Evaluation Of Microbiological Quality And Aflatoxin M_1 (AFM_1) Contamination Of Milk Powder Samples

Sold In Nigeria Market

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Abstract: Milk, a natural liquid food, is one of the most nutritionally complete foods, adding high-quality protein, fat, milk sugar, essential minerals, and vitamins to diet. Milk could also be a source of contaminants such as microorganisms and aflatoxin M_1 (AFM_1). Aflatoxins are important toxins whose consumption could cause food borne diseases. The microbiological quality and Aflatoxin M_1 (AFM_1) contamination of twenty five milk powder samples (10 brands) imported, branded and sold in Nigerian market were evaluated. The total heterotrophic, coliform, Bacilli counts ranged from 2.0 - 8.2 $x10^1$cfu/g, 1.0 - 4.0cfu/g and 3.0 – 4.1 x 10^1cfu/g respectively. There was no detection of *Salmonella/ Shigella, Vibrio*, lactic acid bacteria, *Staphylococcus* and *E. coli*, Yeast and mould in any samples. The bacteria isolates found in the samples were *Bacillus subtilis, B. lincheniformis, B. cereus, Proteus mirabilis* and *Proteus vulgaris* among which *B. subtilis* had the highest frequency of occurrence (48.8%). Fungi were also not detected in the samples. The microbial loads of the milk samples were found to be lower than the specified standard limits (10^2 - 10^3 cfu/g for bacteria) as recommended by United State Food and Drug Administration (USFDA). The AFM_1 level ranged between 0.13 ± 0.01^y – 3.75 ± 0.01^a ppb (n = 25) and was found in all the samples tested. In approximately 80% of the samples, level of contamination was above the permissible concentration (0.5ppb) as specified by European Union (EU). About 20% contain AFM_1 at level below tolerance limit specified by FDA. There were significant differences ($P\geq0.05$) in the mean values of AFM_1 in the samples from the same brand. The detection of AFM_1 in the milk powder samples could be of public health significance and hence there is an urgent need for concerned regulatory bodies to impose necessary measures to safeguard health of consumers. In conclusion, while the microbial load of milk powder samples did not pose public health problem, the level of AFM_1 contamination called for serious attention in the country.
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Introduction

Milk is the lacteal secretion, practically free from colostrums which are obtained through complete milking of one or more healthy cows (Vasavada , 1988). Milk, a natural liquid food, is one of the most nutritionally complete foods, adding high-quality protein, fat, milk sugar, essential minerals, and vitamins to diet.

Production of milk powder is a simple process carried out on a large scale. It involves the removal of water at the lowest possible cost under stringent hygiene conditions while retaining the desirable natural properties of the milk; color, flavor, solubility and nutritional value. The conventional process for milk powders production involved: Collection of raw milk, pasteurization and centrifugation in the dairy factory. Followed by preheating, evaporation, spray drying, packaging and storage (Pearce, 2000)

Milk in the mammary gland at the site of its production does not contain bacteria. Milk becomes contaminated with bacteria that live as commensal micro floral on the teat canal, the duct that conduct milk from the mammary gland to the orifice. Bacteria such as *Bacillus cereus*, *Listeria monocytogenes*, *Yersinia enterocoltica*, *Salmonella* spp, *Escherichia coli* 0157:11 and *Campylobacter jejuni* associated with milk borne diseases have been reported 1(Vasavada , 19883; Alan and Heather , 1990). *Staphylococcus aureus* has been isolated from most samples of raw milk (Riadh , 2005) .

Also milk could also be a source of toxic substances such as aflatoxin M_1 (AFM_1). Aflatoxins are a group of naturally occurring toxins produced by moulds such as *Aspergillus flavus*. When some animals ingest aflatoxin B_1 (AFB_1) - contaminated feed, it is metabolized to AFM_1 and transferred to food materials such as milk and eggs. Aflatoxin M_1 (AFM_1) is the hydroxylated metabolite of AFB_1, found in the milk of humans and animals. They may be found in milk products obtained from livestock that have ingested contaminated feed (Polan *et al.,* 1974; Frobish et *al.,* 1986;(Rustom, 1997;Park, 2002; Bullerman , 1979; Chu , 1991). Although the potency of AFM_1 is less than that of its parent compound, it is also known to be hepatotoxic and carcinogenic (Bullerman , 1979;Chu , 1991).

