

Comparative inhibitory effects of sodium and potassium chloride salts on *Staphylococcus aureus* from fermented *Pentaclethra macrophylla*

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Abstract

Aim: The study was conducted to evaluate comparatively the individual effects of sodium (NaCl) and potassium (KCl) chloride salts against *Staphylococcus aureus* isolated from fermented *Pentaclethra macrophylla*.

Method: A total of twenty (20) traditionally fermented food samples (*Pentaclethra macrophylla*) were purchased from a market in Umuahia metropolis. Isolation and identification of *Staphylococcus aureus* were conducted using standard microbiological techniques. *Staphylococcus aureus* was identified using standard morphological and biochemical tests. The inhibitory effect of both salts (NaCl and KCl) were analyzed on modified nutrient agar and nutrient broth (Media + 5% salt conc., Media + 10% salt conc., Media + 15% salt conc., Media + 20% salt conc.). The study measured culture growth using optical density readings at 425nm over 48 hours with different NaCl and KCl concentrations, while growth on plates was visually assessed on colony counter.

Results: The highest optical density recorded as a result of contact with varying concentration of sodium chloride was 0.48 at 5% concentration after 48hours incubation while the least was 0.04 at 20% concentration after 12hours incubation while the highest optical density recorded as a result of contact with varying concentration of potassium chloride was 0.41 at 5% concentration after 48hours incubation while the least was 0.01 at 20% concentration after 12hours incubation.

Conclusion: Higher salt concentrations resulted in osmotic shock, suppressing growth in *Staphylococcus aureus*, but increased salt concentration partially alleviated growth inhibition over time.

Keywords: *Staphylococcus aureus*; *Pentaclethra macrophylla*; Inhibitory effect; Sodium Chloride; Potassium Chloride

1. Introduction

Staphylococcus aureus is one of the important human pathogens involved in food-related diseases and a common cause of community-associated infection (Fridkin *et al.*, 2005; Normanno *et al.*, 2007). This organism proliferates in food and releases one or more heat-stable enterotoxins, causing food-borne illnesses. *S. aureus* is the most common cause of infections in hospitalized patients and has been a major concern for well over a century (Ekrami *et al.*, 2011)

Staphylococcus aureus has been responsible for food poisoning incidents in many types of food, including ready to eat (RTE) salads, ham, and sausage. With proper conditions, *S. aureus* can grow in food and produce enterotoxins. Staphylococcal enterotoxins (SEs) are heat stable and can cause foodborne intoxication, even following cooking of the food. According to the Centers for Disease Control and Prevention, there were 495 cases of *S. aureus* foodborne

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outbreaks in 2004, with the majority of staphylococcal food intoxications linked to meats and prepared foods (CDC, 2006).

Fermented *Pentaclethra macrophylla* is popularly known as Ugba (or Ukpaka as it is called in Igbo language) also popularly called African salad, it is a ready-to-eat food. Ugba is a proteinaceous delicacy consumed by millions of people in the South-Eastern zone of Nigeria (Njoku and Okemadu, 2017). Ugba production is locally done through a mixed wild bacteria fermentation of the sliced, boiled and soaked African oil bean seeds. Microbial population of Ugba is introduced through the air, water, utensil, banana leaves or the handler; no starter culture is used for the traditional method. Microorganisms involved are predominantly *Bacillus*, *Micrococcus* and *Lactobacillus*. Other organisms isolated from Ugba include *Pseudomonas*, *Staphylococcus*, *Enterobacter*, *Leuconostoc*, *Corynebacterium* and *Alkaligenes* (Njoku *et al.*, 2010; Enujiugha, 2009; Sanni *et al.*, 2011). The problem of occurrence and growth of pathogens in Ugba, like most other fermented food products, cannot be overruled as the general hygienic conditions of the processors, the equipment used, water and other raw materials cannot be said to be free of potential pathogens.

During growth of *S. aureus*, enterotoxins are produced in low amounts at exponential stage, but production increases in late exponential and stationary phases. Thus, significant growth of *S. aureus* has to take place in the food before toxic levels of enterotoxin are formed. In a range of inoculated foods, enterotoxin was not detected in any sample with less than 10^7 *S. aureus* CFU/g. Bergdoll reported that as few as 10^5 CFU/g *S. aureus* were required for staphylococcal enterotoxin production. However, although many food samples with greater than 10^7 *S. aureus* CFU/g were positive for enterotoxin (0.1 mg of enterotoxin per 100 g), several samples with counts of 10^9 to 10^{10} CFU/g of *S. aureus* were negative for enterotoxin (Notermans and van Otterdijk, 2009).

