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Microbiological quality of sachet packed cocoa based beverage marketed in Ibadan, Nigeria

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ABSTRACT

Objective: To assess the microbiological qualities of seven brands of sachet packaged cocoa based beverages in Ibadan city.

Methodology and results: Sachet packed cocoa beverages were purchase from three different market outlets in Ibadan. They were serially diluted and evaluated by pour plate technique on nutrient agar, mannitol salt agar, Maconkey agar and potato dextrose agar. Isolates obtained were identified by cultural, morphological and physiological characteristics. Those that were suspected to produce enterotoxin among the isolates were screened for enterotoxin production. Microbial load of the beverages samples were monitored for 5 days after opening. The sugar content of the samples was also determined and the osmo-tolerant ability of the isolates was established. Total bacterial count obtained from the samples ranged from $1.8x10^1$l – 4.1×10^1 cfu/ml, the coliform count ranged from 2 – 5 cfu/gm while the yeast and mould count ranged from 3.1×10^1– 1.4×10^2 cfu/ml. Bacterial and mould counts progressively increased during the 5 days period of storing the leftover samples at room temperature. Twenty three strains of bacterial isolates were obtained, twelve of which were *Bacillus* sp., two strains each of *Salmonella* and *Micrococcus. Staphylococcus* sp and *Escherichia coli* were also identified. Fungal isolates obtained were *Aspergillus flavus, Aspergillus niger* and *Rhizopus* sp. Sugar content of the samples was between 78 - 103 mg/gram while the growth of bacterial isolates reduced at higher glucose concentration (8 & 10%). One of the *Salmonella* sp caused production of enterotoxin in suckling mice.

Conclusion and application of findings: Results from this study showed that the content of sachet packed cocoa beverages should be consumed at once or stored in clean air tight tin after opening to avoid contamination by potentially harmful microorganisms.

Key words: Powdered cocoa beverage, microbiological quality, enterotoxin, and sugar content.

INTRODUCTION

Instant foods are convenience products that require very little effort to reconstitute or cook prior to consumption. Ground cocoa based beverages are classified as instant foods that contain free cocoa powder simply mixed with sugar and (or) milk powder. They are sources of high calorie intake and mineral nutrition and thus they are common drinks in Nigeria (Shittu and Lawal, 2007). Among the essential minerals contained in cocoa based beverages are iron, calcium, phosphorus and vitamins A, B, B_2 and D (Urbansky, 2008).

The process of fermentation of cocoa is accompanied by potential introduction of yeast, mould and bacteria (Jones, 1984; Oyetunji, 2006). These microorganisms are introduced when hands and feet are used to extract cocoa seeds from the pods, although most of these microorganisms may be destroyed during the drying process. Spore formers can still survive under drying temperature. Ostovar and Keeneye (1973) and Carr *et al*. (1979) reported the isolation of fungi and *Bacillus* after exposing cocoa beans to drying at high temperature. Other genera of bacteria isolated from fermented cocoa beans are *Leuconostoc*, *Micrococcus*, *Lactobacillus*, *Zymomonas* and *Actionomycetes* (Carr *et al.,* 1979). Lehrian and Patterson (1983) isolated yeast of the genera C*andida, Debaromyces, Pischia, Rhodotorula, Schizosaccharomyces* and *Trichospora* from fermented cocoa.

During the manufacture of cocoa beverage, the beans are roasted at 103 - 145^{o}C for about 1½ hours to further reduce the moisture content to between 2.5 – 4% (Anonymous, 1981; Urbansky, 2008). Cocoa based powdered beverage is a food with low water activity that may not support the growth of many microorganisms except for spore forming genera of microorganism such as moulds, *Bacillus*, and *Clostridium* (Frazier and Westhoff, 1978). Some of the ingredients used in processing cocoa powdered beverages like egg, malt and cocoa provide favorable conditions for microbial growth (Pelczar *et al.,* 1993).

In the past few years different brands of cocoa based beverage (powdered) have been produced by multinational and cottage food industries in Nigeria. These are packaged in mini sachets that come in attractive forms and sold at affordable prices so that most Nigerian families can have access to such nourishing instant food. However most of these products are produced by unrecognized industries without the involvement of a food scientist or a microbiologist to ascertain their safety and quality. Also, some of these beverages are packaged in mini sachets made of low density polyethylene which is fragile and thus may be broken easily in transit. Some packets are not properly sealed and they are exposed even before being sold, thus impairing the safety of such product. Sometimes these beverages may not have received enough heat treatment and the raw materials may have been exposed to dust carrying various microorganisms (Oyetunji, 2006).