More recently, aflatoxin exposure early in life has been associated with impaired growth, particularly

stunting (Gong *et al.,* 2002)
. Therefore, the presence of AFM_1 in milk and dairy products may pose a threat, mainly towards children who are considered to be the major consumer of milk and dairy products in many countries (Williams *et al.,* 2004).
This study was carried out to evaluate the microbiological quality and AFM_1 contamination of milk powder samples in Nigeria.

1. Materials and Methods

2.1 Collection of samples

Twenty five milk powder samples of different commercial brands (10 market brands) were randomly purchased from different markets in Nigeria. All the samples were imported (but packed in tin and sachet) and they were packed in Nigeria. The samples were stored in sterile plastic bag at -20^{O}C.

2.2 Laboratory Analyses

Microbiological analysis

Microbiological analysis of the samples was done using the procedure of the American Public Health Association, APHA, (1992). 1ml of the diluents (10^0, 10^1 and 10^2) were plated onto nutrient agar medium for total heterotrophic bacteria counts; MacConkey agar was used for total coliform counts; MRS agar for total lactic acid bacteria count; EMB agar for *E. coli*; and thiosulphate citrate bile salt sucrose agar for total *Vibrio* counts; *Salmonella* and *Shigella* agar for total *Salmonell*a and *Shigella* count; Trypticase soy agar for total *Bacilli* count; yeast extract agar for total yeast count, and Sabouraud dextrose agar with 1% streptomycin for total fungi count. The plates were incubated at 37^{o}C for 24hrs except for Yeast extract agar plates and SDA plates which were incubated at 28±2 for 3-7 days. Colonies were selected randomly and were characterized using morphological and biochemical tests. The identification of the microbial isolates was based on classification scheme proposed by Harrigan and McCance (1976), Buchanan and Gibbson (1974) and Collin and Lyne (1995). The identification was based essentially on morphological and biochemical reactions. Fungal isolates were identified based on their morphological and cultural characteristics as recommended by Sampson *et al.*, (1984) and Frazier and Westholf (1998).

2.3 Mycotoxin analysis

The AFM_1 analyses were performed using enzyme-linked immunosorbent assay (ELISA) kit (Ridascreen, R-Biopharm AG, Darmstadt, Germany) which is a competitive enzyme immunoassay based on antigen-antibody reaction. All chemicals used in the experiments were of analytical grade. For the determination of AFM_1 content of the samples, 10g of each powered sample was extracted with 20 mL methanol: water (70:30) using a shaker at room temperature. After centrifugation, complete removal of upper cream layer was done by aspirating using a Pasteur pipette. One hundred micro liter skimmed portion was directly applied on the AFM_1 test plate i.e. wells coated with antibodies to AFM1 and after mixing, incubated for 60 min. at room temperature in dark. Then wells were washed with buffer solution. In the next state, 100 µl of enzyme conjugate washed with buffer and 50 µl of the enzyme substrate and 50 µl of chromogen were added to wells and incubated for 30 min. at room temperature in the dark. Enzyme conjugate converted the chromogen to a blue product and then 100 µ1 of the stop solution was added to wells which lead to a yellow discoloration of the chromogen.

The optical densities were measured at 450 nm by using an ELISA 96-well microplate reader (Sunrise Gmblt, Tecan, Austria). The optical densities (OD) were then compared to those of the standards. AFM1 concentration in each sample was expressed as parts per billion (ppb).

2.4 Statistical analysis

The results of the analysis are expressed as mean ±SD. Data were analyzed by ANOVA using SAS. Sequential differences among means were calculated at the level of $P \leq 0.05$, using Duncan Multiple Range Test (Duncan, 1956).