However, tolerance of *Staphylococcus aureus* to high concentrations of NaCl in liquid medium has been reported, with no damage or shrinkage observed in its cellular structure, while in the case of *E. coli*, cell injury occurred. NaCl was therefore reported to affect the morphology of *E. coli* and *S. aureus*, while having a milder effect with respect to cell damage, especially on *S. aureus* (Hajmeer *et al.*, 2006). A low salinity leads to an immediate influx of small solutes thus relieving physical stress, in contrast, high salinity leads to water efflux which is counterbalanced by an increase of compatible solutes such as proline, glutamate, glycine betaine, ectoine and trehalose (Krämer, 2010). Acetate, lactate and citrate sodium salts have shown an inhibitory effect on the growth of some food spoilage bacteria and antimicrobial activity against foodborne pathogens, including *Staphylococcus aureus* and *Yersinia enterocolitica*, *Listeria monocytogenes*, *Escherichia coli* (Lee *et al.*, 2012; McWilliam and Stewart, 2015) as well as *Clostridium botulinum*. Moreover, a limited antimicrobial capacity has been reported for some organic acid salts against lactic acid bacteria during meat spoilage (Fux *et al.*, 2005). The aim of this study is to comparatively examine the inhibitory effects of sodium and potassium chloride salts on *Staphylococcus aureus* from fermented *Pentaclethra macrophylla*.

2. Materials and methods

2.1. Isolation, identification, purification and preservation of *Staphylococcus aureus*

2.1.1. Method of Sample Collection

The fermented *Pentaclethra macrophylla* (ugba/ukpaka) samples were purchased from the market and then transported to the Microbiology Laboratory.

2.1.2. Preparation of Media

The media used for the isolation were prepared according to the manufacturer's instruction by dissolving the required amount of the powder in a known volume of diluted water and autoclaved at 121°C for 15mins. The media used were Nutrient agar and mannitol salt agar (MSA).

2.1.3. Serial Dilution

Ten-fold serial dilutions of the samples were prepared by taking 1ml of 100% stock solution into 9ml of distilled water using sterile needle and syringe. This gave dilution factors of $10^1, 10^2, 10^3$.

2.1.4. Inoculation and Incubation of Culture Media

The sterilized culture media were inoculated with a loopful from the dilutions factor using flamed wire inoculating loop and then incubated at 37°C for 24-72 hours (Cheesbrough, 2006).

2.1.5. Identification of Isolates

Morphological characterization and biochemical characterisation were the two stages of the identification process for isolates.

Morphological Characterization

The isolates' cellular morphology was examined under a microscope to achieve this. Gram-stained day-old cultures of the bacterium isolates allowed for the observation and recording of the bacteria's color (purple or pink), shape (cocci or rods), and arrangement (singles, pairs, chains, or clusters).

- Gram staining

On a clean microscope slide, a light suspension of the organism in sterile distilled water was made using a sterile loop. The slide was twice passed over a gas flame to heat-fix the film after it had been air-dried. Next, the slide was given time to cool. After soaking in crystal violet solution for 30 seconds on a staining rack, the slide was washed with running water. The slide was once more completely submerged in Lugol's iodine solution, which was then washed off with running water. Acetone alcohol was applied to the film to decolorize it, and the film was then promptly washed off with running water. Safranin solution was applied to the film and allowed to sit for one minute before being washed off with running water. The slide's film was allowed to dry naturally. The film was then covered with an immersion oil drop, and the 100 oil immersion lens was used to view it under the microscope. Pink indicated a Gram-negative reaction, while dark purple indicated a Gram-positive reaction. Additionally, the cell arrangements and morphologies were noted.

Biochemical Characterization

Conventional biochemical tests were carried out on the bacterial isolates for further identification such as catalase test, oxidase test, coagulase test, indole test, methyl red test.

- *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 37°C, for an overnight. Positive results were indicated by definite clot formation.

- *Catalase test (slide method)*

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 indicated by production of bubbles (Cheesbrough, 2006).

- *Oxidase test*

A few drops of Kovac's oxidase reagent (1% tetramethyl-p-phenylenediamine) were used to wet a piece of filter paper in a Petri dish. A colony of the test organism was transferred to the filter paper using a wire loop, then it was rubbed on the damp area. The synthesis of cytochrome c oxidase was indicated by a purple colour within 30 seconds. (Cheesbrough, 2006).

