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Full Length Research Paper

A survey of the microflora of *Hibiscus sabdariffa* (Roselle) and the resulting "Zobo" juice

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The dried calyx of *Hibiscus sabdariffa* (Roselle) is locally processed into a non-alcoholic drink known as 'Zobo' in Nigeria. This popular drink is quite cheap compared to other bottled soft drinks but its acceptability is still limited because of its very short shelf life (24 h at room temperature). The composition and numbers of the microflora of the dried calyx of the Roselle plant and its resulting juice (Zobo) were examined using standard microbiological methods. The dried calyx obtained from a retail market was processed into juice that was compared with commercially sold (retail) juice. The microorganisms isolated from the dried calyx and the juices included the fungi, *Aspergillus niger, Aspergillus flavus, Rhizopus oligosporus, Penicillium citrinum, Mucor* spp., *Saccharomyces cerevisiae,* and *Candida krusei,* while *Bacillus subtilis*, *Pseudomonas* spp., *Staphylococcus aureus, Streptococcus faecalis, Escherichia coli, Proteus mirabilis, Serratia* spp., *Lactobacillus brevis* and *Lactobacillus fermentum* represented the bacterial isolates. Viable counts ranged from 0.4 x 10^4 to 3.2 x 10^4 cfu/ml. Aerobic and anaerobic bacterial counts were higher in the retail juice (1.1 to 2.2 x10^4 cfu/ml) than in the laboratory-prepared juice (0.8 to 1.4 x10^4 cfu/ml) while the dried calyx had the highest fungal counts of 3.2 x 10^4 cfu/ml. pH of the juices ranged between 2.67 and 2.77 while total titratable acidity values were between 0.02 and 0.08.

Key words: Dried calyx, microflora, viable counts, Zobo juice.

INTRODUCTION

Hibiscus sabdariffa (Roselle) is a vegetable plant of West African origin (Tindall, 1983; David and Adam, 1988) being widely cultivated in West Africa, Asia, Austria and many tropical countries. It has the most widespread acceptance in the Roselle producing areas of the Nigerian Savanna region where it is grown as a vegetable crop (Ibrahim et al., 1998). The Roselle is an annual herbaceous, upright plant growing up to two metres belonging to the family Malvaceae. Its habitat is variable and the leaves also vary in shape and size. The flowers are usually yellowish sometimes occurring with dark red pigmentation at the center (Rice et al., 1990).

The fruits are up to 2.5 cm in length and are surrounded by enlarged fleshy calyxes containing 22-34 seeds per capsule. The seed is dark brown in color, 4-6 cm long and about 0.025 g in weight (Tindall, 1983). Two botanical types of Roselle are recognized. *Sabdariffa var altissima* (a tall, vigorous, practically un-branched plant with fibrous spicy and inedible calyxes mainly cultivated for fiber and *H. sabdariffa var sabdariffa* (a bushy, branched sub-shrub with red or green stem and red to yellow inflate edible calyx (Kocchar, 1981). The young shoots and leaves of the latter Roselle variety are usually cooked and eaten as vegetables while the fleshy, swollen red calyxes and the flowers are used to color and season other foods as well as in the preparations of a fruit drink called 'Zobo' in Nigeria.

The name 'Zobo' is derived from the local Hausa (Northern Nigeria) name for the Roselle plant that is, 'Zoborodo'. The calyx contains about 8.3% moisture, 4% citric acid, 1.5% pigment (mainly anthocyanin), 6.9% protein and about 9% soluble solids with a pH of about

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2.7 (Roy, 1987; Adenipekun, 1998). 'Zobo' is an indigenous non-alcoholic drink made from a hot water extract of Roselle calyxes. The extract is usually sweetened with sugar and may be flavored with other materials such as ginger, pineapple and strawberry. It is quite popular in Northern Nigeria enjoying patronage at various social gatherings and 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 whose concentrates are mostly imported constituting a drain on the economy.

In spite of the increasing popularity of Zobo juice, one of its greatest limitations for large-scale production is that it deteriorates rapidly. In fact, its shelf life is approximately twenty-four hours following production if not refrigerated. There is dearth of information on the micro-flora associated with both the dried calyx and the resulting Zobo juice, which in addition to other factors could contribute to its spoilage. The aim of the present study was therefore to investigate the microorganisms present in the dried calyx and the resulting Zobo juice with a view to creating a database for research in improving the shelf life of the drink.