3 Results

The microbial load in different milk powder samples is shown in Table 1. Growths were observed on nutrient agar, MacConkey agar and Trypticase soy agar. There was no detection of *Salmonella/ Shigella, Vibrio,* lactic acid bacteria, *Staphylococcus* and *E. coli*, Yeast and mould in any of the samples. The total heterotrophic count ranged from $2.0 - 8.2 \times 10^1$ cfu/g; the highest was recorded in sample D1. There was no observable microbial growth on sample E2. Twenty of the milk samples (80%) were found to have total heterotrophic plate count of ≥10 CFU/g while only five milk samples (20%) were found to have total heterotrophic plate count of ≤ 10 CFU/g. The total coliform counts ranged from 1.0 - 4.0 cfu/g with sample H2 having the highest count. Most of the samples showing high bacteria count were those packed in sachets. The total *Bacilli* count in the milk powder samples ranges from $3.0 - 4.1 \times 10^1$ cfu/g and these organisms were prevalent in all the samples except sample E2. Some of the plates were observed to be covered with microbial growth making it difficult to count (Table 1). The biochemical characteristics opf the isolates is shown in Table 2.

The probable organisms from the milk powder

samples were *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis*, *Proteus mirabilis* and *Proteus vulgaris* as shown in Table 3. The percentage frequency of occurrence of the bacteria isolate is shown in Figure 1 in which *Bacillus subtilis* had the highest frequency of occurrence of 48.48%.

The result of Aflatoxin concentration in the milk powder samples is shown in Table 4. Aflatoxin detection using ELISA technique revealed that 100% of the milk powder samples were contaminated with AFM_I. The AFM_1 concentration ranged from 0.13^y – 3.75^a ppb with sample A3 having the highest concentration. The detected minimum and maximum level were 3.63 – 3.76 ppb in brand A, 0.75 – 0.93 ppb in brand B, 0.25 – 0.27 ppb in brand C, 0.10 – 1.28 ppb in brand D, 0.12 – 3.37ppb in brand E, 0.77 – 1.40ppb in brand F, 0.14 – 1.76ppb in brand G, 0.21 – 1.09ppb in brand H, 0.12 – 0.35ppb in brand I and 0.21 – 1.46ppb in brand J. 80% of the milk samples were contaminated with AFM_1 beyond the specified limit by FDA. The permissible level of aflatoxin in milk as approved by USFDA is 0.5 ppb. 20% of the samples had aflatoxin concentrations below the acceptable limit. There was a significant difference in AFM_1 contamination of the milk powder samples of the same brand.

Table 1: Microbial counts of the milk powdered samples

Brand Code	Sample Code	Total plate count	Total coliform count	Total Salmonella Shigella count	Total fungi count	Total yeast count	Total Vibrio count	Total Staph count	Total E. coli count	Total Bacillus count	Total LAB count
	A 1	14±0.026	-	-	-	-	-	-	-	8 ±0.015	-
	A2	9 ±0.015	-	-	-	-	-	-	-	swarm	-
A	A3	19 ±0.034	-	-	-	-	-	-	-	7±0.030	-
	B1	2 ±0.010	1	-	-	-	-	-	-	3±0.025	-
B	B2	17 ±0.026		-	-	-	-	-	-	8±0.015	-
	C1	64±0.005	-	-	-	-	-	-	-	16±0.04	-
C	C2	73 ±0.017	-	-	-	-	-	-	-	17±0.011	-
	D1	82 ±0.015	-	-	-	-	-	-	-	41±0.03	-
	D2	4 ±0.015	1	-	-	-	-	-	-	8±0.025	-
D	D3	17 ±0.017	-	-	-	-	-	-	-	7±0.015	-
	E1	19 ±0.011	-	-	-	-	-	-	-	9±0.017	-
	E2	-	-	-	-	-	-	-	-	-	-
E	E3	34 ±0.015	-	-	-	-	-	-	-	swarm	-
	F1	46 ±0.020	-	-	-	-	-	-	-	swarm	-
F	F2	23 ±0.011	-	-	-	-	-	-	-	swarm	-
	G1	39 ±0.011	-	-	-	-	-	-	-	swarm	-
	G2	27 ±0.026	-	-	-	-	-	-	-	swarm	-
G	G3	39±0.04	-	-	-	-	-	-	-	swarm	-
	H1	18 ±0.05		-	-	-	-	-	-	swarm	-
	H2	21±0.011	4	-	-	-	-	-	-	swarm	-
H	H3	23 ±0.026	-	-	-	-	-	-	-	swarm	-
	I1	12 ±0.017	-	-	-	-	-	-	-	swarm	-
I	I2	28±0.028	-	-	-	-	-	-	-	swarm	-
	J1	14±0.020	-	-	-	-	-	-	-	swarm	-
J	J2	17 ±0.026	-	-	-	-	-	-	-	swarm	-