- *Sugar Fermentation Test*

A 24-hour-old culture was incubated at 37 °C for 24 hours after being pierced into a sterile triple sugar iron agar slant (TSI). Then it was tested for the generation of glucose, lactose, sucrose, and gas; a positive result for glucose was shown by the test tube's bottom turning red, while a positive result for lactose was indicated by the media appearing yellow. (Cheesbrough, 2006).

- *Methyl red test*

This test, which is positive for mixed acid fermenters, was used to identify enteric bacteria based on their mode of glucose metabolism. A glucose and phosphate buffer-containing solution called glucose phosphate broth was used to inoculate the bacteria, and it was then cultured at 37 °C for 48 hours. By adding five drops of the methyl red reagent,

the pH of the medium was determined. The methyl red reagent was evenly distributed by gently rolling the tube between the palms. Yellow color development was viewed as undesirable, and red color development as positive.

2.1.6. Purification and Preservation of Isolates

The resulting colonies from the culture plates were purified by sub-culturing on a freshly prepared nutrient agar plates. The nutrient agar plates were incubated at 35 °C for 24hrs. The purified isolates were kept on agar slants as stock cultures under refrigeration temperature. The isolates were subcultured and transferred unto fresh agar slants on interval.

2.2. Determination of inhibitory effect of sodium chloride and potassium chloride on *Staphylococcus aureus* isolates

2.2.1. Bacterial Strains

Staphylococcus aureus strains used in this study were previously isolated, purified and preserved. The strains were isolated from fermented *Pentaclethra macrophylla* (ugba/ukpaka).

2.2.2. Media Used

- **Liquid media**

Nutrient broth

- **Solid media**

Nutrient agar

2.2.3. Modification of media

Media modifications were made by addition of Sodium chloride (NaCl) in different concentrations to standard media as follows: Media + 0% NaCl (Control), Media + 5% NaCl, Media + 10% NaCl, Media + 15% NaCl, Media + 20% NaCl. The same modification procedure was replicated with the potassium chloride salt.

2.2.4. Inoculation of Modified Media

A stock culture was prepared by incubation of a tube of nutrient broth containing 0, 5, 10, 15 and 20% NaCl, at 37 °C to obtain stationary phase cells. The inocula for growth studies were prepared by making ten-fold serial dilution and transferring a 0.1ml to a plate and incubating Petri dish for 12, 24, 36, 48hr at 37°C (Stern *et al.*, 2009). The same inoculation procedure was repeated with the potassium chloride salt (KCl) modified media.

2.2.5. Measurements of Growth Pattern

Growth in the liquid media was recorded as optical density (OD). The absorbance of the culture was determined at a wavelength of 425nm using spectrophotometer. The absorbance of each growth cultures with different NaCl and KCl concentration was obtained 12 hourly for 48hours. Growth on the plates was determined by visual observation.

3. Results

Table 1 shows the cultural, morphological and biochemical characteristic of *Staphylococcus aureus* which was isolated from *Pentaclethra macrophylla*. The organism was identified as *Staphylococcus aureus* by its morphological characteristics, pigmentation on media, microscopy and several biochemical tests such as Gram stain, Citrate utilization, Voges-Proskauer (VP) test, Methyl Red (MR) test, Indole production, Oxidase, Catalase, and Coagulase test.

Table 1 Cultural, morphological and biochemical characterization of *Staphylococcus aureus* from *Pentaclethra macrophylla* using conventional method.

Cc	GSR	Ca	Co	Ox	MR	Ind	Mot	Glu	Lac	Suc	Man	Presumptive organism
Golden yellow, slightly raised with smooth edges	Gram positive cocci, grape-like clusters,	+	+	-	+	-	-	A	A	A	A	<i>Staphylococcus aureus</i>

Key: Cc= Cultural characteristics, GSR=Gram stain reaction, Ca=Catalase, Co= Coagulase, Ox= oxidase, Mot=Motility, Ind=Indole, MR=Methyl Red, Glu=Glucose, Lac=Lactose, Suc=Sucrose, Man=Mannitol, A=Acid; G = Gas; AG = Acid and Gas; + = Positive; - = Negative

Table 2 The absorbance rate based on the effect of different concentration of Sodium chloride on *Staphylococcus aureus*

Hours of incubation	Optical density at different concentration				
	Control	5%	10%	15%	20%
12	0.21	0.28	0.21	0.14	0.04
24	0.27	0.36	0.28	0.03	0.2
36	0.37	0.43	0.33	0.32	0.3
48	0.41	0.48	0.44	0.31	0.3

Table 2 shows the absorbance rate based on the effect of different concentration of Sodium chloride on *Staphylococcus aureus*. The highest optical density recorded was 0.48 at 5% concentration after 48hours incubation while the least was 0.04 at 20% concentration after 12hours incubation.

