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Full Length Research Paper

Production and evaluation of nutrient-dense complementary food from millet (*Pennisetum glaucum*), pigeon pea (*Cajanus cajan*) and seedless breadfruit (*Artocarpus altillis*) leaf powder blends

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Millet (M) and pigeon pea (P) were fermented (12, 24, 36 and 48 h) and milled into flours used in formulation of complementary foods. The 0 h sample served as the control. These flours and their blends were analysed for functional properties, chemical composition and microbiological count using standard methods. The flours were blended as B_{55} (M+P flours + breadfruit leaf powder (A) blends, 55:30:15), C_{60} (M+P flours + breadfruit leaf powder blends, 60:30: 10), D_{65} (millet + pigeon pea flours + *Artocarpus altilis* blends, 65:30: 10) and E_{70} (M+P flour blends, 70: 30). The best blend was obtained from the nutritional composition and used for the incorporation of 0 to 15% *A. altilis* powder. The best flours were used for the formulation of the complementary diet. Results showed 36 h samples had the highest protein value (11.84 and 30.88 %) compared to the other flours. Based on this, the flours were blended together and fortified with graded levels of dry milled seedless breadfruit leaf (5, 10 and 15%). The blends showed high protein content B_{55} (11. 84 %), C_{60} (16.91 %), D_{65} (14.59 %) and E_{70} (15.78 %) compared with the single millet flours Mo (9.65%), M_{12} (9.82%), M_{24} (11.57 %), M_{36} (11.84 %) and M_{48} (8.37), and with FAO standard protein value of 10%. The composite flour had very high vitamin A, and calcium content due to the incorporation of the breadfruit leaf powder. The breadfruit leaf powder had 13111.07 IU of vitamin A, and 9.10 mg/100g of calcium. Fermentation increased the bulk density, oil absorption capacity, water absorption capacity, swelling index and least gelation concentration of single flours of M+P flours unlike the blends. The total viable and mould counts were within the same range for both the single and composite flours, and are safe for human consumption when compared with $<10^6$ cfu/g as recommend by microbiological standards.

Key words: *Artocarpus altilis* leaf extract, complementary foods, fermentation, millet, pigeon pea.

INTRODUCTION

Breast milk is the ideal food for infants during the first six months of life. It contains still undiscovered substances that cannot be reproduced artificially and its overall nutrient composition is superior to any alternative, including infant formula. Breast feeding is considered best for infants from nutritional and immunological points of view as well as for protection against *Campylobacter*-associated diarrhoea (Nout and Ngoddy, 1997). In developing countries, most infants show satisfactory growth for the first four to six months when breast milk solely meets the nutritional needs but after this period, it may become increasing inadequate as the nutritional

demands of the infant increases (Nkama et al., 2001). In spite of its superiority, breast milk cannot provide all of the nutrients and calories that allow infants to thrive after the first six months, more nutritious foods should gradually be introduced to different types of semi-solid, solid or complementary foods as they gradually transition from a diet-centered on breast milk.

Complementary foods are foods that are readily consumed and digested by the young children, and that provide additional nutrition to meet all growing child's needs. Complementary foods (commonly known as weaning foods) are formulated to satisfy nutritional needs of infants and young children. A true complementary food would add to the diet nutrients such as iron and zinc, which breast milk has not evolved to provide for older infants as the child gradually outgrows her birth stores (Gabrielle, 2009). Weaning is a gradual process during which breast milk increasingly complemented with other foods that fully meet the young child's needs. It is a process of gradual introduction of other foods into a baby's diet to complement breast milk and progressively replace it and eventually adapting the child to adult diets (Nkama et al., 2001).

In Nigeria, as in most developing parts of the world, most people depend on plant foods for dietary needs particularly cereals and legumes. Such legumes like pigeon pea, cowpea, African yam bean, soya bean among others, serve as good sources of dietary protein. These play important role in the diets of many people including children. Many countries have exploited cheap locally available plant materials from cereals and legumes to developed weaning foods (Nkama et al., 2001).

In Nigeria, the usual first weaning food is "pap" referred to as "akamu" by Igbos, "ogi" by Yorubas or "koko" by Hausas. It could be made from maize (Zea mays), millet (Pennisetum americamum) or guinea corn (sorghum species) or a combination of the cereals (Onofiok and Nnanyelugo, 1998). Fermented cereal has been popularly used as complementary infant food in Nigeria, where it is referred to as "ogi." "Ogi" is a smooth creamy, free flowing thin porridge obtained from wet-milled, fermented maize, sorghum or millet. The preparation involves dilution of the fermented product with water and boiling with constant stirring to desired consistency (Johanson et al., 1995). The major disadvantage of sole cereal gruel is that the starchy nature of these foods makes them bind so much water, thus yielding a bulky gruel with decreased nutrient content, and also cereals have lower protein quality compared to legumes (Marero et al., 1988).

Traditional weaning foods in West Africa are known to be of low nutritive value (Guiro et al., 1987), and are characterized by low protein, low energy density and high bulk, cereal based diets have been implicated in protein-energy malnutrition. Guiro et al. (1987) similarly noted that the traditional millet gruel used for weaning Senegalese children was not energy dense and was insufficient to cover all the nutrient needs of the infant. The problems inherent in the traditional West Africa weaning foods and feeding practices predispose the infants to malnutrition, growth retardation, infection and high mortality. The first Nigeria Nutrition Network of 2002 identified poor feeding practices and/ or shortfall in food intake, as the most important direct factors responsible for malnutrition and illness amongst children in Nigeria.

As in most other developing countries, the high cost of fortified nutritious proprietary complementary foods is always, if not prohibitive, beyond the reach of most Nigerian families. Such families often depend on inadequately processed traditional foods consisting mainly of un-supplemented cereal porridges made from maize, sorghum and millet. The amino acid compositions of the proteins in the cereal grains are generally low in the contents of lysine (Manay and Shadaksharawamy, 2006). The proteins in legumes have a well-recognized deficiency of the essential sulphur-bearing amino acids namely methionine and cysteine, but are comparatively rich in lysine (Ihekoronye and Ngoddy, 1985).

Therefore, blending cereal with legume would help to improve nutrient density of the complementary food and improve nutrients intake, which resulted in the prevention of malnutrition problem. Protein malnutrition is wide spread among the poor of developing and under developed countries. Since animal's protein is beyond the reach of this group, their primary protein supply comes from plant based products. Amongst these pigeon pea or red grain (Cajanuscajan (L) millspaugh) is an important food legumes that can be grown under rainfed conditions with least inputs. Pigeon pea is rich in starch, protein, calcium, magnesium, crude fiber, fat, trace elements and minerals. Beside its high nutritional value, pigeon pea is also used as traditional folk medicine in India, China and some other countries. Literature on this aspect show that pigeon pea is capable of preventing and curing a number of human ailments such as cough, pneumonia, respiratory infections, dysentery, tooth ache, wounds among others (Saxena et al., 2010).

Micronutrient deficiencies are common during infancy, and optional approaches for their prevention need to be identified. Micronutrient deficiency is a challenge, particularly in developing countries and deficiencies of iron and other micronutrients including zinc remain public health problems in these countries (Bhaskaram, 2002).

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The deleterious effects of iron deficiency on cognitive development in infants are well documented, whereas zinc deficiency has been implicated in growth faltering (Brown et al., 2002) and increase morbidity, particularly diarrhoea disease and pneumonia (Bhutta et al., 1999).

The Ministry of Health, Nigeria, 2012 emphasized that a child could get protein energy malnutrition when his food does not contain sufficient amount of protein and energy. Protein-energy malnutrition is a disease of children under 5years caused by inadequate intake of food. This disease can be associated with other communicable diseases such as diarrhoea and respiratory tract infection. Marasmus and kwashiorkor are the severe forms of this disease. A number of cereals and legumes that are readily available have been found to have nutrient potentials that could complement one another if properly processed and blended (Fernandez et al., 2002; Oguntona and Akinyele, 1995). Therefore, it is imperative that efforts to formulate composite blends and scientific studies are carried out to ascertain the nutritive adequacy of these locally available blends (cereal and legume) for possible use as complementary foods, especially by the rural and poor urban mothers during the period of weaning.

Most of the previous works on complementary food production were based on cowpea, soyabean, maize and rice. These materials have also been competitively used for different industries, researchers among others. However, millet is a cereal in which its protein and caloric content are comparable to maize and rice but people have been eating maize and rice due to the knowledge of what they derive from the food in terms of calories and proteins but little have been known about the nutritive of millet. Therefore, the knowledge (that millet's caloric and protein contents are comparable to that of maize and rice) brings millet to lime light as a good quality protein and caloric food source. Millets are group of highly variable small-seeded grasses, widely grown around the world as cereal crops or grains for both human food and feeder. They do not form a taxonomic group but rather a functional or agronomic one. Millets are important crops in the semi-arid tropics as of Asia and Africa (especially in India, Nigeria and Niger Republic), with 97% of millet production in developing countries (McDonough et al., 2000).

Millet, like sorghum is predominantly starchy. The protein content is comparable wheat and maize. Pearl millets are higher in fats. Millets are also relatively rich in iron and phosphorus (FAO, 1995). Finger millet has the highest calcium content among all the food grains. Millets are rich in B vitamins (especially niacin, B_6 and folic acid), calcium, iron, potassium, magnesium and zinc.