TABLE.2 Biochemical characterizations of the isolatess

Lates	Gram	Shape	Catalase	MR	Indole	Motility	Glu	Man	Suc	Mal	Lac	Gal	Fru	Sor	Spore	Probable organism
A	+	Rod	+	-	-	+		-	+	+	-	-	+	-	+	*B. licheniformis*
B	+	Rod	+	-	-	+	-	-	+	+	-	-	+	-	+	*B. licheniformis*
C	+	Rod	+	-	-	+	+	+	+	+	+	+	+	+	+	*B. subtilis*
D1	+	Rod	+	-	-	+	+G	+	+	+	+	+	+	+	+	*B. subtilis*
D2	-	Rod	-	+	-	+	-	-	+	+	+	+	+	-	-	*P. mirabilis*
E	+	Rod	+	-	-	+	+G	+	+	+	+	+	+	+	+	*B. subtilis*
F	+	Rod	+	-	-	+	+	+	+	+	+	+	+	+	+	*B. subtilis*
G3	+	Rod	+	-	-	+	+	+	+	+	+	+	+	+	+	*B. subtilis*
G4	+	Rod	+	-	-	+	+	-	+	+	+	+	+	-	+	*B. cereus*
H1	+	Rod	+	-	-	+	+	+	+	+	+	+	+	+	+	*B. subtilis*
H7	+	Rod	+	-	-	+	+	-	+	+	+	+	+	-	+	*B. cereus*
I1	-	Rod	+	+	-	+	-	-	-	+	+	+	+	-	-	*P. vulgaris*
I4	+	Rod	-	-	-	+	+	+	+	+	-	-	+	+	+	*B. subtilis*
J	+	Rod	+	-	-	+	+G	+	+	+	+	+	+	+	+	*B. subtilis*
K	+	Rod	+	-	-	+	+G	+	+	+	+	+	+	+	+	*B. subtilis*
M	+	Rod	+	-	-	+	-	+	+	+	-	-	+	+	+	*B. licheniformis*
N	+	Rod	+	-	-	+	-	+	+	+	-	+	+	+	+	*B. licheniformis*
O	+	Rod	+	-	-	+	+	-	+	-	-	-	+	-	+	*B. cereus*
P	+	Rod	+	-	-	+	+	-	+	+	+	+	+	-	+	*B. cereus*
Q1	+	Rod	-	-	-	+	+G	+	+	+	+	+	+	+	+	*B. subtilis*
Q2	+	Rod	+	-	-	+	-	+	+	+	-	-	+	-	+	*B. licheniformis*
R2	+	Rod	+	-	-	+	-	+	+	+	+	-	+	-	+	*B. licheniformis*
R3	+	Rod	+	-	-	+	+	+	+	+	+	+	+	+	+	*B. subtilis*
S2	+	Rod	+	-	-	+	+G	+	+	+	+	+	+	+	+	*B.subtilis*
S3	+	Rod	+	-	-	+	-	-	-	+	-	-	+	-	+	*B. cereus*
T1	-	Rod	+	+	-	+	+G	+	+	+	+	+	+	+	-	*P. vulgaris*
T2	+	Rod	+	-	-	+	+G	+	+	+	+	+	+	+	+	*B.subtilis*
U	-	Rod	-	-	-	+	+G	+	+	+	+	+	+	+	-	*B.subtilis*
V2	+	Rod	+	-	-	+	+	+	+	+	-	+	+	+	+	*B. subtilis*
V4	+	Rod	+	-	-	+	-	+	+	+	-	-	+	-	+	*B. licheniformis*
W	+	Rod	+	-	-	+	-	+	+	+	-	+	+	-	+	*B. licheniformis*
X	+	Rod	+	-	-	+	+G	+	+	+	+	+	+	-	+	*B. subtilis*
Y	+	Rod	+	-	-	+	-	+	-	+	-	+	+	-	+	*B. licheniformis*