Table 3 The absorbance rate based on the effect of different concentration of Potassium chloride on *Staphylococcus aureus*

Hours of incubation	Optical density at different concentration				
	Control	5%	10%	15%	20%
12	0.21	0.25	0.22	0.15	0.01
24	0.26	0.32	0.25	0.02	0.01
36	0.37	0.38	0.31	0.31	0.1
48	0.43	0.41	0.43	0.28	0.2

Table 3 shows the absorbance rate based on the effect of different concentration of Potassium chloride on *Staphylococcus aureus*. The highest optical density recorded was 0.41 at 5% concentration after 48hours incubation while the least was 0.01 at 20% concentration after 12hours and 24hrs incubation.

Table 4 shows the colony forming unit based on the effect of different concentration of Potassium chloride on *Staphylococcus aureus*. The colony forming units at different concentration and incubation time ranged from 4.5×10^7 to 1.0×10^6 CFU/ml. The highest count was recorded at 5% after 36hrs incubation time while the least count was recorded at 20% after 24, 36 and 48hrs incubation time.

Table 4 The colony forming unit based on the effect of different concentration of Potassium chloride on *Staphylococcus aureus*.

Hours of incubation	Colony forming unit at different concentration(10 ⁷ CFU/ml)				
	Control	5%	10%	15%	20%
12	1.2	3.6	3.1	0.7	0.2
24	2.0	4.1	3.6	1.0	0.1
36	2.8	4.5	3.5	0.6	0.1
48	1.3	3.0	3.1	0.6	0.1

Table 5 The colony forming unit based on the effect of different concentration of Sodium chloride on *Staphylococcus aureus*.

Hours of incubation	Colony forming unit at different concentration(10 ⁷ CFU/ml)				
	Control	5%	10%	15%	20%
12	1.4	4.2	3.4	0.7	0.2
24	2.2	5.0	4.3	1.0	0.1
36	2.8	4.6	4.5	0.6	0.1
48	1.5	3.2	3.1	0.6	0.1

Table 5 shows the colony forming unit based on the effect of different concentration of sodium chloride on *Staphylococcus aureus*. The colony forming units at different concentration and incubation time ranged from 5.0×10^7 to 1.0×10^6 CFU/ml. The highest count was recorded at 5% after 24hrs incubation time while the least count was recorded at 20% after 24, 36 and 48hrs incubation time

In Figure 1-4, a line graph comparing the inhibitory effects of potassium chloride (KCL) and sodium chloride (NaCL) at various concentrations (5%, 10%, 15%, and 20%, respectively) and various incubation times is displayed.

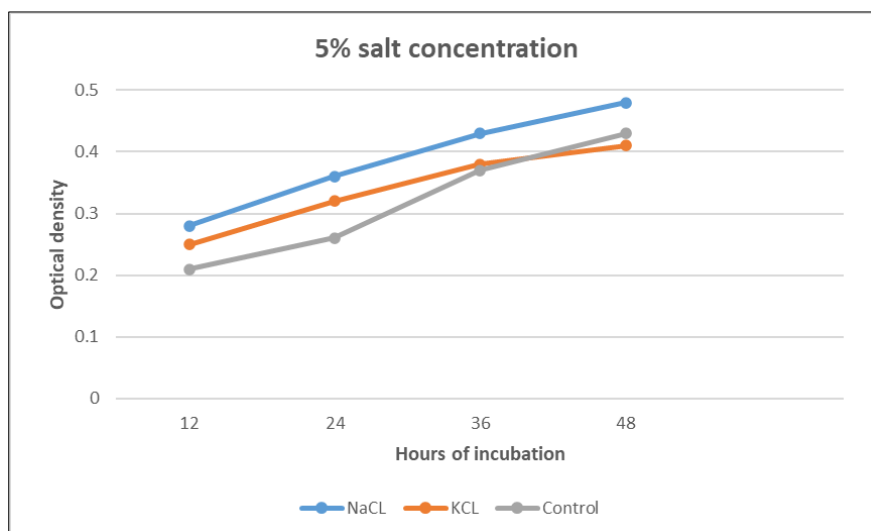


Figure 1 Inhibitory Effect of 5% Sodium Chloride (NaCL) and 5% potassium chloride (KCL) against *Staphylococcus aureus* from *Pentaclethra macrophylla*

