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EFFECT OF SPICES, pH AND TEMPERATURE ON THE SURVIVAL AND MULTIPLICATION OF *SALMONELLA* SPECIES IN ZOBO DRINK

Chude, Charles[1]. Chinwe C. Ejike.[2] and Michael Ikechukwu Nwike.[3]

1. Department of Microbiology, Faculty of Natural Sciences, Chukwuemeka Odumegwu Ojukwu University, Anambra State, Nigeria

2. Department of Medical Microbiology, Chukwuemeka Odumegwu Ojukwu University

3. Nwafor Orizu College of Education, Anambra State, Nigeria

* E-Mail of the corresponding author: ikpower2007@yahoo.com

ABSTRACT

This study was carried out to investigate the effects of spices, pH and temperature on the survival and growth of *Salmonella* species 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 *Salmonella* species 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 (*Zingiber 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 *Salmonella* species. The spices showed pronounced activity against the organism in sterilized and non- sterilized samples of which the activity increased significantly ($P<0.05$) as the concentration increased. The activity of *Zingiber 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. No growth was observed little or no growth at 4^0C. This study has shown that the growth of *Salmonella* species in zobo drink could be controlled using *Zingiber officinale* and *Myristica fragrans* extracts at pH values other than 6, and should best be sold and consumed at refrigeration temperature (4^0C).

Key words: *Salmonella*, zobo drink, *Zingiber officinale*, *Myristica fragrans*

INTRODUCTION

Zobo drink, a non-alcoholic local beverage, is produced from the dried petals of *Hibiscus sabdariffa*. It is locally called “Zoborodo” (Hausa), “Isapa” (Yoruba) and Sorrel in English and is a delicacy in many parts of Nigeria. The *H. sabdariffa* plant, commonly known as Roselle, while native to India and Malaysia is now found in many tropical and subtropical countries of Africa, Asia and the Americas (Adegunloye *et al.*, 2006).It is a dicotyledonous plant belonging to the subclass *Archichlamydea*, Order Malvale and Family, Malvaceae. Zobo drink is prepared first by boiling the dried leaves of the Roselle, followed by cooling and filtration. The filtrate, which is red in colour, is sometimes sweetened to taste with pineapple, orange or sugar and spiced up with ginger. It is further allowed to cool and is best served chilled Zobo is thus a drink available in many local stores where it is marketed in various forms and patronized by a variety of people. Some of its nutritive elements are various amino acids, proteins, carbohydrate, vitamins, and fats among other. Medicinal value of aqueous extracts from the Roselle plant has been reported to include anti-hypertensive, antiseptic astringent diuretic and purgative activities remedy for cancer, abscesses, cough, dysuria, laxative, scurvy and fever. A 2008 United States Department of Agriculture (USDA) study said consuming hibiscus tea or zobo drink lowered blood pressure in a group of pre-hypertensive and mildly hypertensive adults. According to the Journal of Ethno pharmacology, which was published May 2008, hibiscus tea improved the kidney’s ability to filter out waste products which are the uric and oxalic acid, both of which can form kidney stones if they accumulate to excessively high levels (Doughari *et al.*, 2007).

The water used is often obtained from the local water sources often boreholes, streams and in some cases well water is used for preparation. It is these conditions that result in the microbial contamination of the product, coupled with contamination that may result from handling (Oboh and Elusiyan, 2004).

Zobo drink is liable to microbial spoilage if not adequately stored and could act as an important medium for the transmission of pathogenic microorganisms. Many organisms can use the carbohydrate content for their fermentation processes producing undesirable changes in them. The sugar used as a sweetening agent could also contribute to these changes. The microorganisms can be in dormant or semi-metabolic changes, but others are of public health significance since the microbes may be potential pathogens or can produce toxins in food, which can cause illness to consumers Salmonellosis is a very important public health hazard often resulting in high global mortality rate. Salmonellosis is a classic food-borne disease in relative to *S.enterica* serovar Enteritidis in some ingredients, such as sweetening agents and spices have been used for long, as food additives to provide distinctive flavors for foods and beverages around the world. However, the spices have been found to have contaminants the serotypes of *salmonella* responsible for enteric fever typhi and para-typhi account for 6-20 million eases and 200,000 deaths annually, especially in the tropics including Nigeria. (Akinyosoye and Akinyele, 2000)