Table 3: Microorganisms associated with the milk powder samples

Brand code	Sample code	*B. subtilis*	*B. cereus*	*B. licheniformis*	*P. mirabilis*	*P. vulgaris*
	A1	–	+	–	–	
	A2	–	+	–	–	
A	A3	+	–	–	–	
	B1	+	–	–	–	
	B2	–	–	+	–	
B	B3	+	–	–	–	
	C1	+	–	–	–	
	C2	+	–	–	–	
C	C3	–	+	–	–	
	D1	+	–	–	–	
	D2	–	+	–	–	
	D3	–	–	–	+	
	D4	+	–	–	–	
D	D5	+	–	–	–	
	E1	+	–	–	–	
E	E2	–	–	+	–	
	F1	–	–	+	–	
F	F2	–	+	–	–	
	G1	–	+	–	–	
	G2	+	–	–	–	
	G3	–	–	+	–	
	G4	–	–	+	–	
G	G5	+	–	–	–	
	H1	+	–	–	–	
	H2	–	+	–	–	
	H3	–	–	–	+	
	H4	+	–	–	–	
H	H5	+	–	–	–	
	I1	+	–	–	–	
	I2	–	–	+	–	
I	I3	–	–	+	–	
	J1	+	–	–	–	
J	J2	–	–	+	–	

+ = positive, - = negative

Table 4: Total Aflatoxin M_1 concentrations (ppb) in the milk powder samples

Sample Brand	Sample code	AFM_1		
		Minimum	Maximum	Average mean/SD
A	A1	3.63	3.65	3.64 ± 0.01^{c}
	A2	3.68	3.70	3.69 ± 0.02^{b}
	A3	3.74	3.76	3.75 ± 0.01^{a}
B	B1	0.91	0.93	0.92 ± 0.01^{m}
	B2	0.75	0.79	0.77 ± 0.02^{q}
C	C1	0.25	0.27	0.26 ± 0.002^{u}
	C2	0.86	0.88	0.87 ± 001^{n}
D	D1	0.71	0.73	0.72 ± 0.01^{s}
	D2	0.10	0.99	0.69 ± 0.05^{l}
	D3	1.26	1.28	1.27 ± 0.01^{h}
E	E1	0.62	0.64	0.63 ± 0.01^{t}
	E2	3.35	3.37	3.36 ± 0.006^{d}
	E3	0.82	0.84	0.83 ± 0.005^{p}
F	F1	1.38	1.40	1.39 ± 0.002^{g}
	F2	0.77	0.79	0.78 ± 0.01^{r}
G	G1	1.04	1.06	1.05 ± 0.01^{jk}
	G2	0.14	0.16	0.15 ± 0.01^{x}
	G2	1.74	1.76	1.75 ± 0.01^{e}
H	H1	1.08	1.09	1.0833 ± 0.005^{i}
	H2	1.04	1.06	1.05 ± 0.01^{jk}
	H3	0.21	0.22	0.21 ± 0.006^{w}
I	I1	0.83	0.85	0.84 ± 0.01^{o}
	I2	0.12	0.14	0.13 ± 0.01^{y}
J	J1	0.21	0.23	0.22 ± 0.01^{v}
	J2	1.44	1.46	1.45 ± 0.01^{f}

Values followed by the same letter(s) along each column are not significantly different by Duncan's multiple range. Data are means of 3 replicates