Legumes are the species of plants family *Leguminosae*, which are good sources of dietary protein. Examples are cowpea, pigeon pea, soyabean, groundnut, and bambara nut. Pigeon pea has been reported to contain high quality protein and is a good source of amino acids except methionine (Elegbede, 1998). Legumes are rarely used for weaning and are introduced much later (after six months of age) because of the problems of indigestibility, flatulence and diarrhea associated with their use (King et al., 1985). Pigeon peas are an important legume crop of rainfed agriculture in the semiarid tropics. Pigeon peas are both a food crop (dried peas, flour, or green vegetable peas) and a forage/cover crop. In combination with cereals, pigeon peas make a well balanced human food (Akporhonor et al., 2006). Pigeon pea contains high levels of protein and important amino acids (methionine, lysine and tryptophan).

Artocarpus altilis (seedless breadfruit) is 100 feet (30m) tall, with large, spreading branches and a straight trunk with smooth gray bark. Leaves large, 16 to 20 inches (40 to 50cm) wide and 24 to 35 inches (60 to 90cm) long, usually with 5 to 11 deeply out lobes. In Trinidad and the Bahamas, a leaf of *A. altilis* is used to lower blood pressure and relieve asthma. It is also used in the treatment of diarrhea (Zerega et al., 2004).

Cereal gruel processing methods have resulted in the loss of nutrients other than protein. Makinde and Lachance (1976) reported a 95% loss of the original tryptophan in maize during the processing of "ogi". Large losses of niacin during processing which treatment of ogi were reported which could account for the high incidence of pellagra in the area (Onofiok and Nnanyelugo, 1998). This method of processing which leads to micronutrient deficiency in complementary food could be averted by complementation of millet (cereal) with pigeon pea (legume) and fortifying it with *A. altilis* leave powder. Therefore, blending cereal (millet) with legume (pigeon pea) fortified with *A. altilis* leaves would help to improve nutrient density of the complementary food and improved nutrients intake, which resulted in the prevention of problem of malnutrition. The addition of *A. altilis* would also help in the prevention of diarrhoea since it is medicinal.

The main aim of the study is to formulate and evaluate the nutrient-dense complementary food produced from millet, pigeon pea fortified with graded level of bread fruit leaf powder for proximate, vitamin, mineral compositions and functional properties of the flours and their blends as well as the microbiological quality of the formulated complementary food.

MATERIALS AND METHODS

Procurement of raw materials

The seeds of millet and pigeon pea were purchased from Ogige market in Nsukka while the *A.altilis* leaves were obtained from Owere-Obukpa farm in Nsukka, Enugu State.

Processing of millet flour

Millet seeds (3 kg) each were manually cleaned/ winnowed,
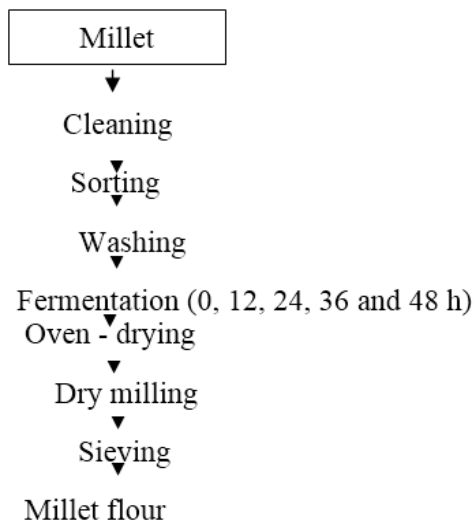
Figure 1. Flow diagram for production of millet flour (Source: Modified method of production of Ogi from Odunfa and Adeyele (1985)).

Figure 2. Flow diagram for the production of pigeon pea flour.

weighed and fermented for 12, 24, 36 and 48 h. The fermentation process was by endogenous micro flora in the seeds at room temperature as described by Onuoha and Obizoba (2001). The fermented grains were oven-dried, milled and sieved by passing through a 1mm pore size sieve to yield flour. The flour was packaged in air tight container and stored for analysis (Figure 1).

Processing of pigeon pea flour

Pigeon pea seeds (2 kg) each were cleaned, weighed and

Figure 3. Flow diagram for the production of *A. alitilis* leaf powder.

fermented for 12, 24, 36 and 48 h. The fermented seeds were oven-dried, milled and were passed through a 1 mm pore size sieve to yield flour. The flour was packaged in air tight container and stored for analysis (Figure 2).

Processing of leaves

A known quantity of *A. atilis* leaves (500 g) were harvested cleaned, oven-dried, milled and sieved with 1 mm sieve to yield leaf powder. The leaf powder was packaged in air tight bag and stored for analysis (Figure 3).

Analysis of samples

The proximate analysis, vitamin and mineral content of each sample (single flour) were determined. This was used as the basis for selecting the best samples. After the initial analysis of each sample, the best two samples from millet flour and pigeon pea flour were blended together with *A. altilis* leaf powder. After blending, the ratios results shown in Table 1. Further analysis were carried out to determine the proximate composition, mineral content, vitamin contents, some selected functional properties and microbial content of the blended samples.

Proximate analysis of millet, pigeon pea flour, *A. altilis* leaf powder, their blends and products

Determination of crude protein

The crude protein content was determined by the micro-Kjeldahl AOAC 920.53 method as described by AOAC (2010).

Determination of moisture content

The hot air oven method as described by AOAC method 925.10 (AOAC, 2010) was used in determination moisture content.

Table 1. Proportion of millet flour, Pigeon pea, flour and *A. altilis* leaf powder used for complementary food formulation.

Millet flour	Pigeon pea flour	*A. altilis* leaf powder
70	30	0
65	30	5
60	30	10
55	30	15

Determination of fat content

This was determined using the Soxhlet extraction with petroleum ether according to AOAC 920.39 method as described by AOAC (2010).

Determination of total ash content

The AOAC 923.03 method described by AOAC (2010) was used in determining the total ash content.

Determination of crude fiber

Crude fiber content was determined by the AOAC 962.09 method described by AOAC (2010).

Determination of carbohydrate

Carbohydrate content was determined by difference as described by Oyenuga (1968) as shown: % carbohydrate = 100 – (% moisture + protein + % ash + % crude fibre)

Determination of mineral content

The mineral content was determined using Atomic Absorption Spectroscopy after wet digestion method as described by Adeyeye and Ajewole (1992).

Determination of vitamin A

Vitamin A content was determined by the procedure described by Jakutowicz et al. (1997).

Determination of vitamin B_1

Vitamin B_1 was determined by the procedure described by Koche et al. (2008).

Microbial analysis

Plate count for bacteria and yeast

Plate count for both bacteria and yeast was done using the method described by Jideani and Jideani (2006).

Determination of some selected functional properties of the blend of millet flour, pigeon pea flour and *A. altilis* leaf powder

Determination of bulk density

The bulk density was determined by the method described by

Nwanekezi et al. (2001).

Determination of viscosity

The viscometer was used in characterizing the flow behavior of each of the sample respectively. The viscosity of porridges from sample flour was determined according to the method described by Sathe and Salunkhe (1981), using the Gallenkamp Universal Torsion Viscometer.

Determination of particle size distribution

The particle size distribution was determined by the method as described by Ihekoronye and Oladunjoye (1988).

Determination of wettability

The wettability of the samples was determined by the method as described by Onimawo and Akubor (2012).

Determination of water /oil absorption capacity

The method described by Onimawo and Akubor (2012) was used in determination of water absorption capacity.

Determination of least gelation concentration

The least gelation concentration was determined according to the method described by Onimawo and Akubor (2012).

Determination of swelling index

Swelling Index was determined using the method of Onimawo and Akubor (2012).

Data analysis and experimental design

The design of the experiment for the blends was done using completely randomized design (CRD). Data analysis was carried out using one-way analysis of variance (ANOVA). Mean separation was done by Duncan's New Multiple Range Test with Statistical Package for Social Sciences (SPSS) version computer software 20. The determinations were done in duplicate and significance was accepted at $p < 0.05$ according to Steel and Torrie (1980).

RESULTS AND DISCUSSION

The discussions are on dry basis.

Effect of fermentation on proximate composition of millet and pigeon pea flour

Table 2 and 3 show the proximate composition of the unfermented and fermented millet and pigeon pea flours. The crude protein content of the unfermented millet was 9.65% while for the various fermentation regimes were 9.82±0.03 % (12 h), 11.57±0.04 % (24 h), 11.84±0.04 % (36 h), 8.37±0.05 % (48 h) as shown in Table 2.

Table 2. Proximate composition (%) of unfermented and fermented millet flour.