In general, infections caused*by Salmonella* causes great economic losses in both developed and developing countries. For Salmonella to cause infection in the host, it has to overcome the host immune factors which include gastric acidity, normal intestinal micro-flora and local intestinal immunity. Where the organism is able to overcome the host factors enteric fever may result. The severity of salmonellosis, which may result in death for some individuals means that manufacturers need to detect contamination before food is released for sale. There is no regulation on the preparation of these foods despite the wide patronage of the people. It might also provide decision makers with information that will help to initiate necessary standards for these and other indigenous drinks that have found their ways to the Nigerian markets. It might also help Nigerians in making choices as to the drinks they patronize (Kreb *et al*., 2000).

MATERIALS AND METHODS

Study Area: Uli is a town located between latitudes 5.47ºN and 5.783ºN and longitudes 6.52ºE and 6.87ºE on the south eastern part of Nigeria. Uli extends west ward to the confluence of the rivers of Atammiri and Eyinja, and across Ushamlake down to the lower Niger region. Uli has rainforest vegetation with two seasonal climatic conditions: the rainy season and the dry season which is characterized by the harmattan between December and February. Uli is characterized by double maxima of rainfall with a slight drop in either July or August known as dry spell or August break. The annual total rainfall is about 1600 mm with a relative humidity of 80% at dawn (UN-HABITAT, 2009).

Sample Collection: This was carried out using modified method of Iheukwumere and Umedum (2014). A total of 12 water samples were aseptically collected in sterilized plastic containers in triplicate from 3 streams used in Uli community, Ihiala L.G.A, Anambra State. The plastic containers were thoroughly washed with detergent and soaked overnight with sodium hypochlorite solution and finally rinsed with distilled water three times. Water samples were collected by lowering the sterile plastic container inside the water body, 30cm deep, allowed to overflow before withdrawing the water sample. Six sampling points were used and the sampling points were approximately 100m away from one another. After collection, the samples were covered and placed in a cooler to maintain the temperature during transportation for laboratory analysis. All samples were analyzed within 5 h of collection and where analyses were to be delayed, samples were refrigerated at 4ºC.

Isolation and Identification of *Salmonella* species:Different strains of the organism used for this work were isolated from boreholes and streams in Uli community. This was carried out by aseptically inoculating 1.0 ml of the water samples on Salmonella-Shigella agar using the pour plate method and incubate at 44.5ºC for 24 h. After 24 h of incubation, the grown colonies were subcultured, characterized and identified using the colony descriptions, microscopic and biochemical characteristics (Arora and Arora, 2008).

Sources of the Spices: The spices (nutmeg and ginger) were collected from Nkwoogbe Ihiala market in Ihiala L.G.A. in Anambra state. The Nutmeg and dried Ginger were collected from five (5) different market women

Processing of Spices: 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 (20g) portion of the powdered spices was extracted by maceration in 200ml of distilled water for 72hours. The resulting extracts were subsequently filtered using whatman 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 *et al*. (2012).

Effects of spices on survival and multiplication of *Salmonella* species: This was carried out using the modified method of Onuorah and Adekeye (1987). For each spice, ten test tubes each containing 15ml 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 *Salmonella species.*, 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 millilitres 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 hours post inoculation and plated in Salmonella Shigella Agar Plates were incubated at 37^0C for 24 hours. 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 *Salmonella* Species in Zobo Drink: This was caused out using the modified method of Ebo *et al*. (2013).Sixty millilitres (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 *Salmonella species* 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 post inoculation, 1ml portion of the zobo drink was removed from each flask and plated on Salmonella Shigella Agar, (SSA), incubated at 37^0C for 24 hours.

Effect of pH on Survival and Multiplication of *Salmonella* Species: This was carried out using the modified method of Ebo *et al*. (2013).Sixty millilitres portion of the locally made zobo drink 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 zobo drink (sterile and non-sterile) to pH 3, 4, 5 and 6 respectively. One milliliter of an overnight culture of *Salmonella* species was inoculated into each of the eight flasks and incubated at 25^0C (room temperature). One milliliter portion of the locally made zobo drink was removed from each flask at 0, 12, 24, 48 and 72 hours post inoculation for the enumeration of *Salmonella* species counts on Salmonella Shigella Agar, (SSA). 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.