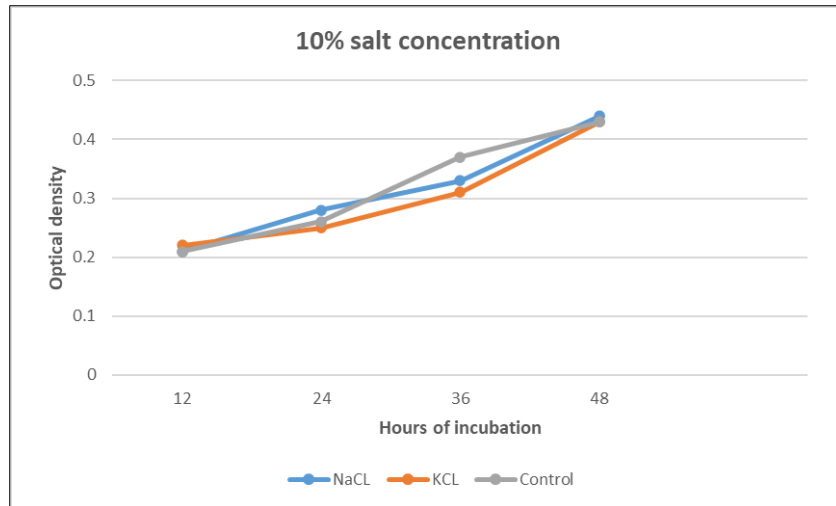


Figure 2 Inhibitory Effect of 10% Sodium Chloride (NaCl) and 10% potassium chloride (KCl) against *Stapylococcus aureus* from *Pentaclethra macrophylla*

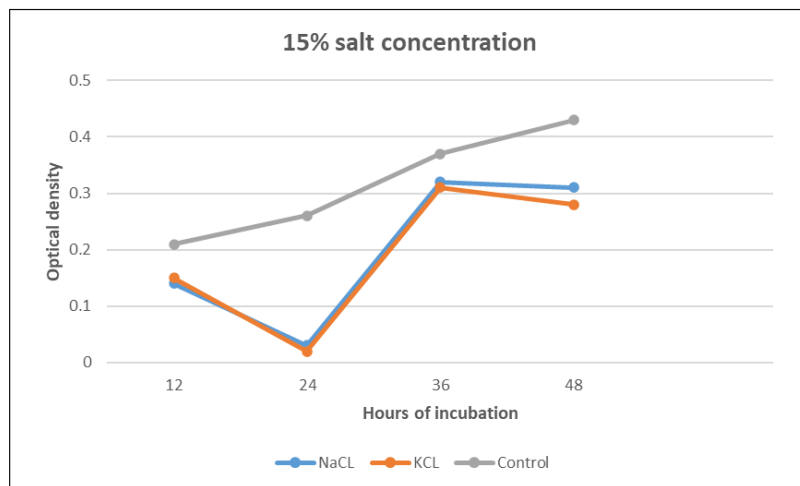


Figure 3 Inhibitory Effect of 15% Sodium Chloride (NaCl) and 15% potassium chloride (KCl) against *Stapylococcus aureus* from *Pentaclethra macrophylla*

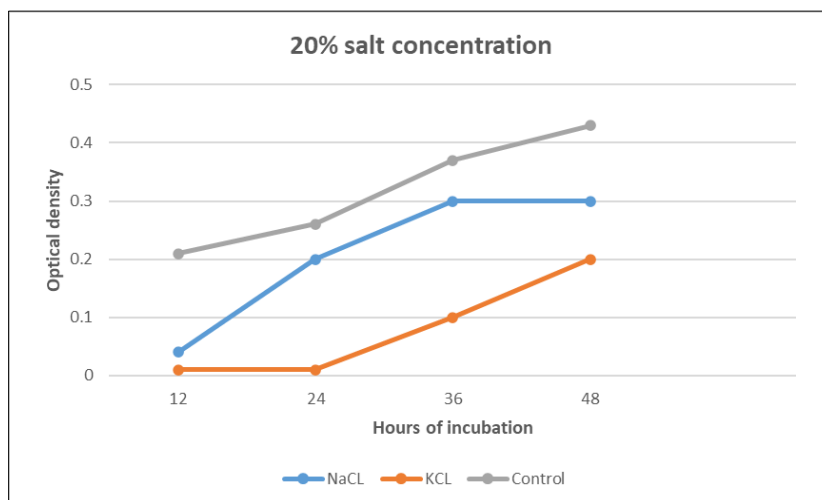


Figure 4 Inhibitory Effect of 20% Sodium Chloride (NaCl) and 20% potassium chloride (KCl) against *Stapylococcus aureus* from *Pentaclethra macrophylla*