Samples	Crude protein	Ash	Fats	Moisture	Crude fibre	Carbohydrate
M_0	$9.65^c \pm 0.00$	$1.06^d \pm 0.02$	$2.50^d \pm 0.55$	$4.20^d \pm 0.02$	$3.45^a \pm 0.05$	$79.19^a \pm 0.78$
M_{12}	$9.82^c \pm 0.03$	$1.35^c \pm 0.20$	$4.60^b \pm 0.06$	$5.68^b \pm 0.05$	$3.43^a \pm 0.22$	$75.14^c \pm 0.11$
M_{24}	$11.57^b \pm 0.04$	$1.67^a \pm 0.01$	$4.96^a \pm 0.03$	$5.22^c \pm 0.07$	$2.96^b \pm 0.15$	$73.65^c \pm 0.00$
M_{36}	$11.84^a \pm 0.04$	$1.53^b \pm 0.00$	$4.82^{ab} \pm 0.09$	$5.84^b \pm 0.03$	$2.72^b \pm 0.15$	$73.36^d \pm 0.19$
M_{48}	$8.37^d \pm 0.05$	$1.09^d \pm 0.02$	$3.77^c \pm 0.08$	$7.32^a \pm 0.06$	$1.13^c \pm 0.01$	$78.56^b \pm 0.27$

Values are means SEM of means of duplicate determinations. Values bearing different super-scripts in the same column are significantly different (p<0.05); Key: M0® unfermented millet flour; M12®12h fermented millet flour; M24® 24 h fermented millet flour; M36® 36 h fermented millet flour; M48 ®48 h fermented millet flour, M®millet flour.

Table 3. Proximate composition (%) of unfermented and fermented pigeon pea flour.

Samples	Crude protein	Ash	Fats	Moisture	Crude fibre	Carbohydrate
P_0	$22.36^d \pm 0.02$	$3.10^c \pm 0.00$	$2.56^a \pm 0.05$	$7.44^d \pm 0.04$	$4.38^a \pm 0.02$	$60.16^b \pm 0.06$
P_{12}	$21.85^d \pm 0.05$	$3.43^b \pm 0.10$	$1.10^d \pm 0.00$	$7.58^c \pm 0.07$	$2.61^b \pm 0.07$	$63.43^a \pm 0.04$
P_{24}	$28.65^b \pm 0.07$	$3.56^a \pm 0.03$	$2.48^{ab} \pm 0.07$	$8.26^b \pm 0.08$	$2.81^b \pm 0.03$	$54.70^c \pm 0.11$
P_{36}	$30.88^a \pm 0.04$	$3.20^c \pm 0.01$	$2.29^b \pm 0.04$	$8.26^b \pm 0.08$	$2.81^b \pm 0.03$	$52.56^d \pm 0.05$
P_{48}	$27.18^c \pm 0.21$	$3.12^c \pm 0.04$	$2.05^c \pm 0.04$	$8.99^a \pm 0.08$	$4.37^a \pm 0.08$	$54.28^c \pm 0.01$

Values are means±SEM of means of duplicate determinations. Values bearing different super-scripts in the same column are significantly different (p<0.05); Key: P_0 .→ Unfermented pigeon pea flour; P_{12}→ 12h fermented pigeon pea flour; P_{24} → 24h fermented pigeon pea flour; P_{36} → 36h fermented pigeon pea flour; P_{48}→ 48h fermented pigeon pea flour, P→pigeon pea flour.

Fermentation for 12 h showed a non-significant (p>0.05) increase in the crude protein level of millet by 1.76% of that originally present. Also, fermentation for 2 4h and 36 h significantly (p<0.05) increased the crude protein level of the millet by 19.89 and 22.69% respectively.

Furthermore, fermentation for 48 h significantly decreased (p>0.05) the crude protein level by 13.26%. The increase in crude protein content of the 36 h fermented millet indicates that 36 h was the optimum fermentation period for protein synthesis by the fermenting micro flora of the sample. The decrease in crude protein level of 48 h could be due to the fact that the fermenting micro-organisms have started using the nutrient in the millet. Also, the decrease in the 48 h crude protein level of the millet is not in agreement with Nwabugwu (2005), who reported increase in the protein content of millet after 48 h fermentation (72 h fermented millet 9.46%). The protein value (Table 3) for the unfermented pigeon pea was 22.36% while for the various fermentation regimes, were21.85 (12 h), 28.65 (24 h), 30.88 (36 h) and 27.18% (48 h). Fermentation for 24, 36 and 48 h increased the crude protein content of the pigeon pea by 28.13, 38.10 and 21.56%, respectively while the 12 h fermentation decrease the protein level by 2.28%. The 36 h fermentation is the optimum fermentation period for pigeon pea and it could be due to the release of free amino acids for protein synthesis (Egbekun, 1998).

The decrease in protein content at 48 h could be probably that microorganisms had used up the nutrient for their growth and metabolism (Ariahu et al., 1999). This decrease did not agree with Onuoha and Obizoba (2001), who reported an increase in the protein content of lima beans after 48 h fermentation. The fat content of fermented millet flour varied from 4.60 (12 h), 4.96 (24h), 4.82 (36h) to 3.77% (48h) with 24 h fermentation having the highest value (4.96 %) and unfermented millet flour had a value of 2.5%. Fermentation significantly (p< 0.05) increased the fat content of the millet flour. The increase in the fat content of millet with fermentation could be attributed to the ability of microorganisms to break up oil cells.

According to Obizoba and Atii (1994), there is an increase in fat content of millet with fermentation. Table 3 shows the fat content of fermented pigeon pea flour. The fat contents of fermented pigeon pea flour varied from 1.10 (12h), 2.48 (24h), 2.29 (36h) and 2.05% (48 h) respectively while the unfermented pigeon pea had the value of 2.56 %. The fat contents were found to be significantly lower in the fermented pigeon pea flour than in un-fermented pigeon pea flour. This decrease in fat contents might be attributed to the increased activities of the lipolytic enzymes during fermentation which hydrolyses fat components into fatty acid and glycerol (Adebowale and Maliki, 2011).

The ash content of the fermented millet flour range

Table 4. Mineral and vitamin contents of fermented and unfermented millet flour.

Samples	Iron (mg/100 g)	Zinc (mg/100 g)	Calcium (mg/100 g)	Vitamin A (IU)	Vitamin B$_1$ (mg/100 g)
M$_0$	7.08^a±0.00	0.45^c±0.03	2.00^c±0.00	33.02^e±0.01	0.36^e±0.02
M$_{12}$	4.19^c±0.02	0.62^b±0.01	2.04^c±0.05	33.29^d±0.78	0.58^d±0.01
M$_{24}$	4.31^b±0.00	0.77^a±0.01	5.00^b±0.00	60.55^b±0.13	0.61^c±0.00
M$_{36}$	2.95^d±0.03	0.77^a±0.01	6.05^a±0.15	48.24^c±0.43	0.68^b±0.01
M$_{48}$	7.07^a±0.05	0.79^a±0.01	4.50^b±0.30	68.35^a±1.76	0.82^a±0.00

Values are means±SEM of duplicate determinations. Values bearing different super-scripts in the same column are significantly different ($p<0.05$); Key: M$_0$→ unfermented millet flour; M$_{12}$→ 12 h fermented millet flour; M$_{24}$→ 24 h fermented millet flour; M$_{36}$ → 36 h fermented millet flour; M$_{48}$→ 48 h fermented millet flour, M→millet flour.

from 1.06 to 1.67%, and unfermented millet flour had a value of 1.06% while highest value was recorded (1.67 %) at 24 h fermentation. Also, the ash content of pigeon pea (Table 3) flour ranged from 3.10 to 3.56% and unfermented pigeon pea flour had a value of 3.10% while 24 h fermentation period had the highest value (3.56%). There were gradual increase in the ash content of both fermented millet flour and fermented pigeon pea flour. There were significant different ($p>0.05$) in the ash content of millet but for the pigeon pea, apart from P$_{12}$ and P$_{24}$, there were no significant difference($p < 0.05$) in the other samples. However, fermentation increased the ash content of both millet and pigeon pea flours.

The fermented samples showed comparable crude fiber content, which varied from 1.13 to 3.45% in millet and unfermented millet sample had the value of 3.45% while in pigeon pea (Table 3) the sample varied from 2.61 to 4.37% and the unfermented pigeon pea had the value of 4.38%.There were slight difference in crude fibre of both millet and pigeon pea. There were decreases in crude fibre of millet as the fermentation time increased but decreased the crude fibre of pigeon pea.

The moisture content of the millet flour ranged from 4.2 to 7.32% with unfermented millet flour having the value of 4.2% and 48h fermentation recorded the highest value (7.32%). Furthermore, the moisture content of the pigeon pea (Table 3) flour ranged from 7.44 to 8.99% with unfermented pigeon pea having the lowest value (7.44%) and 48 h fermentation having the highest value (8.99%). There were gradual increases in moisture content of both millet and pigeon pea with increase in fermentation. The decrease in moisture content of unfermented samples might probably be due to its low dry matter content while the increase in moisture content of both fermented millet and pigeon pea were because of the moisture absorbed during fermentation.

The carbohydrate content of the fermented millet flour ranged from 73.36 to 78.56% while the unfermented millet had the value of 79.19 %. Also, the carbohydrate level of the fermented pigeon pea flour ranged from 52.56 to 63.43%, and the unfermented pigeon pea value is 60.16%. The carbohydrate content of the flour varied with different fermentation periods. The carbohydrate content

of millet fermented for 48h showed a significantly ($p<0.05$) increase than the other fermented millet samples. There was a reduction of carbohydrate content of pigeon pea as the fermentation time increased and in millet after 24 and 36 h fermentation period. This reduction in carbohydrate content could be attributed to possible hydrolysis of complex carbohydrate to simple sugars, which were used for metabolic processes (Nnam, 2001).

