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Effect of Spices, pH and Temperature on the Survival and Multiplication of Staphylococcus aureus in Zobo Drink

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Abstract
This study was carried out to investigate the effects of spices, pH and temperature on the survival and growth of *Staphylococcus aureus* isolated from stream water samples in zobo drink. A total of 12 water samples were drawn from 3 different streams used in Ihiala Local Government Area, Anambra State, and screened for the presence of *Staphylococcus aureus* using pour plate method. The isolate obtained was characterized and identified using their morphological and biochemical characteristics. The effect of spices, pH and temperature on the isolate was determined by subjecting the isolate to 0.25%, 1.25% and 2.5% of spices (*Zigiber officinale* and *Myristica fragrans*), different pH ranges (3-10) and different temperatures (4^0C, 25^0C and 37^0C). Eleven water samples out of twelve samples drawn from the streams showed the presence of *Staphylococcus aureus.* The spices showed pronounced activity against the organism in both sterilized and non sterilized samples of which the activity increased significantly ($P<0.05$) as the concentration increased. The activity of *Zigiber officinale* was significantly ($P<0.05$) higher than that of *Myristica fragrans*. The maximum growth of the isolate was significantly ($P<0.05$) observed at pH 6 and 37^0C. Little or no growth was observed at 4^0C. This study has shown that the growth of *Staphylococcus aureus* in zobo drink could be controlled using *Zigiber officinale* and *Myristica fragrans* extracts at pH values other than 6, and should best be sold and consumed at refrigeration temperature (4^0C).

INTRODUCTION

Zobo is a name derived from Zoborodo which is the local Hausa (Northern Nigeria) name for Roselle hemp plant, *Hibiscus sabdariffa*. Zobo is quite popular especially in Northern Nigeria. Its popularity has recently spread across the entire country because of its purported medicinal value, as well as the increasing cost of other available soft drinks, it is produced from the dried calyces of the roselle plant *Hibiscus sabdariffa* by boiling and filtration (Adegunloye *et al.,* 2006). Hibiscus is made of the following nutritional substances: 1.145 g of protein, 2.61 g of fat, 12.0 g of fiber, 1,263 mg of calcium, 273.2 mg of phosphorus, 8.98 mg of iron, 0.029 mg of carotene, 0.117 mg of thiamine, 0.277 mg of riboflavin and 3.765 m of niacin. Given all this, it can be said that zobo can serve as an excellent food supplement and an aid to boost the body's immune system. It has been proven to reduce blood pressure and cholesterol level. Extracts of the flower is alkaline in nature and has been known to have cleansing properties. It helps to keep the body's pH balanced (without white sugar). The flesh Hibiscus flower is also rich in riboflavin, vitamin C, niacin, carotene, calcium, and iron which are all nutritionally necessary it is caffeine free (Oboh and Elusiyan, 2004).

The mode of packaging or dispensing the juice in nylon or plastic container before retailing, the largely unregulated nature of the trade and poor hygienic practices as well as lack of running water, proper storage and waste disposal facilities at preparation and services point has resulted in poor unsanitary conditions are potential contaminants of the drink by organisms like *Staphylococcus aureus.* The contamination of such organism can cause gastro intestinal illness characterized by diarrhea, abdominal cramps, and vomiting which is an increased risk to public health. Therefore to avoid such contaminations, the containers in which the zobo drink is dispensed must be sterilized, the water used in the process of production should be distilled and the environment where the production is carried out must be clean. The drink should also be stored properly after production more preferably refrigerated (Balola *et al*., 2001).