MATERIALS AND METHODS

Collection of samples

Dried calyxes of Roselle were purchased from a local market in Ogun state, Nigeria and were taken to the laboratory in sterile cellophane bags for analysis. Two samples each of retailed ready-to-drink Zobo juice were purchased from five local hawkers (making a total of ten samples) and taken to the laboratory in sterile bottles for comparative analysis.

Dried calyxes (20 g each) were sorted to remove dirt particles and washed with tap water. The clean calyxes were added to 1 litre of boiling water and left to boil for another 10 min. After cooling, the juice extract was sweetened with 50 g granulated white sugar and bottled until analysis. Five different samples were prepared in duplicates for microbial examination.

Replicate 50 ml samples of retailed Zobo juice and laboratory-prepared Zobo juice were kept in sterile screw-capped bottles and allowed to stay at room temperature (29±1^{o}C) on shelves for up to two weeks. Bottles of each juice sample were picked daily for physico-chemical and microbiological analyses. The laboratory-prepared juice was allowed to cool to room temperature before being analyzed.

Physico-chemical analyses

Samples of both the laboratory-prepared and retail Zobo juices were observed daily for physical changes (color and odor) and chemical changes (pH and total acidity). Color densities of the juice samples were measured using a spectrophotometer while odor was determined by perception with the sense organ upon opening a fresh sample each day. A twenty-five man panel made up of students and staff of the department of Microbiology, University of Agriculture, Abeokuta determined the odor of duplicate juice samples on a 5-point scale of 5 (like extremely) to 1 (dislike extremely). The pH values of the juices were determined by a combined glass electrode and a pH meter (Mettler-Toledo, Essex M3509 Type 340). The pH probe was sanitized each time by swabbing with 95% ethanol prior to placing it in a sample. Duplicate determinations were made in all cases. The total titratable acidity (TTA) of the juices (expressed as percentages of citric acid) was determined by titrating 25 ml of the samples used for pH determination against 1 N NaOH.

Isolation and enumeration of microflora

An amount of 10 g of dried calyx was homogenized in 90 ml sterile peptone water (pH 7.0) to obtain a 1: 10 dilution. Further 10-fold dilutions were prepared from this and 0.1 ml each of appropriate dilutions were plated using the pour plate method (Harrigan and McCance, 1976; Olutiola, 1991). 10-fold dilutions of the juices were also prepared (v/v) daily and plated as described above. Enumeration of the total viable aerobic and anaerobic bacterial counts were carried out using Plate Count Agar (Oxoid CM325, Hampshire, UK) and de Mann Rogossa and Sharpe (MRS) agar (Oxoid CM 361) while Sabouraud Dextrose Agar (SDA, Oxoid CN 41) was used for fungal counts. SDA plates were incubated at 25°C for 72 h for fungi while PCA and MRS plates for bacteria were incubated at 30°C for 48- 72 h. One set each of MRS and PCA plates were incubated under anaerobic conditions stimulated using a CO_2 gas generating kit (Oxoid, Hampshire, UK.).

Characterization of isolates

Characterization of isolated microflora employed cultural and microscopic examination as well as conventional physiological and biochemical tests such as patterns of sugar fermentation, assimilation of sugars, and production of certain enzymes.

At intervals, colonies were randomly picked from incubated plates, purified by repeated sub-culturing before being examined microscopically for Gram reaction (Claus, 1992), cell morphology (using 24 h old cultures), motility, pigmentation and sporulation (Harrigan and McCance, 1976). Biochemical analysis included catalase and oxidase activities, nitrate reduction, patterns of sugar utilization as well as urea and starch hydrolysis (Christensen, 1946; Harrigan and McCance, 1976).

Fungal isolates were stained with cotton-blue lacto-phenol and microscopically observed for cell shape, size and sporulation. Physiological characteristics used for classifying yeasts included ability to 'ferment' certain sugars semi-anaerobically and ability to grow aerobically with various compounds each as sole source of either carbon or nitrogen (assimilation tests) (Kreger-van Rij, 1984; Barnett et al., 1990).

Bacteria and yeast isolates were identified on the basis of the results obtained from biochemical characterization complemented with the API identification kits (API System, France). The results were analyzed using Bergey's manual of systematic bacteriology (Sneath et al., 1986) and the yeast identification program of Barnett et al. (1994). Moulds were identified by their colonial features as well as micro-morphology of their sporulating structures and conidia according to Onions et al. (1981).