Effect of fermentation on the mineral and vitamin contents of fermented millet and pigeon pea flour

Table 4 and 5 shows the mineral and vitamin content of fermented millet and pigeon pea flour. There were a lot of variations in the iron content of millet flour. Fermentation decreased the iron content of millet. The samples were significantly ($p<0.05$) different from each other, it was only the millet flour fermented for 48 h that was not significantly ($p<0.05$) different from the unfermented millet flour. In contrast, there was significant ($p<0.05$) increase in the iron content of the fermented pigeon pea (Table 4). There were gradual increases in the iron content of pigeon pea as the fermentation time increased. Similar increase has been made in fermented soybeans by Van der Riet et al. (1987).

The zinc content of the millet flour was significantly ($p<0.05$) increased by fermentation when compared to the unfermented sample. This increase could probably be due to the removal of antinutrients that might have formed complexes with zinc. A similar increase in the zinc content of the cooked fermented and sprouted millet was reported by Obizoba and Atii (1994). As for the pigeon pea (Table 5) there were variations in their values. There were a non-significant ($p<0.05$) increase after 12 h fermentation of pigeon pea but a sudden significant ($p<0.05$) decrease was observed as the fermentation periods increased.

The calcium content of the fermented millet flour ranged from 2.04 to 6.05 mg/100 g while the unfermented millet flour had 2.00 mg/100 g (Table 4). The calcium content of the fermented pigeon pea ranged from 3.91 to 8.71mg/100g and the unfermented pigeon pea was 3.05

Table 5. Mineral and vitamin content of fermented and unfermented pigeon pea flour.

Samples	Iron (mg/100 g)	Zinc (mg/100 g)	Calcium (mg/100 g)	Vitamin A (IU) activity	Vitamin B$_1$ (mg/100 g)
P$_0$	3.02^d±0.02	0.76^a±0.03	3.05^d±0.30	43.10^c±0.05	0.48^e±0.02
P$_{12}$	3.04^d±0.04	0.77^a±0.01	3.91^c±0.03	12.73^d±0.09	0.69^a±0.00
P$_{24}$	3.73^c±0.03	0.65^{bc}±0.01	7.95^b±0.08	94.82^b±2.09	0.62^b±0.00
P$_{36}$	5.92^b±0.02	0.61^c±0.02	7.98^b±0.05	130.49^a±0.05	0.56^c±0.01
P$_{48}$	11.29^a±0.04	0.69^b±0.02	8.71^a±0.09	126.87^a±0.19	0.54^c±0.01

Values are means±SEM of duplicate determinations. Values bearing different super-scripts in the same column are significantly different (p<0.05); Key: P$_0$→Unfermented pigeon pea flour; P$_{12}$→ 12h fermented pigeon pea flour; P$_{24}$→ 24 h fermented pigeon pea flour; P$_{36}$ → 36 h fermented pigeon pea flour; P$_{48}$→ 48 h fermented pigeon pea flour, P→pigeon pea flour.

mg/100g. Fermentation significantly (p<0.05) increased the calcium content of millet. There were gradual increases in the calcium content of pigeon pea (Table 5) as the fermentation period increases. The unfermented samples of millet and pigeon pea flour were lower compared with those of the fermented samples. Amoo and Jokotagba (2012) reported a similar decrease in fermentation on the nutritive value of *Aspergillus niger* and *Aspergillus fumigatus* fermented *Huracrepitans* (sandbox tree) seed flour.

The vitamin A activity (precursor of vitamin A, beta-carotene), content of fermented millet flour ranged from 33.29 to 68.35 I.U. while unfermented millet flour was 33.02 I.U. The vitamin A content of fermented pigeon pea (Table 5) ranged from 12.73 to 130.49 I.U. while the unfermented pigeon pea was 33.20 I.U. There were significant (p<0.05) increases in both fermented millet and pigeon pea flour. It was observed that pigeon pea fermented for 12h had a significant (p<0.05) decrease in vitamin A content.

The vitamin B$_1$ content of the fermented millet flour increased by 0.58 mg/100g (12 h), 0.61 mg/100g (24 h), 0.68 mg/100g (36 h) and 0.82 mg/100g (48 h) while the unfermented millet flour was 0.36 mg/100g. However, the values for vitamin B$_1$ content of fermented pigeon pea ranged from 0.54 to 0.69 mg/100g while the unfermented sample was 0.48 mg/100g. There are also a slight but significant (p<0.05) increase in vitamin B$_1$ content of both fermented millet and pigeon pea flour. FAO (1995) observed increases in thiamin (up to 90 percent) and riboflavin (to 85 percent) on fermentation of pearl millet batter. There was also a similar increase in thiamin, riboflavin, ascorbic acid, vitamin A and tocopherol in pearl millet germinated for 48h and kilned at 45^{0}C (FAO, 1995).

Effect of fermentation of the functional properties of the single millet flour, pigeon pea flours, breadfruit leaf powder and their blends

The functional properties of unfermented and fermented millet and pigeon pea flours, seedless bread fruit leaf powder and their blends are shown in Table 6, 7 and 8.

The bulk density of fermented millet flour ranged from 0.067 to 0.71 g/ml compared with the unfermented millet flour (0.67 g/ml) (Table 6). The samples M$_{24}$ and M$_{48}$ were significantly different (p< 0.05) from other millet samples. The bulk density of the pigeon pea flour ranged from 0.64 to 0.76g/ml (Table 7).

There were significant (p<0.05) differences among the flour samples. The bulk density of the seedless breadfruit leaf flour and the composite flours ranged from (0.54 to 0.65 g/ml) (Table 8). Fermentation did not have any effect on the bulk density of the millet and pigeon pea. However for the blends, there were no significant difference (p<0.05) they all had the same value. The bulk density values obtained were generally higher (0.67 to 0.71 g/ml) for millet, (0.64 to 0.76 g/ml) for pigeon pea and (0.54 to 0.65 g/ml) for the composite flour blends than that obtained by Edema et al. (2005) for flour from commercially sold soybean (0.38g/ml). However, values obtained from this study were comparable by Okaka and Potter (1979) for cowpea (0.60g/ml) and fall in the range for Bambara groundnut (0.6 to 0.75 g/ml) reported by Onimawo et al. (1998).

The values for water absorption capacity ranges from 1.36 to 2.17 % for millet flour (Table 9), 1.65 to 2.28 % for pigeon pea flour (Table 7), 1.68 to 1.99 for the composite flour blends (Table 11) and 3.67 for the seedless breadfruit leaf flour (Table 8). The unfermented millet flour had a value of 1.52 %, the values for the fermented millet flour increased at 36 and 48 h fermentation periods. The values for pigeon pea flour gradual increased with fermentation periods. As for the composite flour, their values increased with the sample (B$_{55}$) that had the highest quantity (15 %) of breadfruit leaf flour. The increase in values for pigeon pea and the blends was attributed to the fact that fermentation enhanced the hydrolysis of starch, which invariably increased the water absorption capacity of the samples (Onwulata et al., 1998).

The seedless breadfruit leaf flour had the highest value in water absorption capacity. Values obtained from this study are greater than that obtained for flours from soybean (1.12%) as reported by Alfaro et al. (2004) but however, compared with the water absorption capacity

Table 6. Functional properties of unfermented and fermented millet flour.

Sample	Bulk density (g/ml)	Oil absorption capacity (%)	Water absorption capacity (g/g)	Swelling index (%)	Wettability (minutes)	Least gelation concentration	Viscosity at 60 (centipoises)	Particle size distribution
M_0	$0.67^b\pm0.001$	$1.24^c\pm1.765$	$1.52^b\pm0.017$	$1.57^d\pm0.735$	$1.27^e\pm0.015$	$8.00c\pm0.000$	$0.22^d\pm0.002$	$6.11^d\pm0.009$
M_{12}	$0.67^b\pm0.000$	$1.83^a\pm2.080$	$1.36^c\pm0.009$	$1.64^d\pm0.170$	$2.32^d\pm0.020$	$10.25^c\pm0.250$	$0.22^d\pm0.002$	$10.09^b\pm0.004$
M_{24}	$0.71^a\pm0.003$	$1.75^b\pm2.560$	$1.44b^c\pm0.001$	$1.67^d\pm1.925$	$3.57^a\pm0.015$	$15.00^b\pm0.000$	$0.26^c\pm0.001$	$10.78^c\pm0.616$
M_{36}	$0.67^b\pm0.002$	$1.73^b\pm1.200$	$2.08^a\pm0.77$	$1.79^b\pm0.715$	$3.27^b\pm0.050$	$15.00^c\pm0.000$	$0.28^b\pm0.002$	$11.38^a\pm0.440$
M_{48}	$0.61^a\pm0.004$	$1.29^c\pm0.655$	$2.17^a\pm0.050$	$1.89^a\pm0.140$	$2.57^d\pm0015$	$19.25^a\pm0.250$	$0.31^a\pm0.000$	$11.35^a\pm0.0022$

Values are means $\pm$ SEM of duplicate determination. Values carrying different superscript in the same column are significantly different (P<0.05); Key: $M_0\rightarrow$ Unfermented millet flour; $M_{12}\rightarrow$ 12 h fermented millet flour; $M_{24}\rightarrow$ 24 h fermented millet flour; $M_{36}\rightarrow$ 36 h fermented millet flour; $M_{48}\rightarrow$ 48 h fermented millet flour, M $\rightarrow$millet flour.

Table 7. Functional properties of unfermented and fermented single pigeon pea flour.

