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Impact of *Moringa oleifera* Seed extract (MSE) on bacterial load and sensory evaluation of raw milk

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Abstract
This study was carried out to evaluate the impact of Moringa oleifera seed extract (MSE) on bacterial load and sensory evaluation of raw milk. Moringa seed extract (MSE) was added to five treatment groups of 100ml each of raw milk at different levels; Control, 10%, 20%, 30% and 40% which represented T_1, T_2, T_3, T_4 and T_5 respectively. The treated milk samples were examined for microbial load at room temperature and analysis for the microbial load was carried out after 24, 48 and 72 hours. The samples were serially diluted and inoculated using the pour plate method. The result obtained indicated that inclusion of 40% MSE (T_5) reduced average total coliform count significantly ($P<0.05$) at 0, 24 and 48 hours compared to T_2, T_3 and T_4 which indicated no significant difference ($P>0.05$). Nevertheless, the total Coliform counts and total viable bacterial counts were $5x10^3$ and $1.5x10^3$ respectively which was lower than that obtainable from the analyzed raw milk result which was $2.42x10^4$ and $2.73x10^4$. However, sensory evaluation result revealed that 10% Moringa inclusion level scored highest implying most preferred compared to 20%, 30% and 40% inclusion levels. The organisms isolated were clostridium, pseudomonas, *E. coli*, Serratia, Aeromonas, Shigella and Bacillus.

Keywords: Raw milk, moringa seed, bacteria, sensory evaluation

Introduction
Milk is one of the main foodstuffs consumed by humans, and an important starting base for young ones of mammals. Milk is a white liquid nutrient rich food produced by the mammary glands of mammals. As one of the animal product, milk is gotten from Cattle, Goat, Sheep and Buffalo soon after parturition. However, Cow's milk dominates commercial production compared to Goat, Sheep and Buffalo. (Gerosa and Skoet, 2012) [12]. Nigeria is among the largest producer of cow milk in West Africa. Raw milk is used for different purposes such as production of cream, butter, yoghurt, kefir, ice-cream and cheese. Modern industries process milk into casein, whey protein, lactose, condensed milk, powdered milk, evaporated milk etc. Preservation of raw milk from microbial deterioration has traditionally been an important concern in dairy industry even as the demand for partial or complete substitution of synthetic preservative has been on an increase due to their adverse health effects. Consequently, emerging interest in discovering therapeutic plants that could inhibit microbial activities in raw milk for some hours after milking is advocated. (Bukar *et al.*, 2010; Aora *et al.*, 2013) [7]. In recent years, the use of plants as become popular in the food industry thereby focusing on the use of eco-friendly and bio- friendly plants which has antioxidant and antimicrobial effects that enhances shelf-life and safety of raw milk. The possibility of use of plants that are cheap and easily available such as *Moringa oleifera* plays function through pasteurization thereby serving as a cheaper source of microbes-free raw milk (Saadabi *et al.*, 2006) [25].Studies have also proven that diet rich in phenolic antioxidants have many health benefits thus confer longer life expectancy. Phenolic antioxidants are mainly present in leaves and seeds of some plants which prevent free radical mechanism in raw milk. *Moringa oleifera* is a good source of antioxidants and oil which is highly resistant to oxidation (Anwar *et al*., 2007) [3]. Moringa seeds possesses antimicrobial properties such as Lectin, Silvernano-particles, n-4 (α-L-rhamnopyranosyloxy)benzyl carbamate, 4-(β-D-glucopyranosyl-L) benzythiocarboxamide, 4-(α-L-rhamno pyranosyloxy) Benzyl Isothiocyanate, Lectins and Tannin which could flocculate gram-positive and gram-negative bacteria cells (Oluduro *et al.*, 2010, Peixoto *et al.*, 2011, Rim

Jeon *et al.*, 2014 and Dzotam *et al.*, 2016) [18, 20, 23, 10]. The aim of this research therefore is to determine the influence of different levels of Moringa seed extract on bacterial load of raw milk.