Other research works reported by (Ilondu and lloh, 2007), (Akpomedeya and Ejechi,2000), (Akinyosoye and Akinyele,2000) have demonstrated the different methods of zobo preservation. The deleterious role of *Aspergillus niger* and *Penicillium citrinum* in low pH and high sugar content foods have been reported by (Efiuwevwere and Akoma, 2002). The use of chemical preservatives at low allowable concentrations to control the growth of microorganisms in beverages is desirable and gaining research interest worldwide (Efiuwevwere and Akoma, 2001).The effect of plant and spice extracts and salt of sodium on the growth of *Aspergillus* spp. and related fungi were reported by (Kreb *et al*., 2000). Yet there is still need for further research on the preservation of zobo drink against invading pathogens like *Staphylococcus aureus.* Therefore this study is designed to evaluate the effect of spices, pH and temperatures on the growth and multiplication of *Staphylococcus aureus.*

MATERIALS AND METHODS

Sample collection: This was carried out using modified method of Iheukwumere and Uzoh (2014). A total of 12 water samples were aseptically collected in sterilized plastic containers in triplicate from various rivers in Uli community, Ihiala L.G.A, Anambra State. The samples were collected from the river by dipping the bottles about 30-50cm deep invertedly into the river and allowed to flow. After collection, the samples were corked and placed in a cooler to maintain the temperature during transportation for laboratory analysis. The samples were analyzed within five hours of collection, and where analysis was to be delayed, samples were refrigerated at 4^0C.

Isolation and identification of *Staphylococcus aureus:* This was carried out by aseptically inoculating 1.0ml of the sample on Mannitol salt agar, using pour plate method and incubated at 37^0C for 48 h. After 48 h incubation the grown colonies were sub-cultured, characterized and identified using their colony descriptions, microscopic and biochemical characteristics (Arora and Arora, 2008).

Sources of the spices and processing of spices: The spices (nutmeg and ginger) were collected from Nkwo ogbe Ihiala market in Ihiala L.G.A. in Anambra State. The Nutmeg and dried Ginger were collected from five (5) different market women. The spices were washed with distilled water and dried under shade at room temperature at 14 days. The spices were aseptically ground using sterile electric grinder into powdered form.

Extraction and phytochemical analysis: A twenty gram (20 g) portion of the powdered spices was extracted by maceration in 200ml of distilled water for 72 h. The resulting extracts were subsequently filtered using wheatman the NO.1 filter paper and evaporated to dryness at room temperature using electric oven at 30^0C. The phytochemical constituents of the spices were determined quantitatively using the method of Iheukwumere and Umedum (2013).

Effects of spices on survival and multiplication of *Staphylococcus aureus*: This was carried out using the modified method of Onuorah and Adekeye (2000). For each spice, ten test tubes each containing 15 ml of zobo drink were used. Six of the test tubes and their contents were sterilized using an autoclave at 121^0C, 15 PSI for 15 minutes, while the remaining four were left as purchased and considered non sterile. Sterile zobo drink in four test tubes were inoculated with 0.1ml of overnight growth of *Staphylococcus aureus*, and 5ml of 1%spice was added to two of the test tubes making an approximate final concentration of 0.25% in zobo drink. Five milliliters of distilled water was added to the remaining to serve as control. In two of the four test tubes containing non sterile zobo drink, 5ml of 1% spice was added while distilled water was added to the remaining two to serve as second control. All test tubes were incubated at room temperature and 1ml were removed from each test tube at 0, 12, 24, 48 and 72 h post inoculation and plated in Mannitol Salt Agar. Plates were incubated at 37^0C for 24 h. The pH of the zobo drink was also measured at intervals. The same procedure was used 5% spice solution which gave a final concentration of 1.25% and 10% spice solution which gave a final concentration of 2.5% in zobo drink for each spice.