Sample	Bulk density (g/ml)	Oil absorption capacity (%)	Water absorption capacity (g/g)	Swelling index (%)	Wettability (minutes)	Least gelation concentration	Viscosity at 60 (centipoises)	Particle size distribution
P_0	$0.65^c\pm0.003$	$1.08^d\pm7.470$	$1.87^c\pm0.565$	$1.41^d\pm0.245$	$2.99^b\pm0.045$	$10.25^d\pm0.250$	$0.22^a\pm0.001$	$13.85^b\pm0.033$
P_{12}	$0.76^a\pm0.004$	$1.67^c\pm0.225$	$2.25^a\pm0.430$	$3.10^a\pm0.075$	$1.33^a\pm0.000$	$12.00^b\pm0.000$	$0.23^a\pm0.005$	$14.04^a\pm0.008$
P_{24}	$0.69^b\pm0.000$	$1.75^{bc}\pm0.225$	$2.28^a\pm0.014$	$3.07^a\pm0.475$	$1.58^d\pm9.010$	$12.25^b\pm0.0250$	$0.18^b\pm0.001$	$14.55^b\pm0.182$
P_{36}	$0.65^c\pm0.000$	$1.81^b\pm0.805$	$2.03^b\pm0.014$	$2.94^a\pm0.400$	$2.12^c\pm0.300$	$14.25^b\pm0.250$	$0.16^c\pm0.0001$	$12.81^c\pm0.078$
P_{48}	$0.64^c\pm0.002$	$2.26^a\pm2.615$	$1.65^d\pm0.012$	$2.19^c\pm0.015$	$3.22^a\pm0.10$	$18.00^a\pm0.000$	$0.15^c\pm0.003$	$13.04^c\pm0.069$

Values are means $\pm$ SEM of duplicate determinations. Values carrying different superscript in the same column are significantly different (P<0.05).
Key: $P_0\rightarrow$Unfermented pigeon pea flour; $P_{12}\rightarrow$ 12 h fermented pigeon pea; $P_{24}\rightarrow$ 24 h fermented pigeon pea flour; $P_{36}\rightarrow$36 h fermented pigeon pea flour; $P_{48}\rightarrow$ 48 h fermented pigeon pea flour , P $\rightarrow$pigeon pea flour.

Table 8. Functional properties of single breadfruit (*Artocarpus altilis*) leaf powder and the blends of millet, pigeon pea flour and breadfruit leaf powder.

Sample	Bulk density (g/ml)	Oil absorption capacity (%)	Water absorption capacity (g/g)	Swelling index (%)	Wettability (minutes)	Least gelation concentration	Viscosity at 60 (centipoises)	Particle size distribution
A_O	$0.54^b\pm0.150$	$3.17^a\pm2.615$	$3.67^a\pm0.020$	$3.03^{ab}\pm1.285$	$12.96^a\pm0.040$	$33.50^a\pm0.000$	$0.15^{bc}\pm0.000$	$10.76^a\pm0.0333$
B_{55}	$0.65^c\pm0.000$	$2.76^b\pm1.379$	$1.99^b\pm0.005$	$3.16^a\pm4.876$	$3.59^b\pm0.000$	$25.00^b\pm0.000$	$0.61^a\pm0.003$	$10.98^a\pm0.118$
C_{60}	$0.65^a\pm0.000$	$2.27^c\pm0.720$	$1.91^c\pm0.005$	$2.63^{ab}\pm0.080$	$3.14^c\pm0.020$	$20.25^c\pm0.250$	$0.12^c\pm0.001$	$5.17^b\pm0.056$
D_{65}	$0.65^a\pm0.000$	$1.97^d\pm0.970$	$1.72^d\pm0.0001$	$2.63^{ab}\pm0.020$	$3.09^c\pm0.015$	$18.00^{cd}\pm0.000$	$0.14^c\pm0.002$	$5.61^b\pm2.609$
E_{70}	$0.65^a\pm0.001$	$1.32^e\pm0.090$	$1.68^e\pm0.003$	$2.25^b\pm0.695$	$2.51^d\pm0.015$	$17.25^d\pm0.750$	$0.13^c\pm0.003$	$8.85^{ab}\pm0.040$

Values are means $\pm$ SEM of duplicate determinations. Values carrying different superscript in the same column are significantly different (P<0.05). Key: $A_0\rightarrow$ Bread fruit leaf powder; $B_{55}\rightarrow$ Millet + pigeon pea flours + breadfruit leaf powder blends (55:30:15); $C_{60}\rightarrow$ Millet + pigeon pea flours + breadfruit leaf powder blends (60:30:10); $D_{65}\rightarrow$ Millet + pigeon pea flours + breadfruit leaf powder blends (65:30:5); $E_{70}\rightarrow$ Millet + pigeon pea flour blends (70:30).

Table 9. Proximate composition (%) of millet and pigeon pea flour blends and seedless breadfruit (*Artocarpus altilis*) leaf powder.

Samples	Crude protein	Fats	Ash	Moisture	Crude fibre	Carbohydrate
A_0	$24\text{-}27^a\pm0.580$	$1.21^c\pm0.020$	$4.46^a\pm0.245$	$3.74^c\pm0.320$	$11.51^a\pm0230$	$54.87^e\pm0.295$
B_{55}	$23.45^a\pm0.000$	$2.77^b\pm0.035$	$3.48^b\pm0.040$	$3.55^{cd}\pm0.040$	$10.75^a\pm0.030$	$55.99^d\pm0.015$
C_{60}	$16.91^b\pm0.030$	$4.29^a\pm0.025$	$3.01^c\pm0.000$	$4.78a\pm0.090$	$5.03^{bc}\pm0.350$	$65.98^c\pm0.015$
D_{65}	$14.59^d\pm0.250$	$4.85^a\pm0.035$	$3.20^c\pm0.030$	$3.91b\pm0.090$	$4.76^c\pm0.135$	$68.68^b\pm0.075$
E_{70}	$15.78^c\pm0.173$	$2.00^c\pm0.200$	$2.51^d\pm0.015$	$3.39d\pm0.060$	$5.17^b\pm0.035$	$71.17^a\pm0.015$

Values are means $\pm$ SEM of duplicate determinations. Values bearing different superscripts in the same column are significantly different (p< 0.05). Key: $A_0\rightarrow$Breadfruit (*Artocarpus altilis*) leaf powder; $B_{55}\rightarrow$ millet + pigeon pea flours + breadfruit leaf powder blends (55:30:15); $C_{60}\rightarrow$ Millet + pigeon pea flours + breadfruit leaf powder blends (60:30:10); $D_{65}\rightarrow$Millet + pigeon pea flours + breadfruit leaf powder blends (65:30:5);$E_{70}\rightarrow$ millet + pigeon pea flour blends (70:30).

for pigeon pea by Adebowale and Maliki (2011). This result suggests that fermented pigeon pea flour may find application in the production of some baked products.

The values for oil absorption capacity (OAC) ranges from 1.24 to 1.83% (millet flour in Table 6), 1.08 to 2.26% (pigeon pea flour in Table 7), 1.32 to 2.76 % (the blends of millet, pigeon pea flour and seedless breadfruit leaf powder in Table 8 and 3.17% for breadfruit leaf powder (Table 8). The OAC of fermented millet flours are higher than the unfermented millet flour (1.24 %), but there is a gradual decrease in value as the fermentation time increased. However, oil absorption capacity of pigeon pea increase with the increase in fermentation time. As for the blends, the values gradually increased with the increment in the addition of seedless bread fruit leaf powder. Therefore, fermentation increased the oil absorption capacity of the samples. The highest value (3.17%) of seedless breadfruit leaf powder was because of excess fiber in the leaf.

Least gelation concentration is very important in food preparations. Least gelation concentration of millet flour ranged from 8.00 to 19.25% (Table 6), with highest value recorded with 48 h fermentation period of millet flour. Also, the least gelation concentration for the seed flour of pigeon pea ranged from 10.25 to 18.00% (Table 7), with highest value recorded with 48 h fermentation period of pigeon pea. Thus the least gelation concentration increased with fermentation periods for both millet flour and pigeon pea flour. These values are similar to that reported for great northern bean flour (10 %), cowpea (16%) by Sathe and Salunkhe (1981) and soyabean flour (10 %) by Alfaro et al. (2004). Such variation in the gelling properties of different legume flours may be ascribed to the relative rations of different constituents, proteins, carbohydrates and lipids that make up the flours, suggesting that interactions between such components may also have a significant role in the functional properties (Sathe et al., 1982).

This increase in the least gelation concentration of fermented millet and pigeon pea flours suggest that there was a decreased ability of the fermented millet flour and pigeon pea flour to form a stable gel. This observation

was also noted by the Ihekoronye (1986). However, the values for the blends ranged from 17.25 to 25.00 % with bread fruit leaf powder having the highest value of 33.5 % (Table 8). The values gradual increase with the addition of seedless bread fruit leaf powder to the blends. This increase could probably be because of the low or no starch content of the breadfruit leaf powder.