Methodology

The Study was carried out in Akwa Ibom State University, Nigeria. Akwa Ibom State falls within the rainforest zone of the humid tropics which is characterized by hot and humid climate. It is in the Niger Delta region of the South-South coastal part of Nigeria, lying 70m above sea level, located between latitude 4 º 58/ and 5 º 08/N and longitude 8º 02/and 9º 47/E, temperature range between 25.0 ºC and 26.0 ºC with average relative humidity of 75-80% while mean annual rainfall vary from 2250mm to 2926mm in a year (SLUS-AK, 1994) [29].

Raw milk was randomly collected from three (3) Bunaji cows at the livestock teaching and Research farm of the university. The milk was collected at 8.00am hours and immediately transferred to the laboratory before treated with different levels of Moringa Seed Extract.

Moringa seed collection, processing and preparation of extract

70 grams of Moringa seed was bought from Uyo main market in Akwa Ibom State, Nigeria. The seeds were dehulled and sundried for (3) three days before ground into powder and stored in an air-tight container. Twenty (20) grams of the powder was scooped and introduced into 100ml of distilled water, shaken vigorously and left for about 1 hour. The suspension was thereafter filtered using a Whatman filter paper No. 1. (Olayemi and Alabi 1994).

Preparation of treatment samples

Raw milk collected was shared into 5 containers of 100ml each labeled 1 to 5. The Moringa extract was subsequently added to 4 of the 100ml milk quantity at the rate of 10ml, 20ml, 30ml and 40ml and labeled as T_1 (without Moringa-control) T_2, T_3, T_4, T_5 treatment groups respectively. Each container was shaken to mix thoroughly before subjected to organoleptic and microbiological tests. Also, 100ml from the collected milk sample was pasteurized and subjected to microbiological tests.

Organoleptic taste

A panel of 9 judges was set up to carry out the palatability taste. Each member was given treatment samples to score accordingly.

Microbiological analysis of sample

Culture Media: The culture media used for the analysis were Nutrient and MacConkey agar. Differential media was used for identification of organisms.

Processing of sample: Samples were serially diluted before estimation of microbial-densities. The Total Viable Count (TVC) of bacterial and Total Coliform Count (TCC) were determined by the pouring plate and surface spread method respectively (APHA, 1992) [2] using the media mentioned above.

Incubation and growth for purification: The inoculated plates of Nutrient Agar and MacConkey Agar were incubated at 37^0c for the expected hours respectively. Colonies were counted after the incubated period. Growth from each plate was sub-cultured to have pure isolates for the characterization according to Perterson and Finegold (1990) [21] and Domsch *et al.*, (1980) [9].

Identification of bacteria isolates: The cellular morphology and biochemical characteristics of the microbial isolates were used for the identification of the isolates according to Garrity and Holt, (2001) [11]. Cultural characteristics like shape, colour, elevation, surface, edge as well as microscopic features were used for identification. The Bergey's manual of determinative bacteriology by Buchanan and Gibbons (1974), was used to compare the characteristics with the result obtained.

Gram Staining: A wet mount of each isolate was prepared, stained with crystal violet for 60 seconds, 10dine was then added for 60 second, the wet mount was subsequently flooded with 95% ethanol for 30 seconds, washed and SAFRANIN was added to counter stain for 1 min. It was rinsed with water, air dried and examined under a light microscope using x100 oil immersion objective lens. Gram positive organism appeared purple, violet while gram negative organisms appeared red or pink (Momea, 2004).

Catalase Test: The test demonstrated the presence of catalase enzyme characterized with the release of oxygen from hydrogen peroxide (H_2O_2). A drop of 3% of H_2O_2 solution was added to the microscope slide and a loopful of the organism was made to touch the drop of H_2O_2. Catalase production gave prompt effervescence of oxygen while absence of effervescence showed negative reaction (Sohani and Sanjeeda, 2012) [27].

Motility test: Methods as described by Sohani and Sanjeeda (2012) [27], was used. The test tubes were inoculated with the test organisms aseptically and were incubated at 37^0c for 3-5 day for motility test, the turbidity from the surface movement down ward indicated to that the organisms were motile.

Indole test: This was carried out through the addition of 0.5ml of Kovac's reagent on the test organisms for the examination of red colour change after 10mins. The observation of red colour indicated positive while negative was indicated by no colour charge.

Oxidase Test: Filter paper was used for this test. At loop full test of organism was placed on the filter paper and soaked with the oxidase reagent, the presence of purple colour indicated positive while the reverse negative.