RESULTS

The occurrence of *Salmonella* species 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(91.66%) samples were positive. All samples drawn from stream B and C were positive to *Salmonella* species whereas 3 samples out of the 4 samples drawn from stream A were positive. Table 2 shows the morphological characteristics of *Salmonella* species on Salmonella Shigella Agar plates. *Salmonella* species was further characterized using its biochemical characteristics and fermentation of certain sugars and sugar alcohols. *Salmonella* species appeared dark on the Salmonella Shigella agar plates with smooth edge and raised elevation. The isolate also reacted positive to Gram test, catalase test and coagulase test; it reacted negative to motility test, H2S, citrate, VP, MR and oxidase tests. *Salmonella* species was able to ferment lactose, galactose, mannitol and maltose sugars, and was unable to ferment inositol, xylitol and sorbitol sugars.This study showed the phytochemical constituents of *Zingiber officinale* and *Myristica* fragrans in Table 3. The phytochemical analysis of *Zingiber officinale* and *Myristica f ragrans* revealed the presence of alkaloids, tanins, saponins, phenolics, steroids, glycosides and flavonoids. 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 *Salmonella* species when compared to the positive controls. 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 *Salmonella* species 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 *Salmonella* species after 72 h whereas non-sterilized sample (blank control) showed significant counts of *Salmonella* species 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. No growth was recorded from sterilized (Bank control) samples whereas significant numbers of colonies were recorded from sterilized (Positive control) samples. Among the non-sterilized samples, no growth was recorded after 0h among the test samples whereas significant number of colonies was 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; the negative control (blank) also showed significant growth.

Table 1: Occurrence of *Salmonella* species in stream water wamples in Uli Community.

Stream sample	Positive sample (%)	n=12 Negative sample (%)	Total sample (%)
A	4 (33.33)	0(0)	4(33.33)
B	4(33.33)	0(0)	4(33.33)
C	2(16.67)	2(16.67)	4(33.33)
Total	10	2 (16.67)	12(100)

n = total number of water samples A = Atammiri B = Ubahudara C = Aluora

Table 2: Characteristics and Identity of *Salmonella enterica ser.* Typhimurium

Parameter	*S.enterica ser.* Typhimurium
Appearance on SS-agar	colorless and dark at the center
Elevation	raised
Edge	entire
Gram reaction	−
Morphology	rod
Motility	+
Catalase	+
H_2S production	+
Indole	−
Citrate	+
VP	−
MR	+
Oxidase	−
Lactose	−
Galactose	+
Inositol	−
Xylitol	+
Mannitol	+
Dulcitol	−
Sorbitol	+
Maltose	+

SS-Agar = *Salmonella-Shigella*-Agar, VP = Voges proskauer

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 *Salmonella Species* on Sterilized Zobo Drink

	Count(CFU/ml)			
Spice	0 h	24 h	48 h	72 h
ZO (1%)	0	0	10	30
ZO (5%)	0	0	12	18
ZO (10%)	0	0	0	7
MF (1%)	0	0	20	30
MF (5%)	0	0	22	25
MF (10%)	0	0	4	10
C_1	0	0	0	0
C_2	0	20	30	56

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 *Salmonella* species on non-sterilized zobo drink.

	Count(CFU/ml)			
Spice	0 h	24 h	48 h	72 h
ZO (1%)	0	12	20	30
ZO (5%)	0	1	7	21
ZO v (10%)	0	0	3	10
MF (1%)	0	8	15	20
MF (5%)	0	3	9	23
MF (10%)	0	0	11	15
C_1	16	20	28	35
C_2	30	41	42	47

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 *Salmonella* species on sterilized zobo drink

Temperature (^{0}C)	Count(CFU/ml)			
	0 h	24 h	48 h	72 h
4	0	0	0	0
25	0	0	0	3
37	0	0	8	20
C_1	0	0	0	0
C_2	0	20	25	48

C_1= Sterilized sample incubated at 4^0C, C_2= Sterilized sample inoculated with the test isolate

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

Temperature (^{0}C)	Count(CFU/ml)			
	0 h	24 h	48 h	72 h
4	0	0	0	0
25	0	2	6	11
37	0	10	16	26
C_1	10	15	18	27
C_2	27	35	42	50

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

Table 8: Effect of pH on the survival and multiplication of *Salmonella* species 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	5	12
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	37	53