The viscosity at 60°C of the fermented and unfermented millet and pigeon pea flours are shown in Table 6 and 7. The viscosity of the fermented millet ranged from 0.22 to 0.31 cp while the unfermented was 0.22 cp (Table 6). There was a small but significant (p<0.05) increase in the viscosity started from the 24 h fermented millet. This was not in agreement with Mensah et al. (1991), who observed reduction in viscosity of fermented maize. However, there was a reduction in the viscosity of fermented pigeon pea flour from 24 h (0.18 cp), 36 h (0.16 cp) and 48 h (0.14 cp) while there was no significant (p<0.05) different in the unfermented pigeon pea (0.22 cp) and 12 h fermented pigeon pea flour (0.23 cp in Table 7). This reduction in viscosity of pigeon pea flour is due to the activities of amylase that broke starch down into simpler sugars, thus reducing viscosity (Mensah et al., 1991). Also, for the blends, the values ranged from 0.12 cp (C_{60}), 0.13cp (E_{70}), 0.14 (A_0), 0.14 (D_{65}) and 0.16 cp (B_{55}) (Table 8). The sample B_{55} had higher viscosity value than other samples and this could be due to the higher quantity of seedless breadfruit leaf powder in the blends. There was also reduction in the viscosity of other blends. This reduction in viscosity would produce thin porridges, which would be good for infant formula.

The swelling indices of fermented and unfermented millet, pigeon pea flours and their blends are presented in Tables 6, 7 and 8 respectively. The fermented millet ranged from 1.64 to 1.89% while the unfermented millet was 1.57% (Table 6). The value for unfermented pigeon pea was 1.41% while the fermented pigeon pea flour ranged from 2.19 to 3.10% (Table 7). There were significant (p<0.05) increase in the values of both fermented millet and pigeon pea flours. The swelling index of the blends ranged from 2.25 to 3.16% (Table 8).

Table 10. Mineral and vitamin contents of millet and pigeon pea flour blends.

Samples	Zinc (mg/100 g)	Calcium (mg/100 g)	Iron (mg/100 g)	Vitamin A (1U) activity	Vitamin B1 (mg/100 g)
A0	5.54a±0.000	9.01c±0.055	5.54c±0.011	1311.07a±6.173	0.69a±0.003
B55	0.68b±0.000	1.98e±0.108	11.54b±0.350	839.53b±2.236	23.61c±0.170
C60	0.37d±0.003	2.97d±0.020	2.92e±0.100	645.21c±4.305	25.98b±0.075
D65	0.42c±0.008	49.96b±0.0195	23.54a±0.505	69.72d±0.160	28.51a±0.160
E70	0.23e±0.004	53.89a±0.945	4.91a±0.184	38.44a±0.320	23.05d±0.070

Values are means ±SEM of duplicate determination values carrying different superscripts in the same column are significantly different (p<0.05); Key: $A_0 \rightarrow$ Breadfruit (*Artocarpus altilis*) leaf powder; $B_{55} \rightarrow$ Millet + pigeon pea flours + Breadfruit leaf powder blends (55:30:15); $C_{60} \rightarrow$ Millet + pigeon Pea flours + Breadfruit leaf powder blends (60:30:10); $D_{65} \rightarrow$ Millet + pigeon pea flours + Breadfruit leaf powder blends (65:30:5) ; $E_{70} \rightarrow$ Millet + Pigeon Pea flour blends (70:30).

The swelling index for the blends varied, and did not follow a particular trend. This could probably be due to the various quantity of seedless breadfruit leaf powder in the blends. These increases in swelling indices of millet, pigeon pea flour and their blends as the fermentation time increases, made the bond relax, thereby causing starch granules to imbibe water in swell and a low molecular weight amylase solubilize and leach out into the aqueous medium (Oke and Bolarinwa, 2012).

The time for wettability of fermented and unfermented millet, pigeon pea, seedless breadfruit leaf and their blends are shown in Table 6 to 8. It ranged from 2.32 to 3.57 min (fermented millet) and 1.27 minutes (unfermented millet, Table 6), 1.33 to 3.22 minutes, (fermented pigeon pea flour) and 2.99 minutes (unfermented pigeon flour Table 10) 2.51 to 3.59 minutes for the blends and 12.96 for the seedless breadfruit leaf powder in Table 8. There were significant (p<0.05) different in the samples. This could probably be due to the effect of fermentation period which have affected the flour. As for the seedless breadfruit leaf powder (which had the highest value), it could be probably due to the fact that it is a leaf and cannot absorb moisture very fast in the water. It affects other blends which had had the high value of wettability.

The particle size distribution of the fermented and unfermented millet, pigeon pea and their blends are shown in Table 6, 7 and 8 respectively. The fermented millet increased gradually as the fermentation period increased while the value for the unfermented millet (6.11) was lower than the fermented samples (Table 6). There was also significant (p<0.05) increases in the value of pigeon pea flour from P_{12} (14.04) and P_{24} (14.55) but a sudden decrease as the fermentation period increase P_{36} (12.81) and P_{48} (13.04) while the value for the unfermented pigeon pea was 13.85 (Table 7). There was significant increase and decrease in the value of the blend which ranged from 5.17 to 10.98 (Table 8) while the seedless breadfruit leaf powder was 10.76 (Table 8). This could probably be due to the varying amounts of incorporation of the seedless bread fruit leaf powder in the blends.

Effect of the incorporation of the seedless breadfruit (*A.altilis*) leaf powder on the proximate composition of millet, pigeon pea flour blends

Table 9 presents the proximate composition of millet flour pigeon pea flour and seedless breadfruit leaf powder blends. The protein content of the blends ranged from 23.45 (B_{55}), 16.91 (C_{60}), 14.59 (D_{65}) and 15.78% (E_{70}), respectively while the seedless breadfruit leaf powder had 24.27% in Table 9. There were significant (p<0.05) increases in the protein level of the blends with respect to the samples that had the highest quantity of breadfruit leaf powder. This protein level of seedless breadfruit powder was high (24.27%) compared to the protein level (16.91%) of the blends. This showed that the leaf had an appreciable amount of protein. This result agreed with Badra (1993) who recorded crude protein values of between 21.03 and 29.70% for *Celosia laxa, Corchorus olitorius (Ewedu)* and *Amaranthus caudatus* (velvet flower or foxtail amaranth) according to Dike (2010).

Sample D_{65} had the highest level of fat content while the seedless breadfruit leaf powder had the lowest level of fat content. The fat content of the breadfruit leaf powder did not agree with the fat content of the *Gnetaceae* leave (7.52 %) according to Dike (2010). The ash level of seedless breadfruit leaf powder was 4.46% and it was higher compared to the ash level of the blends. There was significant (p<0.05) difference of the ash content of the blends. The ash content of sample B_{55} was higher than others. This was probably due to high level (15%) of seedless breadfruit leaf powder in the sample.

There were significant (p< 0.05) differences in the moisture level of the blends and the breadfruit leaf powder respectively. This could probably be due to the various levels of millet and bread fruit in the blends. The moisture content of the samples were low A_0 (3.74%), B_{55} (3.55%) C_{60} (4.78%), D_{65} (3.91%) and E_{70} (3.39%). A similar result was obtained from weaning food produced from the blends of pearl millet, cowpea and groundnut which recorded (4.5 %) moisture content.

The crude fibre of the sample B_{55} (10.75%) was higher

than the other blends because it contained the highest quantity of the breadfruit leaf powder (11.51%) crude fibre. There were significant ($p<0.05$) increases in the carbohydrate content of the blend starting from sample E_{70} (71.17%), D_{65} (68.68%), C_{60} (65.98 %) and B_{55} (55.99 %) while the breadfruit leaf (A_0) had the lowest level of 54.87%. Sample E_{70} had the highest level of carbohydrate. This was probably due to the highest level of millet and increment followed a particular trend. Sample E_{70} (millet 70 % + pigeon pea 30 %) appeared to be rich in carbohydrate. The possible reason for this is that the flour is sediment of starch, proteins and other nutrients which are concentrated in the residue. A similar result was obtained with sorghum traditional complementary food and its composite flours (Nnam, 2001). The proximate values for the blends were higher than the millet flour which was normally used as a traditional complementary food.

Effect of the incorporation of the seedless breadfruit (*A. altilis*) leaf powder on the mineral and vitamin contents of millet and pigeon pea flour blends

The mineral and vitamin contents of millet, pigeon pea flour and seedless breadfruit leaf powder blends are shown in Table 10. The zinc content of the blend ranged from 0.23 to 0.68 mg/100 g while the seedless breadfruit leaf powder had 5.54 mg/100 g. Sample B_{55} with the highest quantity of breadfruit leaf powder had the highest value of 0.68 mg/100g while E_{70} (without no seedless breadfruit leaf powder) had the lowest value of 0.23 mg/100 g. There were significant ($p<0.05$) among the samples. The calcium level of the blends ranged from 1.89 to 53.89 mg/100g while the breadfruit leaf powder was 9.01 mg/100 g. There were significant ($p<0.05$) different among the samples. Sample E_{70} (which contains no seedless breadfruit leaf powder) had the highest level of calcium content (53.89 mg/100g).

The iron content of the blends ranged from 2.92 to 11.45 mg/100g while the seedless breadfruit leaf powder had 5.54 mg/100 g. However, sample B_{55} had the highest level of iron. The levels of zinc, calcium and iron of the breadfruit leaf powder were low. Few scientists, Badra (1993) and Dike (2009), also recorded low concentration of these minerals in *Amaranthus caudatus* (velvet flower), *Celosia laxa* (silver spinach or woolflower) among others.