Statistical Analyses

One-way analysis of variance (ANOVA) was performed to test for differences in pH, TTA, color and total viable counts while bivariate correlations were carried out to determine the correlations among the same parameters using SPSS10.0 for Windows.

Table 1. Total viable counts (cfu/ml) of microflora of dried calyx and Zobo juices.

Sample	Total aerobic bacterial counts	Total anaerobic bacterial counts	Total fungal counts
Dried calyx	$1.7x10^4{}_b$	$0.4x10^4{}_a$	$3.2x10^4{}_c$
Retailed juice	$2.2x10^4{}_c$	$1.1x10^4{}_c$	$1.2x10^4{}_b$
Laboratory prepared juice	$1.4x10^4{}_a$	$0.8x10^4{}_b$	$0.9x10^4{}_a$

Values followed by the same subscript are not significantly different.

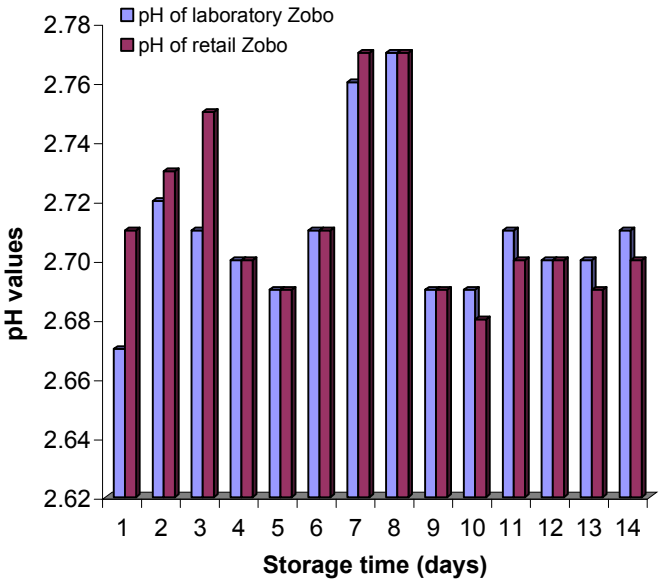

Figure 1. pH changes during the storage of Zobo juices (storage temperature = 29 ± 2°C).

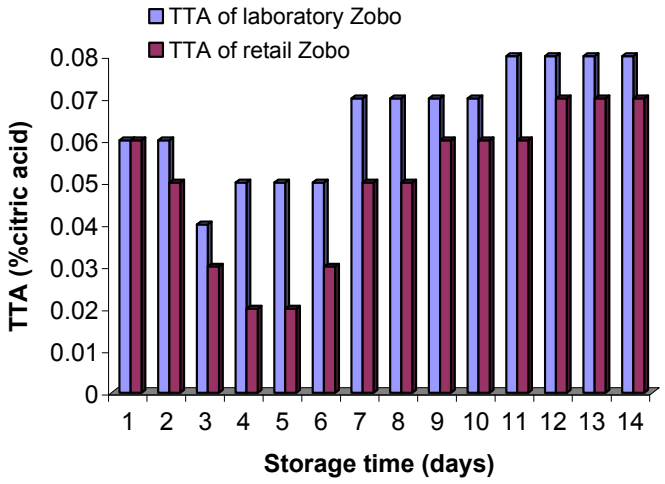

Figure 2. Total titratable acidity (TTA) changes during the storage of Zobo juices (storage temperature = 29 ± 2°C).

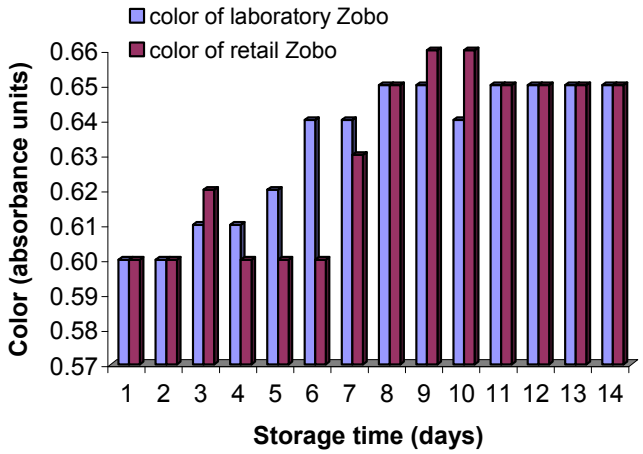

Figure 3. Color changes during the storage of Zobo juices (storage temperature = 29 ± 2°C).