Voges-proskaver test: To the 2-5ml suspension of the tested organism, 0.6ml of the Voges-Proskaver reagent. A was added followed by 0.2ml of Voges-Proskaver B. The suspension of the tested organisms was shook and allowed to sit for 15mins. The observation of a pink to red colour indicated positive while yellow or colourless indicated negative.

Data Collection: Data was collected at the expiration of 24 hours incubation period of each sample inoculated in different media plates. The different colony growth was counted and recorded thrice as Total Viable Bacterial Count (TVBC) and

Total Coliform Count (TCC) as the samples were kept for 72 hours duration.

Statistical analysis: Statistical analysis was performed using IBM SPSS version 20.Data was analyzed utilizing one way analysis of variance (ANOVA) with the test of assumption of homogenous of variance. Post hoc analysis for pair wise comparison was done for statistical significant difference. Least significance difference (LSD) was used where the assumption of homogenous of variance was assumed and Games-Howel where assumption was violated. Mean difference was considered significant at p-value less than 0.05 (P<0.005).Pair wise comparison between groups were indicated in superscripts, values with the same superscript was not significant while the values with different superscript were significant at p-value less than 0.05

Results and Discussion

Table 1: Average Total Viable Bacterial Count (cfu/ml) at different concentration of Moringa seed extract (MSE) across 72 hours duration

	Treatment					
Duration	**T1 (control)**	**T2 (10%)**	**T3 (20%)**	**T4 (30%)**	**T5 (40%)**	**SEM**
0 hour	198.00	124.33	82.34	65.00	17.33	2.76
24 hours	273.00a	198.67	156.67a	105.00a	36.33a	2.64
48 hours	289.00a,b	285.67	284.67a,b	273.67a,b	261.33b	0.28
72 hours	307.33b	297.67	296.33b	291.33b	257.00c	0.53

[a, b, c] means with same superscript within groups are not significant while means with different superscript are significantly different at (P<0.05). T_1 = control, T_2 =10% inclusion of MSE, T_3 =20% inclusion of MSE, T_4 =30% inclusion of MSE and T_5 =. 40% inclusion of MSE

The results in Table 1 indicate impact of moringa seed extract on average total viable bacterial count and total coliform count of milk. The average colony count at different concentration of Moringa seed extract across 72 hours duration. At T_2 concentration, the mean colony count increased with duration although the difference between the means was statistically not different (P> 0.05). At T_3 concentration, the mean difference of the colony count was significant (P < 0.001), on post-hoc analysis the mean count at 72 hours was significantly higher than 24 hour but not higher than 48 hours. Result from table 2 showed the mean Coliform count at different MSE concentrations across the duration of 72 hours. The average Coliform count across the duration in the first three treatments (T_2, T_3 and T_4) was non-significant (P> 0.05), but however, became significant at T_5 as it showed an ascending order of Coliform increase. The colony counts at 48 and 72 hours were significantly higher than 24 hours duration; this finding suggests that the microorganisms might have been suppressed by the MSE at 0 and 24 hours.

Furthermore, increase in microbial load of milk as shown in Table 1 and 2 as duration increased is supported by the investigation by Masika and Afoloyan (2002) who reported that gram negative bacteria are more resistant to water extracts because as reported by most researchers; Paz *et al.*, (1995), Martini *et al.*, (1998) and Yibeltat *et al.*, (2013), water extracts of plants do not have much activity against bacteria. Perhaps water extract is different from other solvents which have compounds that may interact antagonistically in their overall activities. In addition to this, the active compounds of plant materials are not readily extractable in water. Nevertheless, the reduced activity of water extracts against microbes is in agreement with previous studies that showed that aqueous extracts of plants generally exhibit little or no antimicrobial activities (Ashafa *et al.*, 2009, Aiyegoro *et al.*, 2012) [5, 1].