4. Discussion

The study evaluates comparatively the inhibitory effects of sodium and potassium chloride salts on *Staphylococcus aureus* from fermented *Pentaclethra macrophylla* (Ugba). Traditionally fermented Ugba were purchased from local markets for isolation of *Staphylococcus aureus*. Among the organisms isolated, the isolate identified as *Staphylococcus aureus* based on its cultural and morphological characteristics and also based on its biochemical reaction using conventional methods was selected. From this study the presence of *Staphylococcus aureus* in fermented *Pentaclethra macrophylla* compares favourably with the reports of other researchers like Anyanwu *et al.*, 2016 who evaluated microbiological and nutritional qualities of fermented ugba (*Pentaclethra macrophylla*) sold in Mbaise, Imo State, Nigeria and isolated different organisms including *Staphylococcus aureus*. Ogbulie *et al.* (2014); Okorie *et al.*, (2017); Isu and Njoku, (1997) and Nwagu *et al.*, (2010) also isolated *Staphylococcus aureus* from their respective study.

Staphylococcus aureus are more commonly associated with the skin and hence are easily disseminated through handling; thus, the organism can gain entry into the Ugba by direct contact with human skin or air droplets from sneezing. Also, addition of salt would selectively favour the growth of *Staphylococcus aureus* which is known to be salt tolerant (Adam and Moss, 1999). *Staphylococcus aureus* has however, previously been reported to be involved in the fermentation of Ugba though their numbers decreasing after 72 hours of fermentation (Ogueke *et al.*, 2010). This may account for their presence in the present study.

In this study, the inhibitory effect of the chloride salts (NaCl and KCl) and microbial growth was monitored via optical density measurements and colony count as a function of contact time in the presence of progressive chloride salt concentrations. The optical density recorded as a result contact time with 5% concentration of sodium chloride indicates that the turbidity was higher in comparison with the control. Turbidity in liquid media is a function of growth and increase in cell biomass. Hence, in this study, 5% sodium chloride displayed no inhibitory effect against *Staphylococcus aureus* instead it provided a more suitable growth environment for *Staphylococcus aureus*. According to Nanjani and Soni, (2014), Staphylococcal halotolerance makes these bacteria capable colonizers of environments with low water content and high salinity, which gives them a competitive advantage over many other microorganisms in these niches (Onyango and Alreshidi, 2018). Similar growth rate was noticed for Potassium chloride salt, although the growth rate at 5% concentration for sodium chloride was greater. At higher concentration and longer incubation period both salt tend to reduce the growth rate of the organism but didn't inhibit their growth completely. The results of this current study compares favourably with result of Erwin and Haight (1973), Who carried out a study to evaluate the lethal and inhibitory effects of sodium chloride on thermally stressed *Staphylococcus aureus* and reported that Sodium ion was incorporated as the active agent of nutrient media containing potassium chloride and glycerol (osmotic equivalence) produced neither inhibition nor the lethal effect. Similarly, Ganjian *et al.*, (2012) and Abu-Ghazaleh (2016) reported in an increase in growth rate at lower concentration and a rapid decrease at high concentration.

S. aureus has the ability to detect external salt-stimulated signaling and activate the stress response, which changes its physiological and metabolic states and increases its capacity to survive (Krämer, 2010). Feng *et al.*, (2022) in their study on the Response and Survival Mechanisms of *Staphylococcus aureus* under High Salinity Stress in Salted Foods combine both phenotypic identification, transcriptomic and metabolomic analyses to clarify this mechanism of *Staphylococcus aureus* tolerance in high salt environment recorded that that biofilm formation, virulence, transfer system, and osmotic regulation were the main changes that occurred in *S. aureus* under high salinity stress

5. Conclusion

Higher salt concentrations of the medium increase its osmolarity, which is likely to have resulted in hyper osmotic shock to *Staphylococcus aureus* cells causing growth suppression. Consequently, it can be concluded that increasing the salt concentration in the medium partially inhibited the growth of *S. aureus* at longer incubation period.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to disclosed.

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