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Effect of Spices, pH and Temperature on the Survival and Multiplication of Staphylococcus aureus in Locally Made Soya Milk 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 Soya milk 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 Soya milk 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

Soya milk (Doujiang) originated in China, probably during the early Han dynasty (202 BCE to 9 CE) after the rotary millstobne was introduced and became widely used to grind wheat. It did not become widely used in China until the 1800s, when it was discovered that extended heating made it taste better and easier to digest (Frazier and Westhoff, 2010). Soya milk is an inexpensive and remarkably versatile high protein food made from soya beans. It is a white liquid made from the seed. Unlike most other protein foods, milk is entirely free from cholesterol and low in fat (especially saturated fats). The quality of protein is as high as that found in chicken. It is also good for dieters as this contains low calories. It is an excellent food for babies, children, elderly people and pregnant and lactating women since it contains vegetable protein which is very nutritious and easy to digest (Lawrence *et al*., 2016).

Soy milk is naturally lower in sugar content than regular milk. Cow's milk has about 12 grams of sugar per cup as opposed to only 7 grams in soy milk. This is why a cup of whole soy milk has only 80 calories, which is the equivalent of skim milk. In addition, the monounsaturated fatty acid in soy milk can inhibit your intestinal absorption of fat, which is another great advantage for weight loss (Ihekonye and Ngoddy, 2004). Drinking soy milk also gives you an extra dose of fiber, keeping your feeling fuller for longer time. Soy milk is a rich source of phytoestrogen, a unique plant hormone that can inhibit the production of testosterone in men (Riaz, 2006). Reduced testosterone levels can significantly cut the risk of prostate cancer. Studies have shown that men who eat a soy-rich diet are less likely to develop prostate hypertrophy or prostate cancer. During menopause, a woman's natural production of estrogen drops to a minimum. The sudden reduction of estrogen creates a number of health problems for postmenopausal women. Postmenopausal women have higher risks of heart disease, diabetes and obesity. They are also more vulnerable to depression, mood swings, insomnia and other psychological disorders. The phytoestrogen in soy is an effective estrogen replacement. Regular intake of soy is a great way to prevent and alleviate these postmenopausal syndromes (Laswai *et al*., 2009).

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 and potential contaminants of the drink by organisms like *Staphylococcus aureus* and is an increased risk to public health (Palacios *et al*., 2009) which can cause bloody diarrhea, fever, abdominal cramps and vomiting.This study was undertaken to determine the effect of spices, pH and temperature on the survival and multiplication of

Staphylococcus aureus in locally made soya milk drink

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 30-50 cm 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 (5 h) of collection, and where analysis was to be delayed, samples were refrigerated at 4^0C.

Isolation and identification of *Staphylococcus aureu*: 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 subcultered, characterized and identified using their colony descriptions, microscopic and biochemical characteristics.

Sources of the spices and Processing of 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. 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. A twenty gram (20 g) portion of the powdered spices was extracted by maceration in 200 ml 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) and Ogiehor *et al*. (2008). For each spice, ten test tubes each containing 15ml of soya milk 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 soya milk 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 soya milk 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 soya milk 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 1 ml were removed from each test tube at 0, 12, 24, 48 and 72 hours post inoculation and plated in Mannitol Salt Agar. Plates were incubated at 37^0C for 24 hours. The pH of the soya milk 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 soya milk drink for each spice.

Effect of temperature on survival of *Staphylococcus aureus* in soya milk drink: This was caused out using the modified method of Onuorah and Adekeye (2000) and Ogiehor *et al*. (2008). Sixty millilitres (60 ml) portion of the soya milk drink was dispensed each on 250 ml flasks. Three flasks with their content were sterilized using an autoclave at 121^0C. 15 psi for 15 minutes while the remaining three flasks were left unsterilized as purchased. Two flasks each from sterilized and unsterilized soya milk 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 soya milk drink were added 1ml of distilled water each. The flasks were incubated at 4^0C (refrigerator), 25^0C (room tempetrature) and 37^0C (incubator). At 0, 12, 24, 48 and 72 post inoculation, 1ml portion of the soya milk drink was removed from each flask and plated on Mannitol Salt Agar (MSA), incubated at 37^0C for 24 h.

Effect of pH on survival and multiplication of *Staphylococcus aureus*: This was carried out using the modified method of Onuorah and Adekeye (2000) and Ogiehor *et al*. (2008). Sixty millilitres portion of the locally made soya milk was dispensed each in eight 250 ml flasks. Four flasks with their content were sterilized using an autoclave at 121^0C, 15 psi 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 millilitre of an overnight culture of *Staphylococcus aureus* was inoculated into each of the eight flasks and incubated at 25^0C (room temperature). One millilitre 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 statistical analysis of the valuable data generated from this study was examined using SPSS package program version 20.0. Data were analyzed by one-way Analysis of Variance (ANOVA) to determine the significant difference of the mean values at 95% confidence limit. Pair wise comparison of mean

was done by Least Significant Difference (LSD) (Iheukwumere and Umedum, 2013).