RESULTS

Total viable aerobic and anaerobic counts of microflora associated with the dried calyx of *Hibiscus Sabdariffa* and its juices (retail and laboratory-prepared) are shown in Table 1. Counts from 0.4 to 3.2 x 10^4 cfu/ml. Aerobic and anaerobic bacterial counts were higher in retail or commercial Zobo juice (1.1 to 2.2 x 10^4 cfu/ml) than in the laboratory-prepared juice (0.8 to 1.4 x 10^4 cfu/ml) while the dried calyx had the highest fungal counts of 3.2 x 10^4 cfu/ml. Differences in viable counts for all samples were significant. Correlations between counts of anaerobic bacteria and fungi were also significant.

The pH of the juices ranged between 2.67 and 2.77 (Figure 1) while total titratable acidity (TTA) values were between 0.02 and 0.08 (Figure 2). Color change as measured by the spectrophotometer was between 0.60 and 0.66 (Figure 3). There were significant differences in TTA values obtained for both the laboratory-prepared and retail juice samples examined daily as well as changes in the color of the laboratory-prepared Zobo juice.

Over a hundred isolates were randomly picked from inoculated plates. Characterization results showed that some isolates were very similar in their characteristics

Table 2. Distribution of microflora in dried calyx and Zobo juices.

Isolate	Dried Calyx	Retail Zobo		Laboratory Zobo	
		Fresh	2 wks old	Fresh	2 wks old
Bacillus subtilis	+	+	+	–	–
Pseudomonas putida	+	–	–	–	–
Staphylococcus aureus	+	+	–	–	–
Streptococcus faecalis	–	+	+	+	–
Lactobacillus brevis	+	+	+	–	+
Lactobacillus fermentum	–	+	+	+	–
Proteus mirabilis	–	+	–	–	–
Serratia spp.	–	+	–	–	–
Escherichia coli	–	+	–	–	–
Saccharomyces cerevisiae	+	+	+	+	+
Candida krusei	+	–	+	–	+
Rhizopus oligosporus	+	–	–	–	–
Mucor spp.	+	–	–	–	–
Aspergillus niger	+	+	+	–	–
Aspergillus flavus	+	+	+	+	+
Penicillium citrinum	+	–	–	–	–

and on merging those that had the same features, sixteen different organisms including bacteria, yeasts and moulds emerged as the representative isolates. Bacteria included both Gram-positive and Gram-negative rods and cocci. These were *Bacillus subtilis* (Gram-positive, endospore-forming rods), *Staphylococcus aureus* (Gram-positive, coagulase positive cocci occurring in bunches), *Streptococcus faecalis* (Gram-positive, catalase and oxidase negative cocci occurring in chains), lactic acid bacteria (Gram-positive, asporogenous, catalase and oxidase negative glucose fermenting rods) represented by hetero-fermentative rods, *Lactobacillus brevis* and *Lactobacillus fermentum* (which were differentiated by the ability of *Lactobacillus brevis* to ferment inulin and reduce nitrate while *Lactobacillus fermentum* did not). Gram-negative rods included *Pseudomonas putida* (aerobic, oxidase positive rod which does not liquefy gelatin but hydrolyses arginine) and members of the family Enterobacteriaceae (facultatively anaerobic, oxidase negative, catalase positive, motile rods able to produce acid aerobically from glucose) represented by *Proteus mirabilis* (Indole and lactose negative), *Escherichia coli* (Indole and lactose positive) and *Serratia* spp. (red pigmentation on culture medium). Two yeasts species were isolated and identified as *Saccharomyces cerevisiae* and *Candida krusei* differentiated by the ability of *Saccharomyces cerevisiae* to ferment galactose, sucrose and raffinose while *Candida krusei* did not. Moulds isolated were *Rhizopus oligosporus* and *Mucor* spp. (whitish to grayish fluffy colonies differentiated by the presence of root-like rhizoids at the base of the sporangiophores of *Rhizopus oligosporus* and the presence of large columella in *Mucor* spp.), *Aspergillus niger, Aspergillus flavus* and *Penicillium citrinum* (differentiated by the swollen apex of the conidiophores of the aspergilli). Table 2 shows the distribution of the isolates in the samples examined. Only two organisms, *Saccharomyces cerevisiae* and *Aspergillus flavus*, were isolated from all samples with all other isolates being randomly distributed in the samples. However, the retail Zobo juice had a very wide variety of the isolated organisms. Although the types of micro-flora in all juices samples reduced after a storage time of two weeks, numbers of micro-flora were found to increase (Figure 4a,b) with the total elimination of some organisms such as enterics (*Proteus, Serratia* and *E. coli*), *Pseudomonas putida* and *Staphylococcus aureus.*