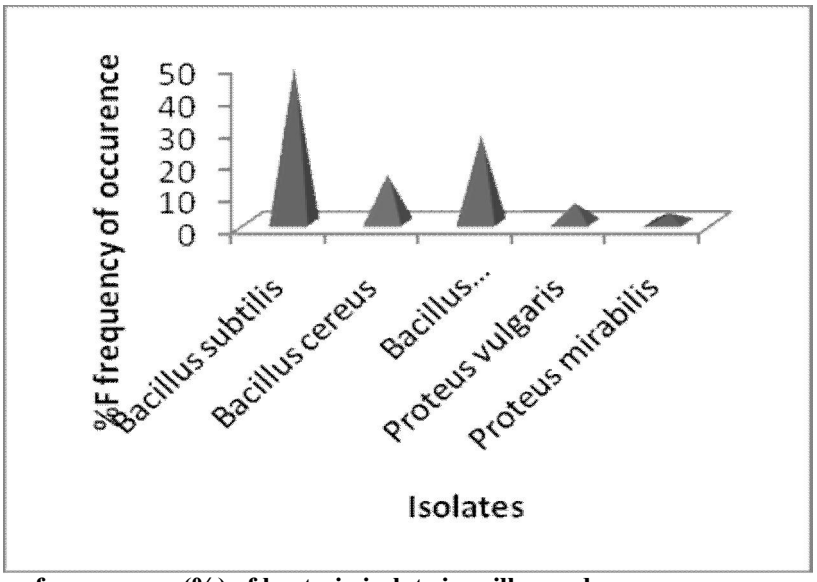

Figure 1: Frequency of occurrence (%) of bacteria isolate in milk powder

4.0 DISCUSSION

Milk powder as a good source of many nutrients consumed by both adult and children must be of good microbiological quality. Milking done under aseptic condition must be practically free from bacterial flora. Presence of different microorganisms in freshly produced milk may be due to the care employed in milking, cleaning, and handling of utensils (Alan and Heather , 1990). The result obtained shows that the sampled milk powder harbors a wide range of microorganisms. Generally, the overall assessment of the milk samples indicated that the microbial loads were within the permissible limits stated by New Zealand FDA [20], (10^{-2} - 10^{-3} cfu/g). The low incidence of microbial contamination found in this study indicates, consequently, a low contamination of the milk samples. The bacteria isolates encountered indicate possible contamination either during milking operation, transportation, storage, processing or packaging. The contamination could as well be from the environment and/ or inadequate handling and unsanitary conditions (ICMSF, 2005).

The bacterial isolates; *Bacillus subtilis*, *Bacillus cereus and Bacillus licheniformis* have been reported in the past to be found in feeds and because they are spore forming bacteria, the spores can survive the passage through the alimentary tract of dairy cow, and are excreted with feces (Klijn *et al.,* 1995; Cocolin *et al.,* 2004;Le Bourhis *et al.,* 2005).

Species of *Bacillus* are associated with the spoilage of heat-treated dairy product thereby reducing the shelf-life (Te Giffel *et al.* 1997). The spores of *Bacillus* species are ubiquitous and can be isolated from plants, beddings materials, concentrated feeds, roughages and cattle feces (Le Bourhis *et al.,* 2005).. Several studies have indicated that silage is also a significant source of contaminating milk with Bacillus spores (Vissers *et al.,* 2007b), which is due to growth of spore-forming bacteria in poorly conserved silages. Microbiological and physicochemical quality of powdered soymilk has been reported (Adebayo-Tayo *et al.,* 2009)

The presence of *Proteus* species which belong to the family of Enterobacteriaceae in the milk sample is indicative of poor sanitary condition or contamination especially of fecal nature (Collins and Lyne 1984). *Proteus sp.* has been reported as causative agent of opportunistic infection in humans and urinary tract infection, wound infection, pneumonia and septicemia and these calls for concern (Prescott *et al.,* 1992).

About 80% of the milk sample analyzed showed the presence of aflatoxins in a range higher than acceptable level set by USFDA (2001) . Though there was no trace of fungal growth in the milk samples, yet aflatoxins were detected in the milk samples. This could be as a result of feeds used in feeding the cow which might have been contaminated with aflatoxins. This result is in agreement with the report of Kiessling *et al*., (1984) who stated that the presence of mycotoxin in dairy products reflects the contamination of feedstuff. It has been stated by USFDA (2001) that the maximum aflatoxin concentration in feeds for feeding cow should not exceed 20 ppb. The concentration of aflatoxin in feeds varies with location, because it is influenced by weather conditions during harvest and feed storage practice. Due to the fact that aflatoxin are not visible neither do they have a particular flavor, therefore it is not easy to convince consumers about their existence in food. Aflatoxins have been detected in food from different researches being carried out by different individual (Bankole and Mabekoje (2003); Adebayo–Tayo BC, *et al.* (2006); and Adebayo-Tayo BC*, et al.,* (2008)

CONCLUSION

In conclusion, while the microbial load of milk powder samples do not pose public health problem, the level of AFM_1 contamination call for serious attention in the country. The presence of AFM1 in the milk powder samples can pose a public health hazard which call for a need for controlling aflatoxin contaminated feedstuff and the use of contaminated feedstuff should be prohibited. The detection of AFM_1 in the milk powder samples could be of public health significance and hence there is an urgent need for concerned regulatory bodies to impose necessary measures to safeguard health of consumers. Further research work should be carried out on milk powder and other milk products on sale in Nigerian market.

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