Cocoa powder beverages especially the cheaper ones packed in sachets are used by street hawkers to prepare hot beverages sold as breakfast to consumers or prepared as chilled beverage drink and sold as thirst breakers to the populace on hot afternoons. This study aimed to carry out microbiological evaluation of the cocoa based powder beverages to ascertain their quality and any potential health risk they may pose to consumers.

MATERIALS AND METHODS

Collection of samples: Twenty eight samples of seven brands of the cocoa based powder beverages were purchased from three market outlets in Ibadan (Sango, Bodija and Dugbe). The samples were obtained at three week intervals over a period of twelve weeks. The brands used were 'Balovita' (BL), 'Bournvita' (BV), 'Choconat' (CC) 'Galovita' (GL), 'Kemovita' (KM), 'Milo' (ML), and 'Suco' (SC)

Enumeration of microorganisms: Bacterial and fungal isolates were enumerated using Plate Count Agar (PCA) for total aerobic bacteria, MacConkey Agar (MAC) for Coliforms; Mannitol Salt Agar (MSA) for *Staphylococcus* species; Potato Dextrose Agar (PDA) for moulds and yeasts; and Nutrient Agar (NA) for general bacteria isolates. All the media were Oxoid brand. Aerobic bacterial and fungal isolates were cultured by dilution and direct plating technique.

For enumeration of Coliforms and *Staphylococcus*, 1gm of sample was directly introduced into each of the sterile Petri dishes. Petri dishes were then over laid with sterile cool molten MSA and MAC for enumeration of *Staphylococcus* and Coliforms, respectively. They were all incubated at 37^{0}C for 36 hours for bacteria and 28 $\pm$ 2^{0}C for 72 hours for fungi. The isolates from the plates were repeatedly sub-cultured on fresh PDA and NA for fungi and bacteria, respectively, until pure culture of each isolate was established. Each of the samples was opened for five (5) days and the microbial

load enumerated after 24, 72, and 120 hours of storage.

Bacterial isolates were identified by morphological and biochemical tests as described in Bergey's Manual of determinative bacteriology (Holt *et al*., 1994) while fungal isolates were identified by cultural and microscopic observations and compared with compendium of soil fungi (Domsch and Anderson 1980).

Physiological study on the Isolates: Osmotic tolerance of bacterial isolates was evaluated using the method of Harrigan *et al.* (1976). The quantitative estimation of sugar content of cocoa beverage samples was done using DNSA method according to Miller (1979).

Immunological test for identification of isolates: Immunological tests were carried out on some of the identified *Salmonella* spp using *Salmonella* polyvalent O, H and Vi specific and non specific antisera, separately. Each isolate was prepared from overnight culture in 0.5ml of sterile saline solution (0.85%) in a test tube using a sterile loop. A loopful of each culture suspension was placed on sterile glass slides and mixed with a loopful of each antiserum separately by tilting the slides to and fro for 30 seconds. The slides were observed under a good source of light against a dark background with the naked eye. Clumping within 60 seconds was taken as positive. Isolates positive for the three antisera were identified as *S. typhi* while those positive for antisera O and H were identified as *Salmonella* sp.

Enterotoxin activity test: A modification of the infant mouse Enterotoxin Activity Test originally described by Dean *et al*. (1972) was used for this test, to determine the presence of heat stable (ST) toxin in bacteria broth cultures. The isolates used for this test were *Staphylococcus* sp, *Escherichia coli*, and *Salmonella* sp. They were grown in broth cultures consisting of 2% (w/v) peptone (Difco), 0.3% (w/v) Magnesium Chloride and 1% (v/v) horse serum (Manninen *et al.,* 1982). Thereafter the cultures were incubated at 42^0C for 10 hours without shaking and then for another 10 hours with shaking at 100rpm on a G24 environmental incubator shaker. After incubation, cultures were centrifuged at 1,200 rpm for 30 minutes on a table top Beckmann centrifuge and the supernatant was used immediately for the test. Four suckling mice were each inoculated orally with 0.1ml broth culture supernatant from each of the eight isolates tested.

Another set of two mice received oral inoculation of sterile broth as negative control while two other sets of mice were inoculated with 0.1ml supernatant broth of *E. coli* E7539/77 which serves as positive control. Inoculated mice were kept at room temperature for 24 hours and then killed with chloroform. The abdomen was opened and small intestines removed and weighed. The ratio of the gut to the remaining body weight was determined and a value of 0.09 and above was taken as positive for enterotoxin production.

Data analysis: The data obtained were analyzed by determination of mean of each replicate values.

