCl, for 48 hr at 37°C. The inocula for growth studies were prepared by transferring a loopful of the stock culture to a plate and a tube containing 0 ml of nutrient broth and incubating the tube and Petri dish for 17, 48, 72, 96 hr at 37°C (Stern et al, 1979).

Growth in the liquid media was recorded as optical density (OD), which was determined at 600 nm with a Jenway 6200 spectrophotometer.
Growth on the plates was determined by visual observation.

Biochemical methods:

Tube Coagulase test:

Half a milliliter of \(1:5\) diluted human plasma in normal saline was placed in a small sterile agglutination tube, then \(0.5\) ml of \(18-24\) hours old broth culture was added and incubated at \(73^\circ C\), for an overnight. Positive results were indicated by definite clot formation.

Catalase test:

A drop of \(3\%\) aqueous solution of hydrogen peroxide was placed on a clean slide. A small amount of the bacteria under test was placed on the hydrogen peroxide drop using a glass rod. Positive results were indicted by production of bubbles.

Oxidase test:

A strip of filter paper soaked in \(1\%\) solution of tetramethyl - P - phenelene diamine dihydrochloride was dried in an oven. Then it was placed using a sterile forceps on a clean slide. A discrete colony was picked with a sterile bent glass rod and rubbed over the surface of the strip. A purple colour was recorded as positive to cytochrome oxidase within one minute.

Oxidation / fermentation test (O/ F):

Two tubes of Hugh and Leifson’s medium were inoculated with the test culture. One was covered with a layer of sterile soft paraffin to a depth of
about \(1-2\) cm. The tubes were then incubated at \(37^\circ C\) and examined daily up to \(41\) days. Fermentative organisms were indicated by change in colour to yellow in both tubes.

\[\text{Urease test:}\]

The slant surface of urea agar was streaked with the test cultures and incubated at \(37^\circ C\) for \(42-84\) hours. A red colour was indicative of production of \(NH_3\).

\[\text{Sugar fermentation test:}\]

The sugar medium was inoculated with bacterial growth in peptone water, incubated and then examined daily for up to \(7\) days. Acid production was indicated by the development of pink colour in the medium. If the colour remains unchanged then no acid was produced.

\[\text{Novobiocin sensitivity test:}\]

Standard disc diffusion method was used to carry out the sensitivity of the test organism to antibiotic. Novobiocin sensitivity disc of Oxoid (\(5\ \mu g\)) was used. A plate of nutrient agar was dried in the incubator for \(3\) minutes then a diluted suspension of the organism was poured onto the surface of the medium. Excess fluid was aspirated and the plate was allowed to dry again for \(3\) minutes using sterile forceps, the antibiotic disc was gently applied on the plate and incubated at \(37^\circ C\) for \(4\) hours. The zones of inhibition were measured in mm to determine whether the organism was sensitive or
CHAPTER FOUR

RESULTS AND DISCUSSION

This work was undertaken to study the effect of NaCl on \textit{S. aureus} growth. The antibacterial effect of salt has been extensively studied and, in food, salt has been used as preservative since ancient times.

In this study the growth tests were carried out on \textit{S. aureus} isolated from cheese and milk, where the isolates were tested at different concentrations of sodium chloride on solid media (nutrient agar) and liquid media (nutrient broth) at 37°C.

\textbf{The effect of NaCl on \textit{S. aureus} growth:}

\textbf{Growth on solid media:}

Cell growth was observed decrease as NaCl level was increased. This result agrees with Genigeorgis and Sadler (1991).

\textbf{Growth at \% NaCl:}

In absence of sodium chloride cultures of \textit{S. aureus} were able to grow well (Plate 1 and 2).
Plate 1: Growth of *S. aureus* isolated from milk at ٪ NaCl.

Plate 2: Growth of *S. aureus* isolated from white cheese at ٪ NaCl.
Growth at ٪٥ NaCl:

There was good growth when compared to growth on higher NaCl concentrations (Plate ٥ and ٦). Abd Alsamed, (٠٠٠٢) found that S. aureus could grow well in range between ٪٥ and ٪٨ NaCl.

Growth at ٪٠١ NaCl:

A reduction in cell density was noticeable at ٪٠١ NaCl compared with ٪٥ NaCl or ٪٨ NaCl (Plates ٩ and ١٠). This result is in agreement with Jay (١٩٩٠) who mentioned that most strains of S. aureus grew well in ٪٠١ NaCl.

Growth at ٪٥١ NaCl:

Poor growth was observed when compared with growth at ٪٠١ NaCl (Plates ٧ and ٨). The result obtained in this study was in agreement with Momoun (٥٠٠٢) who found that S. aureus grew at concentrations ٪٥٠٠-٪٥١ NaCl.