Effect of temperature on survival of *Staphylococcus aureus* in zobo drink: This was caused out using the modified method of Ebo*et al*. (2013). Sixty milliliters (60ml) portion of the zobo drink was dispensed each on 250ml flasks. Three flasks with their content were sterilized using an autoclave at 121^0C. 15psi for 15 minutes while the remaining three flasks were left unsterilized as purchased. Two flasks each from sterilized and unsterilized zobo drink were inoculated with 1ml portion of overnight growth culture of *Staphylococcus aureus*while the remaining ones i.e the other remain test tubes of the sterilized and unsterilized zobo drink were added 1ml of distilled water each. The flasks were incubated at 4^0C (refrigerator), 25^0C (room temperature) and 37^0C (incubator). At 0, 12, 24, 48 and 72 h post inoculation, 1ml portion of the zobo drink was removed from each flask and plated on Mannitol Salt Agar (MSA), incubated at 37^0C for 24 hours.

Effect of pH on survival and multiplication of *Staphylococcus aureus*: This was carried out using the modified method of Ebo *et al*. (2013). Sixty milliliters portion of the locally made soya milk was dispensed each in eight 250ml flasks. Four flasks with their content were sterilized using an autoclave at 121^0C, 15psi for 15 minutes while the remaining four flasks were left unsterilized as purchased. Sterile 3N HCl was used to adjust the pH of the two sets of the locally made soya milk (sterile and non-sterile) to pH 3, 4, 5 and 6 respectively. One milliliter of an overnight culture of *Staphylococcus aureus* was inoculated into each of the eight flasks and incubated at 25^0C (room temperature). One milliliter portion of the locally made soya milk was removed from each flask at 0, 12, 24, 48 and 72 h post inoculation for the enumeration of *Stapylococcus aureus* counts on Mannitol Salt Agar (MSA). Changes in pH were also determined. The procedures were repeated by adjusting the pH using sterile 3N NaOH to 7, 8, 9 and 10 respectively.

Statistical Analysis: The data generated from this study were represented as mean ±Standard deviation and then charts. The statistical analysis of data generated from protective study was carried out using students "t" test at 95% confidence limit (Iheukwumere and Umedum, 2013).

RESULTS

The occurance of *Staphylococcus aureus* in stream water samples is shown in Table 1. Out of twelve (12) samples collected from Aloura, Ubahudara and Atamiri streams in Uli community, Ihiala Local Government

Area of Anambra State, 11(91.67%) samples were positive. All samples drawn from stream B and C were positive to *Staphylococcus aureus* whereas 3 samples out of the 4 samples drawn from stream A were positive. Table 2 shows the morphological characteristics of *Staphylococcus aureus* on Mannitol Salt agar plates. *Staphylococcus aureus* was further characterized using its biochemical characteristics and fermentation of certain sugars and sugar alcohols.

This study showed the phytochemical constituents of *Zingiber officinale* and *Myristica* fragrans in Table 3. The study showed that the spices (*Zingiber officinale* and *Myristica fragrans*) were able to show significant ($p<0.05$) protection of the zobo drink against *Staphylococcus aureus* when compared to the positive controls (Table 4 and 5). The positive effects of spices increased significantly ($p<0.05$) as the concentration of the spices used increased. Maximum protection was seen when the concentration of the spices was 10%. No count was recorded at zero (0) hour and after 24 h among the sterilized samples protected with the spices. Also the number of counts recorded increased significantly ($p<0.05$) as the time increased and *Zingiber officinale* (ZO) protected the zobo drink samples against *Staphylococcus aureus* than *Myristica fragrans* (MF) among the sterilized samples. In non-sterilized samples *Myristica fragrans* showed more protection than ZO at their one percent (1%) concentration but the protective effect of ZO became more pronounced than that of MF at their 5% and 10% concentration. The spices protected the sterilized zobo drink than non-sterilized zobo drink, and the (blank control) recorded zero growth of *Staphylococcus aureus* after 72 h whereas non-sterilized sample (blank control) showed significant counts of *Staphylococcus aureus* after 72 h.