RESULTS AND DISCUSSION

Microbial contamination in all the samples were generally high especially those packaged in low density polyethylene sachets, compared to those in aluminum foil sachets. The average bacterial count on opening ranged from 1.8×10^1 to 4.0×10^2 cfu/ml (Table 1). The average staphylococcal count for the samples on opening was between 3.0 to 5 cfu/gm. Average mould counts obtained on opening of samples ranged from 3.1 x to 1.4×10^2 cfu/ml.

The average bacterial count on all the samples progressively increased during storage. The average bacterial count after 24 hours of storage was between 3.3×10^1 to 1.4×10^1 cfuI/ml, while after 120 hours, it ranged from 6.3×10^2 to 3.2×10^4 cfu/ml (Table 2). Progressive increases in coliform, staphylococcal, moulds and yeasts counts were also recorded from 24 to 120 hours. This contamination level is comparable to that obtained by Te Giffel *et al*. (1996) and Oyetunji (2006), of 10^3 – 10^5 cfu/g for bacterial and 1.8×10^2cfu/g for fungal contaminants in powdered beverages.

The progressive increase in the microbial load during storage of the samples could be due to activation of spores present in the samples which germinated into vegetative cells on exposure of samples to desirable condition of growth. Also once opened the sachets could not be properly/tightly covered to prevent exposure to further contamination during storage. This shows that the habit of storing the leftover of these products in such sachets instead of air tight containers after use is hazardous because prolonged storage exposes them to microbial contamination.

Table 1: Average microbial load of powdered cocoa beverage samples on opening.

Samples	Average Total Aerobic Bacterial Count (cfu/g)	Average Total Staphylococcus count (cfu/g)	Average Total coliform count (cfu/g)	Average Total Yeasts & Mould Count (cfu/g)
Balovital (BL)	31	N.D.	2.0	**23**
Bournvita (BV)	18	N.D	N.D	**ND**
Chochonat (CC)	30	N.D.	3.0	**1**
Galovita (GL)	41	5.0	3.0	**4**
Kemovita (KM)	42	3.0	5.0	**3**
Milo (ML)	21	N.D.	N.D.	**1**
Suco (SC)	**34**	**N.D.**	**N.D**	**3**

N.D. – Isolate not detect

Table 2: Average microbial load in powdered cocoa beverage samples during storage of open sachets for up to 120 hours.

Sample	Average Total Aerobic Bacterial Count Cfu/g(10^2)			Average Total Staphylococcus Count Cfu/g(10^2)			Average Total Coliform count Cfu/g(10^2)			Average Total Fungal count Cfu/g(10^2)		
	24h	72h	120h	24h	72h	120h	24h	72h	120	24h	72h	120
BL	0.6	4.2	5.8	0.1	1.1	1.5	0.04	1.91	1.92	2	2.8	90
BV	0.31	4.5	6.3	0.1	1.1	1.7	0.03	0.15	0.42	ND	2	8
CC	0.6	8.3	28.7	1.2	2.13	9.8	0.15	1.37	5.22	1.2	8.6	51
GL	5.26	51.82	319.6	2.1	4.0	15.2	0.15	2.19	5.20	4	6.4	53
KM	14.1	90	150	1.2	1.61	4.6	0.18	2.16	4.33	3.1	34	120
ML	3.6	31.8	70.9	0.3	2.0	10.1	0.05	0.49	0.94	0.12	0.4	10
SC	1.1	9.2	15.8	0.3	1.6	8.3	0.06	1.92	5.22	0.1	2	19

ND – Isolates not detected

A total of twenty three bacterial isolates were obtained and characterized. They included catalase positive spore forming bacteria of the genus *Bacillus;* others were *Escherichia coli*, *Staphylococcus* sp, *Micrococcus* sp and *Salmonella* sp. The *Bacillus* species were identified as *B. megaterium* from samples of 'GL', 'BV' and 'ML', *Bacillus sphaericus* from the samples of 'BV'. *B. licheniformis* was obtained from samples of 'SC' and 'BL'. Two other species of *Bacillus* were obtained from 'BV', 'GL', 'BL', 'SC', and 'ML' samples that were used for this study as shown in Table 3. An *E. coli* strain was obtained from 'BL', 'CC', and 'KM' samples and *Salmonella* sp were obtained from 'GL' and 'KM' samples. *Staphylococcus* sp. was isolated from samples of 'GL', 'KM' and 'CC' while species of *Micrococcus* were obtained from 'GL', 'KM' and 'ML' samples. Moulds obtained from the samples were identified as *Aspergillus flavus*, *A. niger* and *Rhizopus* sp. *A. flavus* was obtained from samples of 'SC' and 'MC' while *A. niger* was isolated from samples 'KM' and 'CC' and *Rhizopus* sp. from 'BV' samples. Some of these moulds have been identified as mycotoxin producers (Lee *et al*., 2003; Oyetunji, 2006). Apart from the danger of food poisoning which may be caused by the microorganisms isolated, these organisms also reduce nutrient content of the food (Shittu and Lawal, 2007; Urbansky, 2008).