Growth at ٪٠٢ NaCl:

No visible growth occurred at this concentration of salt (Plates ٩ and ١٠). Jay (١٩٩٠) mentioned that some strains of staphylococci are able to grow in up to ٪٠٢ NaCl. Nunheimer and Fabian (١٩٤٠) reported that ٪٠-٪٢ NaCl salt was inhibitory for growth, while ٪٠-٪٢ was found to be germicidal.
Plate 3: Growth of *S. aureus* isolated from milk at 5% NaCl.

Plate 4: Growth of *S. aureus* isolated from white cheese at 5% NaCl.
Plate 5: Growth of *S. aureus* isolated from milk at $\frac{1}{2}$ NaCl.

Plate 6: Growth of *S. aureus* isolated from white cheese at $\frac{1}{2}$ NaCl.
Plate V: Growth of *S. aureus* isolated from milk at 10% NaCl.

Plate VIII: Growth of *S. aureus* isolated from white cheese at 10% NaCl.
Plate ٩: Growth of *S. aureus* isolated from milk at ٪۰.۲ NaCl.

Plate ١٠: Growth of *S. aureus* isolated from white cheese at ٪۰.۲ NaCl.
Growth in liquid media:

As shown in Table 3 and Fig. 1, growth of *S. aureus* isolated from milk in 5% and 10% NaCl was only slightly lower than growth in control media containing no salt. However, the NaCl concentration of 15% and 20% brought about drastic reduction in growth as reflected by low optical density values. An interesting phenomenon is noticed here. Reduction of OD was observed after 21 hours but more drastically after 42 hours, then there was growth at 63 hours in concentrations of 15% and 20% of NaCl.

Apparently, first some strains of the organism grew but lysed at 84 hours. But then some more salt-tolerant strains took over and grew to high level before lysing.

With respect to the bacteria isolated from cheese (Table 4 and Fig. 2) the effect of the 5% NaCl was low when compared to the control containing 7% NaCl. But the concentrations of 15%, 10% and 20% NaCl brought about great reduction in growth.

It is noted that the isolate from white cheese attained in the absence of salt and in the presence of 5% NaCl about twice (about 6.0 OD units) the maximum growth (82.0 OD units) reached by the isolated from milk.

The same phenomenon observed with the milk isolate indicating the selection of more salt-tolerant strains at 15% and 20% NaCl, was also observed with the cheese isolate but only with respect to growth at 84 hr lysis at 84 hr.
We can say that both isolates were able to grow even at 10% and 20% NaCl although the growth was not much.

Table 3: The relationship between growth(OD) of *S. aureus* isolate from milk and NaCl concentration:

<table>
<thead>
<tr>
<th>Concentration of NaCl</th>
<th>OD at 12 hr</th>
<th>OD at 24 hr</th>
<th>OD at 36 hr</th>
<th>OD at 48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>0.2</td>
<td>0.28</td>
<td>0.17</td>
<td>0.11</td>
</tr>
<tr>
<td>5%</td>
<td>0.18</td>
<td>0.27</td>
<td>0.13</td>
<td>0.08</td>
</tr>
<tr>
<td>10%</td>
<td>0.11</td>
<td>0.25</td>
<td>0.13</td>
<td>0.04</td>
</tr>
<tr>
<td>15%</td>
<td>0.14</td>
<td>0.33</td>
<td>0.12</td>
<td>0.11</td>
</tr>
<tr>
<td>20%</td>
<td>0.04</td>
<td>0.019</td>
<td>0.02</td>
<td>0.03</td>
</tr>
</tbody>
</table>

OD: Optical density at 600 nm.
Table 4: The relationship between growth (OD) of *S. aureus* isolated from cheese and NaCl concentration:

<table>
<thead>
<tr>
<th>Concentration of NaCl</th>
<th>OD at 12 hr</th>
<th>OD at 24 hr</th>
<th>OD at 36 hr</th>
<th>OD at 48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>0.30</td>
<td>0.60</td>
<td>1.04</td>
<td>0.28</td>
</tr>
<tr>
<td>5%</td>
<td>0.33</td>
<td>0.65</td>
<td>0.25</td>
<td>0.13</td>
</tr>
<tr>
<td>10%</td>
<td>0.15</td>
<td>0.26</td>
<td>0.11</td>
<td>0.08</td>
</tr>
<tr>
<td>15%</td>
<td>0.02</td>
<td>0.05</td>
<td>0.15</td>
<td>0.02</td>
</tr>
<tr>
<td>20%</td>
<td>0.01</td>
<td>0.02</td>
<td>0.23</td>
<td>0.01</td>
</tr>
</tbody>
</table>