DISCUSSION

The Zobo juice produced from the dried calyx of *H. sabdariffa* were very acidic with low pH values of between 2.67 and 2.77. In the absence of comparative literature on the present study, the pH values obtained, when compared with the pH of vegetable juices (between 4 and 6) is quite low (Frazier and Westhoff, 1986). Changes in the pH values obtained during storage were not significant while changes in TTA values were significant. The inverse relationship between TTA and pH was also not very noticeable. These observations could be due to the presence of inherent buffering factors in the

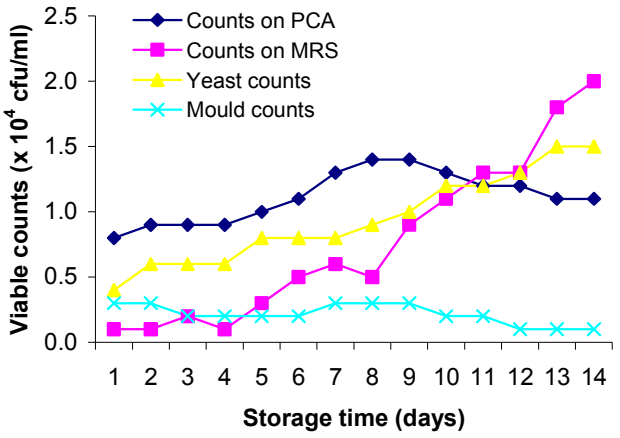

Figure 4a. Viable counts of microflora during storage of laboratory prepared Zobo juice. PCA = Plate Count Agar; MRS = deMann Rogosa Sharpe medium.

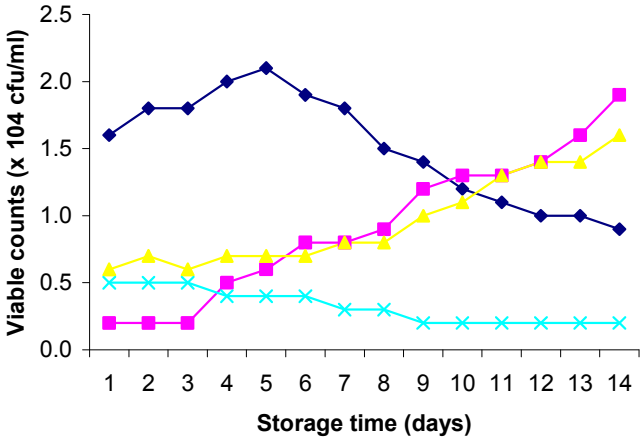

Figure 4b. Viable counts of microflora during storage of retail Zobo juice. PCA = Plate Count Agar; MRS = deMann Rogosa Sharpe medium.

samples. Some food materials are able to resist changes in pH and are said to be buffered. Buffering is common in foods such as milk and meats because of their proteins and is not often noticed in food materials like vegetables (Jay, 1978; Frazier and Westhoff, 1986). TTA values of the samples appeared constant around day 4 and then began to increase steadily after day 6 of storage.

No possible explanations could be proffered for the significance in color changes of the laboratory-prepared juice samples with storage time. Deterioration was identified by change in odor after four days of storage with the fresh aroma of the juice being replaced by sharp fermentative odor, which increased in intensity with storage time.

The counts of viable microorganisms were generally low compared with counts of microflora in related food materials (Frazier and Westhoff, 1986; Prescott et al., 1999). The high acidity of the juices could account for the low numbers and few types of organisms isolated although most of the isolates have been found associated with food (Stainer et al., 1987; Prescott et al., 1999).

No enteric organisms were found in the dried calyx but *Serratia spp, Proteus mirabilis* and *Escherichia coli* were present in the juices. The presence of this group of microorganisms in the juices is an indication of feacal contamination most probably from the water used for the production of the juices. Edema et al. (2001) reported the presence of such organisms in drinking water in the same town of Abeokuta, Nigeria, where the present study was also conducted. The enterics were however not present in the juice samples after two weeks of storage. This could be as a result of microbial succession with the lactic acid bacteria and yeasts dominating the microflora and suppressing or eliminating other organisms. Such suppression by the lactic acid bacteria have been reported by various workers (Nout et al., 1989; Mensah et al., 1991; Svanberg et al., 1992; Kingamkono et al., 1995; Olasupo et al., 1997). Spores of microorganisms such as moulds and endospore formers are commonly present in air and as such they occur on the surfaces of food materials as contaminants. The occurrence of *Staphylococcus aureus* in the retail juice samples could be indicative of inadequate precautionary measures during processing, production and/or packaging.