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

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

			Count(CFU/ml)	
pH	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	0	4	11
7	0	5	7	15
8	0	0	0	4
9	0	0	0	0
10	0	0	0	0
C_1	0	0	0	0
C_2	12	17	20	25

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

DISCUSSION

A thirst quenching beverage zoborodo popularly called zobo can be free from microbial contamination especially from organisms like *Salmonella* species, which is a gram positive, non-spore forming, facultative anaerobic, spherical and non-motile bacterium, when processed and packaged under hygienic conditions. *Salmonella* speciescontamination in zobo drink could possibly be through the processing method which usually involves the use of hands. The organism is responsible for staphylococcal food poisoning which appears around 3hours after ingestion, with common symptoms like vomiting, abdominal cramp, nausea and diarrhea. In severe cases, symptoms like headache, muscle cramp and transient changes in blood pressure and pulse rate may occur (Lowy, 2000; Alo et al., 2012). This study revealed that eleven samples(11) out of twelve (12) stream samples collected from three(3) streams in Uli community, Ihiala Local Government Area of Anambra state showed the occurrence of *Salmonella* speciesin the streams as seen in Table 1. The presence of *Salmonella* speciesin those streams could be traced from the fact that people swim, wash and bath in those streams. *Salmonella* specieshas earlier been isolated from stream samples according to (Iheukwumere and Uzoh, 2014).

The phytochemical analysis of *Zingiberofficinale* and *Myristicafragrans* revealed the presence of alkaloids, tannins, saponins, phenolics, steroids, glycosides and flavonoids. These phytochemical constituents could be responsible for the antimicrobial activities of the extracts (Iheukwumere and Umedum, 2013)

The spices *Zingiberofficinale* and *Myristicafragrans* showed pronounced activity against *Salmonella* specie. This could be attributed to the phytochemical constituents of the spices. Similar conclusion was drawn by (Iheukwumere and Umedum, 2013). *Zingiberofficinale*proved to inhibit *Salmonella* speciesthan *Myristicafragrans* at higher concentrations. This could be attributed to the potency of the phytochemical constituents present in *Zingiberofficinale* as reported by (Iheukwumere and Umedum, 2013) It is therefore evident that *Zingiberofficinale*as a spice is recommended in the production of zobo drink due to its anti-microbial effect on *Salmonella* species.(Adesokan*et al*., 2013).

The three temperatures (4, 25 and 37)^{0}C used in the study are temperature to which zobo drink is exposed to during refrigeration storage, at room temperature by hawkers and during sale in hot weather when zobo drink is most popular (Ebo*et al*., 2013). The study revealed that with zobo drink kept at refrigeration temperature, the population of contaminating *Salmonella* speciesis insignificant in the zobo drink compared to the number of

growth observed at 25^0C and 37^0C. The number of growth observed at 37^0C was dominant than that of the growth observed at 25^0C. The decline at 4^0C could be due to the fact that *Salmonella* speciessurvives in the temperature range of (7^0C – 48^0C) as reported byAgelollotiet *al*.(2000). The growth observed at 37^0C could be due to the fact that *Salmonella species*grows optimally at 37^0C (Agelollotiet *al*., 2000)

The effect of pH was studied at pH 3, 4, 5,6 ,7 ,8 ,9 and 10 i.e pH 3-6 representing acidity range and pH 7- 10 representing alkalinity range (Eboet *al*., 2013). It was observed that *Salmonella* speciesdid 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 *Salmonella* speciessurvives optimally ranges from 6-7 (Paniker, 2006). This study revealed that the pH of the zobo may inhibit the growth of *Salmonella* species, if contaminated by the organism as the pH is not conducive the organism. The consumers of the zobo drink may consume at refrigeration temperature which doesn't support the growth and multiplication of *Salmonella* species. Also, addition of spices in conjunction with good sanitary practices may be useful providing a good safety margin for zobo drink which might have been contaminated during preparation, sale and storage (Onuorah et al., 2000).

CONCLUSION

This study has revealed the presence of *Salmonella* species in samples collected from atamiri, ubahudara and aroura streams in Uli community, Ihiala Local Government Area of Anambra state of which *Salmonella* specieswas indicated in 91.67% of the samples collected. This study has shown that the growth of *Salmonella* species inzobo drink could be controlled using *Zingiberofficinale* and *Myristicafragrans* extracts at pH values other than 6, and should best be sold and consumed at refrigeration temperature (4^0C).

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