The isolation of *Salmonella* sp and *E. coli* in some of the samples is a pointer to the fact that some of the samples may not have been exposed to enough heat to reduce the moisture content considerably as reported by Oyetunji (2006). Also, they may have been produced under dirty and unhygienic environment, or they may not have been sealed properly. These microorganisms and *Staphylococcus* sp are potential enterotoxin producing species (Dean *et al*., 1972; Lapeyre *et al*., 1988)

Table 3: Frequency of occurrence of bacterial isolates in samples

Isolates	Samples								
	GL	BL	KM	SC	CC	BV	ML	Frequency of occurrence	**Percentage of occurrence**
***Micrococcus* sp**	3	2	3	-	-	-	-	8	**28.2**
Micrococcus	-	-	-	-	-	-	4	4	**14.2**
***Staphylococcus* Sp.**	4	-	4	-	4	-	-	12	**42**
Escherichia coli	-	2	2	-	4	-	-	8	**28.2**
***Salmonella* sp**	2	-	-	-	-	-	-	2	**7.1**
***Salmonella* sp**	-	-	2	-	-	-	-	2	**7.1**
Bacillus megaterium	4	-	-	-	-	4	4	12	**42**
Bacillus licheniformis	-	4	-	4	-	-	-	8	**28.2**
Bacillus sphaericus	-	-	-	-	-	4	-	4	**14.2**
***Bacillus* sp.**	**4**	**4**	**4**	**4**	**-**	**2**	**3**	**21**	**75**

'Balovita' (BL), 'Bournvita' (BV), 'Choconat' (CC) 'Galovita' (GL), 'Kemovita' (KM), 'Milo' (ML), and 'Suco' (SC)

Quantitative estimation of sugar content of samples showed that the range was between 78 and 103mg/gm in the cocoa based powdered beverages, with 'ML' having the lowest glucose content and 'BL' having the highest. Other beverages which included BV, CC, GL, KM and SC had 101, 98, 85, 85 and 90mg/gm, respectively. Shittu and Lawal (2007) also reported that most cocoa based powdered beverages produced in Nigeria generally have high sugar content. Between 52 - 90% sucrose is added to sweeten and to further mask the residual astringency and bitterness of cocoa taste. Shittu and Lawal (2007) further submitted that only 30% of cocoa based powder beverage producers abide with the 85% (Sucrose) stipulated by the Standard Organization of Nigeria (SON).

Table 4 shows the enterotoxin activity values of the isolates tested with *Salmonella* sp isolates from 'Kermovita' having a value of 0.109 a value higher than the standard value of 0.09 according to FAO (1997) which shows that the isolate can produce enterotoxin. Other isolates had values lower than 0.09 activity but the *Salmonella* sp isolates from 'Galovita' had 0.08, a value very close to 0.09. The *Staphylococcus aureus, E. coli* and other *Salmonella* sp isolates obtained from the samples used in this study did not produce enterotoxin.

Table 4: Enterotoxin activity test in some toxin producing bacterial strains

Isolates	M.G.W. (gm)	M.B.W(gm)	E.A.V
***E. coli* (from CC, BL, KM)**	0.117	2.634	**0.044**
***Salmonella* sp from (KM) sample.**	0.323	2.970	**0.109**
***Salmonella* sp (from GL)**	0.263	2.986	**0.088**
***Staphylococcus* sp. (from GL, CC & KM samples)**	0.205	2.941	**0.069**
***E. coli* E7539/77 (Positive control)**	0.235	1.603	**0.147**
Sterile uninoclated broth (Negative control)	**0.231**	**3.335**	**0.069**

Data represent the mean values of 3 replicate tests; MGW – Mean Gut Weight; MBW – Mean Body weight; EAV – Enterotoxin Activity value; (Ratio MGW: MBW) Balovita' (BL), ' 'Choconat' (CC) 'Galovita' (GL), 'Kemovita' (KM),

CONCLUSION

Results from this study showed that the bacterial count for all the samples on the first day was within the standard recommended for dry consumable food hence were safe for consumption. Isolation of enterotoxigenic species of *Salmonella* in some of the products indicates that they can cause human infection. Use of hot water for preparation of these beverages produced by local cottage industries is recommended. Further, it is recommended that the content of a sachet should be consumed preferably within one day after opening. In the case of larger sachets, the remnants should be stored in a dry, sterile clean air- tight container.

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