The study showed that among the sterilized samples, no growth was observed at 4^0C. At 25^0C, 5 colonies were recorded after 72 h whereas at 37^0C, significant colonies were recorded after 48 h and 72 h (Table 6 and 7). No growth was recorded from sterilized (Blank control) samples where as significant number of colonies were recorded from sterilized (Positive control) samples. Among the non-sterilized samples, no growth was recorded after 0 h among the test samples where as significant number of colonies were recorded from both non-sterilized (Blank control) and non-sterilized (Positive control) samples. Maximum growth was observed at 37^0C for both sterilized and non-sterilized samples whereas the least growth was observed at 4^0C. The inhibitory effect at 4^0C was significant ($p< 0.05$) most when compared to 25^0C, 37^0C and positive control. The study showed that among the sterilized samples, no growth was observed except at pH 6 and that of positive control (inoculated sterilized samples without pH adjustment) (Tables 8 and 9). Similar, results were recorded for non-sterilized zobo drink samples, only that the negative control (blank) also showed significant growth.

Table 1: Occurrence of *Staphylococcus aureus* in stream water sample in Uli community

	N=12		
Stream sample	Positive sample (%)	Negative sample (%)	Total sample (%)
A	3(25)	1(8.33)	4(33.33)
B	4(33.33)	0(0)	4(33.33)
C	4(33.33)	0(0)	4(33.33)
Total	11(91.67)	1(8.33)	12(100)

N = Total number of water samples
A =Aloura
B = Ubahudara
C= Atamiri

Table 2: Characteristics and identity of *Staphylococcusaureus*

Parameter	*Staphylococcus aureus*
Appearance on mannitol salt agar	Golden yellow
Elevation	Raised
Edge	Smooth
Gram reaction	+
Morphology	Coccus
Motility	-
Catalase	+
H_2S	-
Citrate	-
VP	-
MR	-
Oxidase	-
Coagulase	+
Lactose	+/-
Galactose	+
Inositol	-
Xylitol	-
Mannitol	+
Dulcitol	+
Sorbitol	-
Maltose	+

VP = Voger Proskauer
MR = Methyl red
H2S = Hydrogen Sulphide

Table3: Phytochemical constituents of the spices

Parameter	*Zingiber officinale* (mg/ 100g)	*Myristica fragrans* (mg/100g)
Alkaloids	10.12	3.17
Tannins	4.38	0.64
Saponins	0.81	1.58
Phenolics	1.32	0.92
Steroids	0.02	0.04
Glycosides	1.08	0.32
Flavonoids	5.62	1.82

Table 4: Effect of spices on the survival and multiplication of Staphylococcus aureus on sterilized zobo drink

	Count(CFU/ml)			
Spice	0 h	24 h	48 h	72 h
ZO (1%)	0	0	12	32
ZO (5%)	0	0	14	20
ZO (10%)	0	0	0	8
MF (1%)	0	0	22	38
MF (5%)	0	0	20	24
MF (10%)	0	0	0	12
C_1	0	0	0	0
C_2	0	22	35	68

ZO= *Zingiber officinale* (Ginger)
MF= Myristica fragrans (Nutmeg)
C_1= Sterilized sample
C_2= Strerilized sample inoculated with the test isolate

Table 5: Effect of spices on the survival and multiplication of *Staphylococcus aureus* on non-sterilized zobo drink

	Count(CFU/ml)			
		24 h		
Spice	0 h		48 h	72 h
ZO (1%)	0	15	25	32
ZO (5%)	0	2	10	25
ZO (10%)	0	0	5	12
MF (1%)	0	14	20	28
MF (5%)	0	5	15	27
MF (10%)	0	0	12	18
C_1	24	28	35	48
C_2	32	40	48	50

ZO=*Zingiber officinale* (Ginger)
MF= Myristica fragrans (Nutmeg)
C_1= Sterilized sample
C_2= Strerilized sample inoculated with the test isolate

Table 6: Effect of temperature on the survival and multiplication of *Staphylococcus aureus* on sterilized zobo drink