Table 2: Average total coliform count (TCC) (cfu/ml) at different concentration of moringa seed extract (MSE) across 72 hours duration

	Treatment					
Duration	**T1 (control)**	**T2 (10%)**	**T3 (20%)**	**T4 (30%)**	**T5 (40%)**	**SEM**
0 hour	198.00	124.33	82.34	65.00	17.33	2.76
24 hours	242.00a	237.67	216.33	99.00	36.33 a	2.89
48 hours	283.33a,b	240.00	175.00	185.67	178. 33b	1.30
72 hours	308.67 b	180.33	257.33	224.00	211.33b	1.28

[a,b,c] means with different superscript are significantly different at (p<0.05). T_1 = control, T_2 = 10% inclusion of MSE, T_3 = 20% inclusion of MSE, T_4 = 30% inclusion of MSE, T_5 = 40% inclusion of MSE.

Table 3: Sensory Evaluation of Raw Milk

Milk sample	Flavour	Colour	Overall acceptability
A (Milk + 10% MSE)	7.3^{a}	7.8^{a}	7.5^{a}
B (Milk + 20% MSE)	6.7^{a}	7.1^{abc}	5.1^{b}
C (Milk + 30% MSE)	5.5^{b}	6.3^{bc}	4.2^{b}
D (Milk + 40% MSE)	5.3^{b}	6.1^{c}	4.5^{b}
E (Milk with no MSE)	7.1^{a}	7.3^{ab}	8.1^{a}

[a,b,c] means on the same row with different superscript are significantly different (P<0.05).

Sensory Evaluation of Raw Milk Fortified with MSE

Result from Table 3, shows the mean values of the sensory scores for raw milk mixed with Moringa seed extract (MSE). The analysis of variance in appendix 4, 5 and 6 showed significant differences for colour, flavour and overall acceptability (P< 0.05). The results however reveals that milk not treated with MSE (E) was more preferred than others. This was followed by milk fortified with 10% of MSE (A), 20% of MSE (B) then 30% MSE (C) and lastly 40% MSE (D). The reason for the consumers scoring milk with 40% MSE inclusion lowest suggests the higher quantity of MSE inclusion which must have affected the taste of the raw milk; however, no adverse effect was caused by MSE on the colour, taste and overall acceptability of raw milk.

Identification of Bacteria and Coliform Isolates from Raw Milk Fortified with MSE

The biochemical characteristics of bacterial isolates from raw milk fortified with MSE samples are represented in Table 4 and 6. The probable organisms identified were Bacillus, Serratia, Aeromonas, Pseudomonas, Shigella, Clostridium and Escherichia. The result supports

Table 4: Identification of Coliform Isolate from Raw Milk Fortified with MSE

Gram reaction	Shape	Catalase	Indole	oxidase	Motility	Probable organism
-	Circular	+	-	-	-	Bacillus
-	Rod	+	-	-	-	Shigella
-	Rod	+	+	+	+	E. coli

Table 5: Identification of Bacteria Isolates Isolate from Raw Milk Fortified with MSE

Gram reaction	Shape	Catalase	Indole	Oxidase	Mortility	Protable organism
-	Curve	+	-	-	+	Serratia
-	Rod	+	+	+	+	Aeromonas

The biochemical characteristics of bacterial isolates from raw milk fortified with MSE samples are represented in Table 4 and 5. The probable organisms identified were Bacillus, Serratia, Aeromonas, Pseudomonas, Shigella, Clostridium and Escherichia. The result supports the findings of Upman (2002) [28] who reported the frequent occurrences of these microbes in milk. From the result, an indole positive and oxidase negative indicated the presence of *Escherichia coli* that possessed a gram negative reaction. The other probable organisms detected such as Bacillus, Clostridium and Shigella tested negative respectively in the indole reagent and gram reaction. This result supports the study by Nwankwo *et al.*, (2015) [17]. It is important to note that the presence of *Escherichia coli* in the result does not necessarily indicate a direct faecal contamination of milk, but is considered to be an indicator of poor hygiene and sanitation during milking and post manipulation. The presence of these bacteria in milk can also be linked to contamination of water used (Chyee *et al.*, 2004) [8]. Also, the presence of Pseudomonas specie and Coliform has been elucidated by Kletor (1984) which supports this finding.

Conclusion

In conclusion, the results obtained showed that Moringa Seed Extract at 40% could reduce the microbial load of raw milk from 0 to 24 hours. It is therefore recommended that more than 20g of Moringa Seed Meal could be used in preparing the seed extract.

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