RESULTS

Occurance of *Staphylococcus aureus* in stream water sample in Uli, community: 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(91.66%) samples were positive.

Characteristics and identity of the isolate : Table 2 shows the morphological characteristics of *Staphylococcus aureus* on Mannitol Salt agar plates.

Phytochemical constituents of the spices: This study showed the phytochemical constituents of *Zingiber officinale* and *Myristica* fragrans in Table 3. The phytochemical analysis of *Zingiber officinale* and *Myristica fragrans* revealed the presence of alkaloids, tanins, saponins, phenolics, steroids, glycosides and flavonoids.

The effects of spices on the survival and multiplication of *Staphylococcus aureus* in sterilized and non sterilized soyamilk drink: The study showed that the spices (*Zingiber officinale* and *Myristica fragrans*) were able to show significant ($p<0.05$) protection of the soya milk drink against *Staphylococcus aureus* 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 soya milk 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 soya milk drink than non-sterilized soya milk 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 effects of temperature on the survival and multiplication of Staphylococcus *aureus* in sterilized and non-sterilized soyamilk: The study showed that among the sterilized samples, no growth was observed at 4^0C. At 25^0C, 5 colonies were recorded after 72h whereas at 37^0C, significant colonies were recorded after 48h and 72h. 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 0 h 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 effects of pH on survival and multiplication of S.aureus on sterilized and non-sterilized soyamilk: The study showed that among the sterilized samples, no growth was observed except at pH6 and that of positive control (inoculated sterilized samples without PH adjustment). Similarly results was recorded for non-sterilized soya milk 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= Atamir

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))
Alkaloids	10.12
Tannins	4.38
Saponins	0.81
Phenolics	1.32
Steroids	0.02
Glycosides	1.08
Flavonoids	5.62

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

Spice	0h	24h	48h	72h
ZO (1%)	0	0	10	30
ZO (5%)	0	0	12	24
ZO (10%)	0	0	0	4
C_1	0	0	0	0
C_2	0	20	35	50

ZO= *Zingiberofficinale* (Ginger)
MF= Myristicafragrans (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 soyamilk drink.

Spice	0h	24h	48h	72
ZO (1%)	0	12	28	30
ZO (5%)	0	3	15	20
ZO (10%)	0	0	4	16
C_1	25	28	39	40
C_2	35	45	49	60

ZO=*Zingiberofficinale* (Ginger)
MF= Myristicafragrans (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 soyamilk drink

Temperature (^{0}C)	0h	24h	48h	72h
4	0	0	0	0
25	0	0	0	4
37	0	0	10	20
C_1	0	0	0	0
C_2	0	28	35	40

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 soya milk drink

Temperature (^{0}C)	0h	24h	48h	72h
4	0	0	4	8
25	0	5	10	15
37	0	15	18	25
C_1	20	32	38	40
C_2	30	45	48	56

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 soya milk drink

PH	0h	24h	48h	72h
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	0	2	5	10
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	38	49	58

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 soya milk drink

PH	0h	24h	48h	72h
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	0	10	15	20
7	0	0	0	0
8	0	0	0	0
9	0	0	0	0
10	0	0	0	0
C_1	20	25	30	48
C_2	30	48	49	59

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

DISCUSSION

The presence of *S.aureus* in the studied streams could be traced from the fact that people swim, wash and bath in those streams. *S.aureus* has earlier been isolated from stream samples according to Iheukwumere and Uzoh (2014).The phytochemical constituents present in the studied spices could be responsible for the antimicrobial activities of the extracts (Iheukwumere and Umedum, 2013). *Zingiber officinale* proved to inhibit *S. aureus* than *Myristica fragrans* at higher concentrations. This could be attributed 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 soya milk drink due to its anti-microbial effect on *S.aureus.* (Rehman *et al*., 2007; Ogiehor *et al*., 2008).

The insignificant growth of *S. aureus* at 4^0C could be due to the fact that S.*aureus* survives in the temperature range of (7^0C – 48^0C) as reported by Agelotti *et al*. (2001). The growth observed at 37^0C could be due to the fact that *S.aureus* grows optimally at 37^0C (Agelotti *et al*., 2001). It was observed that *S.aureus* did not survive in the alkalinity range but survived in the acidity range and showed significant growth at pH 6. This could be due to the fact that the pH at which *S.aureus* survives optimally ranges from 6-7 (Torres, 2008).

CONCLUSION

This study has shown that the growth of *S.aureus* in soya milk could be controlled using *Zigiber officinale* extracts at pH values other than 6 and 7, and should best be sold and consumed at refrigeration temperature (4^0C).

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