Table 10 also presents the vitamin composition of millet flour, pigeon pea flour and bread fruit leaf powder. The vitamin A activity content of the blends ranged from 38.44 to 839.53 IU while the value of vitamin A activity content of seedless breadfruit leaf powder was 1311.07 IU. Also, the vitamin B_1 content of the blends ranged from 23.05 to 28.5 mg/100g while seedless breadfruit leaf powder had 0.69 mg/100g. There were significant ($p<0.05$) differences in both vitamin A and B_1 contents of the blends. The vitamin A contents followed a particular trend. It was observed that the incorporation of the seedless breadfruit

Table 11. Total viable counts (TVC) and mould count of fermented unfermented pigeon pea flour, millet flour, breadfruit (*Artocarpus altilis*) leaf powder and the blends.

Sample	TVC (cfu/g)	Mould count (cfu/g)
P_0	3.5×10^4	-
P_{12}	4.3×10^4	2.0×10^3
P_{24}	3.4×10^4	2.0×10^3
P_{36}	2.7×10^4	2.0×10^3
P_{48}	1.9×10^4	1.0×10^3
M_0	4.4×10^4	1.0×10^3
M_{12}	4.8×10^4	1.0×10^3
M_{24}	3.6×10^4	3.0×10^3
M_{36}	2.9×10^4	1.0×10^3
M_{48}	1.9×10^4	-
A_O	2.1×10^4	1.0×10^3
B_{55}	2.9×10^4	2.0×10^3
C_{60}	2.0×10^4	2.0×10^3
D_{65}	2.9×10^4	1.0×10^3
E_{70}	3.2×10^4	3.0×10^3

Values are means $\pm$ standard of error of means of duplicate determination. key:$\rightarrow$ No growth; $P_0\rightarrow$ Unfermented pigeon pea flour; $P_{12}\rightarrow$ 12 h fermented pigeon pea flour $P_{24}\rightarrow$ 24 h fermented pigeon pea flour; $P_{36}\rightarrow$ 36 h fermented pigeon pea flour $P_{48}\rightarrow$ 48 h fermented pigeon pea flour; $M_0\rightarrow$ Unfermented millet flour $M_{12}.\rightarrow$ 12 h fermented millet flour; $M_{24}\rightarrow$ 24 h fermented millet flour; $M_{36}\rightarrow$ 36 h fermented millet flour; $M_{48}\rightarrow$ 48 h fermented millet flour; $A_0\rightarrow$ Bread fruit leaf powder; $B_{55}\rightarrow$ Millet + pigeon pea flours + bread fruit leaf powder blends (55:30:15) ; C_{60} $\rightarrow$ Millet + pigeon pea flours + bread fruit leaf powder blends (60:30:10); $D_{65}\rightarrow$ Millet +pigeon pea flours + bread fruit leaf powder blends (65:30:5); $E_{70}\rightarrow$ Millet + pigeon pea flour blends (70:30).

leaf powder increased the vitamin content. However, the vitamin B_1 content had increases in its value as the quantity of the breadfruit leaf powder was reduced. Therefore, the vitamin A content of breadfruit leaf powder was very high 1311.07 1U while that of vitamin B_1 was very low 0.69 mg/100g. Blending of M_{36} and P_{36} increased the vitamin A content and lowered vitamin B_1 content.

Total viable and mould counts of fermented and unfermented pigeon pea flour, millet flour, breadfruit (*A.altilis*) leaf powder and the blends

The total viable count of the fermented and unfermented pigeon flours, millet flours, unfermented breadfruit leaf powder and the blends of millet (M_{36}), pigeon pea (P_{36}) and breadfruit leaf powder are shown in Table 11.

The total viable count for the fermented pigeon pea flour ranged from 4.3×10^4 (P_{12}), 3.4×10^4 (P_{24}), 2.78×10^4 (P_{36}) and 1.9×10^4 cfu/g (P_{48}) while the unfermented

pigeon pea was 3.5×10^4 cfu/g (P_0). Also, the values for fermented millet flour ranged from 5.8×10^4 (M_{12}), 3.6×10^4 (M24), 2.9×10^4 (M_{36}), 1.9×10^4 cfu/g (M_{48}) while that of unfermented millet flour was 4.4×10^4 cfu/g (M_0). It was observed that P_{12} and M_{12} had the highest values 4.3×10^4 cfu/g and 5.8×10^4 cfu/g, respectively.

This may be because the 12 h fermentation was favourable for the microorganisms to thrive on. After 12 h fermentation period, the microbial count of both pigeon pea and millet flours started decreasing gradually as the fermentation time was increasing. This could probably be due to increase in acidity and other metabolites which made the environment unbearable for the microorganisms to survive thereby leading to decrease in the microbial count with progression of fermentation.

The seedless breadfruit leaf powder (A_0) was 2.1×10^4 cfu/g while the values for the blends varied from 2.0×10^4 cfu/g to 3.2×10^4 cfu/g. There were significant ($p<0.05$) differences in the microbial count of the blends and this could probably be due to varying ratio of millet and seedless breadfruit leaf powder in the blends. The International Microbiological Standard recommended that the limit of bacteria contaminants for food of less than 10^6 cfu/g (Anonymous, 1974).

Mould count for the fermented millet flour is shown is Table 11. The fermented millet ranged from 1.0×10^3 cfu/g to 3.0×10 cfu/g while the unfermented millet flour had 1.0×10^3 cfu/g. Also, the fermented pigeon pea flour ranged from 1.0×10^3 cfu/g to 2.0×10^3 cfu/g while the unfermented pigeon pea flour had 1.0×10^3 cfu/g. There was no growth in P_0 and M_{48}. However, for the blends, they were also at the same range of 1.0×10^3 to 3.0×10^4 cfu/g.

CONCLUSION

Based on the proximate composition, it was observed that M_{36} (millet fermented for 36 h) and P_{36} (pigeon pea fermented for 36 h) had high protein 11.84 and 30.88% respectively, and was selected for formulation of complementary food. The composite flours (M_{36}/P_{36}) showed appreciable amounts of protein, ash, crude fibre, calcium, iron, vitamin A and B_1 over the single flours used in the preparation of traditional complementary food. The low microbial count (below the acceptable safe level) was reported. From the bulk density, oil absorption capacity, wettability and viscosity, it was observed that single flours could be used for weaning formulation. This study showed that nutrient dense complementary food could be produced from millet and pigeon pea fortified with seedless breadfruit leaf powder than using millet flour alone.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

The composition and extractability of thermo - molded wheat gluten bio-plastics

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The study was aimed to evaluate the effect of thermo-molding on gluten and gluten fractions composition and its extractability with 1.0 ml 0.05 M sodium phosphate buffer (pH 6.8) with 2.0% sodium dodecyl sulfate (SDS) at 130 and 150°C for 5 and 25 min. The gluten, gliadin and glutenin composition as affected by thermo-molding was analysed with reverse phase high performance liquid chromatography (RP-HPLC) and amino acid was analysed with acid hydrolysis using high-performance anion-exchange chromatography with integrated pulsed amperometric detection (HPAEC-IPAD). The extractability and composition were significantly affected. Gluten extracts were found in equivalent proportions; gliadin (51%) and glutenins (49%). Gluten, gliadin and glutenin subunits (GS) extracts were significantly affected and a reduction with increased molding condition explains the occurrence of polymerization reactions due to SH-disulfide interchange reactions. The glutenin fraction contains 15% gliadin while the gliadin fraction contains 2.5% glutenin after extraction with 60% ethanol. The extractability of the gliadin types and GS decreased with increasing molding time and temperature.

Key words: Gluten, gliadin, glutenin, extractability, composition, thermo-molding, amino acids.

INTRODUCTION

Wheat is among the most important staple food crops in the world with different cultivars classified according to physical hardness, agronomic properties, color of the kernel and end-use characteristics. Wheat proteins are separated by the Osborne fractionation via successive extractions with solvents (Delcour and Hoseney, 2010; Veraverbeke and Delcour, 2002). Gliadins and glutenins are functional gluten proteins in wheat found in more or less equal amounts (Goesaert et al., 2005; Belitz et al., 2009). In contrast to non-gluten proteins, gluten proteins are poorly soluble in water or dilute salt solutions due to

low amino acids content with ionizable side chains and high contents of non-polar amino acids. Cysteine, a minor amino acid in glutens is extremely important for its structure and functionality in disulfide bonds within a protein or between different proteins (Wieser, 2007).Glutenins,a heterogeneous mixture of disulfide-linked polymers (80,000 – Millions in MW) of GS is insoluble in alcohol. Glutenins are categorized into;

(1) HMW-GS (x- and y- types); a minor component (10%) of the total weight with three structural domains of N- and

C-terminal and more than four cysteine residue of repetitive sequence of proline, glutamine and glycine (Delcour and Hoseney, 2010; Wieser, 2007; Veraverbeke and Delcour, 2002).

(2) LMW-GS (B-, C- and D-types); makes up 20% of the total protein, less characterized group than the HMW-GS. The B- and C-type LMW-GS are sulfur-rich prolamins, while the D-type LMW-GS are sulfur-poor prolamins (Veraverbeke and Delcour, 2002).