	Count(CFU/ml)			
Temperature (^{0}C)	0 h	24 h	48 h	72 h
4	0	0	0	0
25	0	0	0	5
37	0	0	10	25
C_1	0	0	0	0
C_2	0	24	30	54

C_1= Sterilized sample
C_2= Strerilized sample inoculated with the test isolate

Table 7: Effect of temperature on the survival and multiplication of *Staphylococcus aureus* on non-sterilized zobo drink

	Count(CFU/ml)			
		24 h		
Temperature (^{0}C)	0 h		48 h	72 h
4	0	0	5	10
25	0	8	14	20
37	0	12	18	28
C_1	20	30	35	40
C_2	28	37	44	58

C_1= Sterilized sample
C_2= Strerilized sample inoculated with the test isolate

Table 8: Effect of pH on the survival and multiplication of *Staphylococcus aureus* on sterilized zobo drink

pH	Count(CFU/ml) 0 h	24 h	48 h	72 h
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	0	3	7	15
7	0	0	0	0
8	0	0	0	0
9	0	0	0	0
10	0	0	0	0
C_1	0	0	0	0
C_2	0	22	35	0

C_1= Sterilized sample
C_2= Strerilized sample inoculated with the test isolate

Table 9: Effect of pH on the survival and multiplication of *Staphylococcus aureus* on non-sterilized zobo drink

pH	Count(CFU/ml) 0h	24 h	48 h	72 h
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	0	10	17	20
7	0	0	0	0
8	0	0	0	0
9	0	0	0	0
10	0	0	0	0
C_1	22	25	35	48
C_2	32	44	47	51

C_1= Sterilized sample
C_2= Strerilized sample inoculated with the test isolate

DISCUSSION

The presence of *Staphylococcus aureus* in the studied streams could be traced from the fact that people swim, wash and bath in those streams. *Staphylococcus aureus* has earlier been isolated from stream samples according to Iheukwumere and Uzoh (2014).

The phytochemical analysis of *Zingiber officinale* and *Myristica fragrans* revealed the presence of alkaloids, tannins, saponins, phenolics, steroids, glycosides and flavonoids. These phytochemical constituents could be responsible for the antimicrobial avtivities of the extracts (Iheukwumere and Umedum, 2013)

The pronounced activities of the spices against *Staphylococcus aureus* could be attributed to the phytochemical constituents of the spices. Similar conclusion was drawn by Iheukwumere and Umedum (2013). *Zingiber officinale* proved to inhibit *Staphylococcus aureus* than *Myristica fragrans* at higher concentrations, due to the potency of the phytochemical constituents present in *Zingiber officinale* as reported by Iheukwumere and Umedum (2013. It is therefore evident that *Zingiber officinale* as a spice is recommended in the production of zobo drink due to its anti-microbial effect on *Staphylococcus aureus* (Adesokan *et al*., 2013).

The insignificant growth of *Staphylococcus aureus* observed at 4^0C could be due to the fact that the organism survives in the temperature range of 7^0C – 48^0C as reported by Donlan and Costerton (2002). The growth observed at 37^0C could be due to the fact that *Staphylococcus aureus* grows optimally at 37^0C (Agelolloti *et al*., 2000). It also was observed that *Staphylococcus aureus* did not survive in the alkalinity range but survived

in the acidity range and showed significant growth at pH 6, though the normal pH of the zobo drink was ranged 3-4. This could be due to the fact that the pH at which *Staphylococcus aureus* survives optimally ranges from 6-7 (Donlan and Costerton, 2002). This study showed that the pH of the zobo may inhibit the growth of *Staphylococcus aureus* if contaminated by the organism since the pH is not conducive for the growth of the organism.

CONCLUSION

This study has shown that the growth of *Staphylococcus aureus* (contaminant introduced from water sample) in zobo drink could be controlled using *Zingiber officinale* and *Myristica fragrans* extracts (preferably *Zingiber officinale*) at pH values other than 6, and should best be stored and consumed at refrigeration temperature (4^0C).

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