In conclusion, the microflora of *H. sabdariffa* were isolated and identified. The low numbers of the microorganisms is an advantage whereas the presence of sporulating organisms such as moulds and *Bacillus subtilis* is not. This knowledge of the microflora associated with the dried calyx and Zobo juice of . *sabdariffa* will serve as a guide in the design of appropriate preservation techniques for the juice, the focus of ongoing research by some of the authors.

REFERENCES

Adenipekun IT (1998). Extraction and colours of Roselle (*Hibiscus sabdariffa*) juice. M. Sc. Thesis, University of Ibadan, Ibadan.

Barnett JA, Payne RW, Yarrow D (1990). Yeasts: Characteristics and Identification. 2nd Edition. Cambridge University Press. 1002 pp.

Barnett JA, Payne RW, Yarrow D (1994). Yeast identification program. Cambridge University Press.

Christensen WB (1946). Urea decomposition as a means of differentiating *Proteus* and Para-colon organisms from each other and from *Salmonella* and *Shigella* types. J. Bacteriol. 52: 461.

Claus DC (1992). A standardised gram staining procedure. *World* Journal of Microbiolol.and Biotechnol. 8: 451- 452.

David G, Adam P (1988). Crops of the drier regions of the Tropics. ELBS Edt.

Edema MO, Omemu AM, Fapetu OM (2001). Microbiology and physico-chemical analysis of different sources of drinking water in Abeokuta, Nigeria. Nig. J. Microbiol. 15 (1): 57-61.

Frazier WC, Westhoff DC (1986). Food Microbiology TMH Edition 540pp.
Harrigan WF, McCance ME (1976). Laboratory methods in food and diary microbiology. Academic Press, London. p. 452.
Ibrahim RS, Miko HM, Althea CC (1998). Effect of N.P.K fertilization on the yield of Roselle. Paper presented at the 16th Annual Conference of HORTSON, Abeokuta, Ogun state. Nigeria
Jay JM (1978). Modern food microbiology 2nd Edition Litton Edu. Pub. Inc., N.Y. pp 254- 260.
Kingamkono R, Sjogreen E, Svanberg U, Kaijser B (1995). Inhibition of different strains of entero-pathogens in lactic fermenting cereal gruel. World J. Microbiol. Biotechnol. 11: 299-303.
Kreger-van Rij NJW (Editor) (1984). The yeasts: A taxonomic study. 3rd Edition Elsevier science. Publishers, Amsterdam.
Mensah PPA, Tomkins AM, Draser BS, Harrison TJ (1991). Antimicrobial effect of fermented Ghanaian maize dough. J. Appl. Bacteriol. 70: 203-210.
Nout MJR, Roumbouts FM, Havellar A (1989). Effect of accelerated natural lactic fermentation of Infant foods ingredients on some pathogenic microorganisms. International J. Food Microbiol., 8: 351 -361.
Olasupo NA, Olukoya DK, Odunfa SA (1997). Assessment of a bacteriocin-producing *Lactobacillus* strain in the control of spoilage of a cereal based African fermented food. Folia Microbiol. (Praha), 42(1), 31-34.
Onions AHS, Allsopp D, Eggins HOW (1981). Smith's introduction to Industrial Mycology. 7th Edt. Edward Arnold, London. 398pp
Prescott LM, Harley JP, Kleen DA (1999). Food Microbiology 5th Edition, McGraw Hill N.Y. pp 325-627.
Rice RP, Rice IW, Tindall HD (1990). Fruits and Vegetables Production in Warm Climates. Macmillan Press Limited. (Pp40-65)
Sneath PHA, Mar NS, Sharpe ME, Holt JG (Edt.).(1986). Bergey's Manual of Systematic Bacteriology. Volume 2 Williams and Wilkins co. Baltimore.
Stainer RY, Ingram J, Wheellis ML, Painter M (1987). General Microbiology. 5th Edt.
Svanberg U, Sjogren E, Lorri W, Svennerholm AM Kaijser B (1992). Inhibited growth of common entero-pathogenic bacteria in lactic-fermented cereal gruel. World J. Microbiol. Biotechnol. 8: 601-606.