OD: Optical density at 600 nm.
Fig. 1: Level of growth of *S. aureus* isolated from milk as affected by different concentrations of NaCl (Data from Table 3).
Fig. 2: Level of growth of *S. aureus* isolated from white cheese as affected by different concentrations of NaCl (Data from Table 4).
changes in pH values of growth medium of S. aureus:

S. aureus can grow over the range of pH 4-9 but its optimum is in the range of pH 6-8 (Jay, 1991). The results obtained in this study, pH 0.6-7.7 for isolate from cheese pH 0.5-7 for S. aureus isolated from milk (Tables 5 and 6), fall within these ranges.

As shown in Fig. 3, growth of S. aureus isolate from white cheese has little effect on the pH value which generally remained slightly below neutrality. In the case of the isolate from milk (Fig. 4) there is first a slight reduction in pH after 21 hours growth followed by a rise in values to slightly below neutrality. In general there was little or no effect of salt on pH value. Therefore, we can conclude that the lysis of the cells after growth in not due to a change in pH.
Table 6 The relationship between concentration of NaCl and pH of medium in which grew *S. aureus* isolated from white cheese:

<table>
<thead>
<tr>
<th>Concentration of NaCl</th>
<th>pH at 12 hr</th>
<th>pH at 24 hr</th>
<th>pH at 36 hr</th>
<th>pH at 48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>6.7</td>
<td>7.3</td>
<td>7.7</td>
<td>7.0</td>
</tr>
<tr>
<td>5%</td>
<td>6.6</td>
<td>6.9</td>
<td>6.8</td>
<td>6.7</td>
</tr>
<tr>
<td>10%</td>
<td>6.1</td>
<td>6.0</td>
<td>6.8</td>
<td>6.2</td>
</tr>
<tr>
<td>15%</td>
<td>6.0</td>
<td>6.1</td>
<td>6.3</td>
<td>6.4</td>
</tr>
<tr>
<td>20%</td>
<td>6.3</td>
<td>6.1</td>
<td>6.1</td>
<td>6.0</td>
</tr>
</tbody>
</table>
Table 6: The relationship between concentration of NaCl and pH of medium in which grew *S. aureus* isolated from milk:

<table>
<thead>
<tr>
<th>Concentration of NaCl</th>
<th>pH at 24 hr</th>
<th>pH at 48 hr</th>
<th>pH at 66 hr</th>
<th>pH at 84 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>7.3</td>
<td>7.0</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>5%</td>
<td>7.0</td>
<td>7.9</td>
<td>6.4</td>
<td>6.4</td>
</tr>
<tr>
<td>10%</td>
<td>6.0</td>
<td>6.0</td>
<td>6.4</td>
<td>6.4</td>
</tr>
<tr>
<td>15%</td>
<td>5.9</td>
<td>6.2</td>
<td>6.3</td>
<td>6.3</td>
</tr>
<tr>
<td>20%</td>
<td>5.9</td>
<td>6.1</td>
<td>6.4</td>
<td>6.0</td>
</tr>
</tbody>
</table>
Figure ٣: The relationship between concentration of NaCl and pH of medium in which grew *S. aureus* isolated from white cheese (Data from Table ٥).
Figure 4: The relationship between concentration of NaCl and pH of medium in which grew *S. aureus* isolated from milk (Data from Table 6).
CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

١. Conclusions:

١. Sodium chloride concentrations of ٥٪ and ٠٠٪ have a little effect on growth of S. aureus isolated from milk.

٢. Sodium chloride concentration of ٥٪ has little effect on isolate from cheese.

٣. Concentrations of ١٠٪ and ٢٠٪ NaCl have a clear inhibitory effect on the growth of isolate from milk.

٤. Isolate from cheese was inhibited by NaCl concentrations of ٢٠٪, ١٠٪ and ٢٠٪.

٥. High concentrations of salt apparently select for more tolerant strains.

٦. There is some growth even at ٢٠٪ NaCl.

٧. Sodium chloride at the concentration of ٥٪, ١٠٪ and ٢٠٪ have little effect on pH of the growth medium.
Recommendations:

1. More research should be carried out on the effect of salt on growth of *S. aureus* specially the salt-tolerant strains.

2. White cheese and other food containing much salt (like fessiekh) should have more than $\frac{2}{3}$ NaCl if possible.

3. As there is growth of *S. aureus* even at $\frac{2}{3}$ NaCl we recommend that food containing salt as preservative should also be refrigerated or pasteurized.
REFERENCES