Gliadins represent a heterogeneous mixture of single-chained proteins (30,000-80,000 in MW) soluble in alcoholic media. Based on their mobility at low pH in gel electrophoresis, three gliadin groups that is, α-, γ- and ω- were identified (Wieser, 2007). The ω-Gliadins have a MW (39,000 to 55,000) composed of high level of glutamine, proline and phenylalanine, low level of methionine but lacked cysteine residues. The α- and γ-gliadins overlaps inMW (28,000 to 35,000) with lesser proline, glutamine than the ω-gliadins and increased level of cysteine residues in the C-terminal domain. The C-terminal domain contains non-repetitive sequences of glutamine and proline than the N-terminal domain (Wieser, 2007; Veraverbeke and Delcour, 2002). In general the α- and γ-gliadins exist in higher and equivalent proportion than ω-gliadins (Wieser, 2007). The N-terminal domain constitutes 40 to 50% of the protein with repetitive sequences of glutamine, proline, phenylalanine and tyrosine.

Proteins are cross-linked by covalent bonds between polypeptide chains within a single protein or between different proteins important for structural and functional properties. The protein cross links are either naturally occuring or formed during processing (Gerrard, 2002). Disulfide bonds between cysteine residues are the dominant crosslinks formed by oxidative coupling of two cysteine residues adjacent a protein matrix in the presence of an oxidant which accepts hydrogen atoms from the thiol groups (Veraverbeke and Delcour, 2002; Gerrard, 2002; Shewry and Tatham, 1997). An intramolecular disulfide bond can be converted into an intermolecular disulfide bond by a sulfhydryl (SH)-disulfide interchange reaction. Other cross-links include isopeptide bonds, dehydroprotein, Maillard reaction and tyrosine derived during thermal processing; chemical treatment in the formation of covalent bonds from the cysteine and lysine residue (Lagrain et al., 2010; Rombouts et al., 2009; Hanft and Koehler, 2005; Gerrard, 2002; Tilley et al., 2001; Singh, 1991).

The MW of gluten aggregates and protein extractability is dependent on the heating condition, hydrostatic pressure and moisture content attributed to oxidation of SH groups and SH-disulfide interchange reactions which forms intermolecular bonds (Kieffer et al., 2007; Lagrain et al., 2005; Singh and MacRitchie, 2004; Weegels et al., 1994). The formation or cleavage of disulfide bonds is stimulated by the application of redox agents (potassium bromate, potassium iodate and reducing agent

dithiothreitol [DTT]) on the gluten proteins. During hydrothermal treatment, protein extractability was higher in the presence of oxidants thus suggesting a decreased cross-linking which hinders gluten polymerization. In the presence of reducing agents (DTT), protein extractability was lower suggesting increased crosslinking which promotes gluten polymerization due to increased levels of free SH groups and less flexibility of glutenin chains (Lagrain et al., 2008, 2007, 2005; Guerrieri et al., 1996).

Gluten bioplastic formation

The need for environmentally friendly, renewable and biodegradable plastics is increasing due to pollution and long term health impacts of synthetic polymers (Zhang et al., 2010; Sun et al., 2008); possess suitable gas barrier properties and nutritional value (Lagrain et al., 2010; Cao et al., 2007); poor moisture barrier properties due to its hygroscopic nature; binds with water through hydrogen bridges modified by thermal and chemical treatments (Sun et al., 2008; Carvalho et al., 2008; 2006; Brauer et al., 2007; Dicharry et al., 2006; Gallstedt et al, 2004; Domenek et al., 2004; Woerdeman et al., 2004). The effect of processing on gluten network formation is determined by the SDS extractability which measures the degree of polymerization: the lower the extractability the higher the degree of polymerization (Sun et al. 2008; Gallstedt et al., 2004; Domenek et al., 2004; Pommet et al., 2003; Micard et al., 2001; Cuq et al., 2000). Besides process conditions, the presence of additives influences extractability (Lagrain et al., 2010; Sun et al., 2007; Dicharry et al., 2006; Gallstedt et al., 2004; Woerdeman et al., 2004).

MATERIALS AND METHODS

Wheat gluten was fractionated into gliadin and glutenin with 60% ethanol and protein composition was analysed with RP-HPLC in the presence of redox agents (SDS) and thermo-molded at 130 and 150°C for 5 and 25 min in a Pinette Press Zenith 2 (Pinette Emidecau Industries, Chalon sur Saône, France) at 5 bars after conditioning for 2 days at 50% relative humidity and 20°C (the moisture was adjusted to 7%). Hydrophilic molecules elute first, followed by hydrophobic molecules which interact stronger with the column (Figure 1). Amino acid analysis was performed after acid hydrolysis of high-performance anion-exchange chromatography with integrated pulsed amperometric detection (HPAEC-IPAD) as described by Rombouts et al. (2009).

RESULTS AND DISCUSSION

Gluten composition and extractability

Thermo – molded gluten and gluten fractions composition and extractability with 1.0 ml 0.05 M sodium phosphate buffer (pH 6.8) and 2.0% sodium dodecyl sulfate (SDS) are presented in Tables 1 to 4. The gliadin (51%) and

Table 1. The extractability and composition of gluten protein before and after thermo - molding at 130 and 150°C for 5 and 25 min analysed by reverse phase –high performance liquid chromatography (RP-HPLC) (expressed in percentage).

Sample	Treatment				
	Control	130°C - 5 min	130°C - 25 min	150°C - 5 min	150°C - 25 min
Gliadin	**50.6 (0.7)**	**28.4 (2.7)**	**11.6 (0.9)**	**8.0 (0.3)**	**6.4 (0.1)**
ω-gliadin	6.2 (1.5)	6.2 (0.6)	3.6 (0.1)	3.3 (0.0)	4.0 (0.1)
α-gliadin	20.6(5.0)	13.3(1.3)	4.5 (0.0)	2.6 (0.0)	1.3 (0.1)
γ-gliadin	17.0 (3.9)	9.0 (0.9)	3.5 (1.0)	2.2 (0.2)	1.1 (0.1)
GS	**49.4 ((8.8)**	**70.8 (4.8)**	**66.5 (0.7)**	**65.6 (1.4))**	**50.8 (1.6)**
D-LMW-GS	2.7 (0.8)	2.5 (0.2)	2.1 (0.0)	2.2 (0.1)	2.1 (0.0)
HMW-GS	11.4 (1.2)	10.5 (0.9)	6.8 (0.1)	6.8 (0.2)	6.6 (0.1)
B/C-LMW-GS	35.7 (6.8)	57.8 (3.8)	57.6 (0.7)	56.6 (1.1)	42.1 (1.5)

The standard deviation is given in brackets

Table 2. The Extractability and composition of glutenin protein before and after thermo-molding at 130 and 150 °C for 5 and 25 min analysed by reverse phase-high performance liquid chromatography (RP-HPLC) (expressed in percentage).

Sample	Treatment				
	Control	130°C - 5 min	130°C - 25 min	150°C - 5 min	150°C - 25 min
Gliadin	**14.7 (0.0)**	**6.5 (0.2)**	**4.1 (0.1)**	**7.2 (0.1)**	**12.6 (1.5)**
ω-gliadin	2.3 (0.0)	2.2 (0.0)	2.1 (0.0)	3.8 (0.0)	7.6 (0.3)
α-gliadin	7.1 (0.0)	2.6 (0.1)	1.2 (0.0)	1.3 (0.0)	2.0 (0.2)
γ-gliadin	5.3 (0.1)	1.7 (0.1)	0.8 (0.1)	2.1 (0.0)	3.0 (1.0)
GS	**85.3 (0.8)**	**86.3 (2.3)**	**81.5 (1.2)**	**92.8 (4.8)**	**32.5 (1.4)**
D-LMW-GS	4.4 (0.1)	4.1 (0.2)	4.3 (0.0)	25.1 (1.7)	1.4 (0.6)
HMW-GS	23.4 (0.1)	19.4 (0.6)	16.5 (0.2)	52.6 (2.2)	13.8 (0.3)
B/C-LMW-GS	57.5 (0.8)	62.9 (1.5)	60.6 (0.9)	52.6 (2.2)	17.4 (0.5)

The standard deviation is given in brackets.

Table 3. The extractability and composition of gliadin protein before and after thermo-molding at 130 and 150 °C for 5 and 25 min analysed by reverse phase-high performance liquid chromatography (RP-HPLC) expressed in percentage.

Sample	Treatment				
	Control	130°C - 5 min	130°C - 25 min	150°C - 5 min	150°C - 25 min
Gliadin	**97.5 (1.3)**	**59.6 (0.8)**	**24.1 (0.1)**	**12.3 (0.5)**	**11.1 (0.1)**
ω-gliadin	14.5 (0.3)	9.4 (0.1)	6.3 (0.1)	5.6 (0.2)	6.5 (0.3)
α-gliadin	45.8 (1.1)	27.4 (0.4)	9.7 (0.3)	3.0 (0.1)	1.7 (0.1)
γ-gliadin	37.2 (0.2)	22.9 (0.3)	8.1 (0.4)	3.7 (0.2)	3.0 (0.3)
GS	**2.5 (0.4)**	**44.2 (2.1)**	**76.2 (3.0)**	**76.0 (6.4)**	**62.6 (0.3)**
D-LMW-GS	0.0(0.0)	1.0 (0.1)	1.3 (0.0)	1.5 (0.3)	1.6 (0.0)
HMW-GS	0.2 (0.0)	4.2 (0.2)	4.9 (0.2)	5.2 (0.5)	5.8 (0.1)
B/C-LMW-GS	2.3 (0.3)	39.0 (2.0)	70.0 (2.8)	69.3 (5.7)	55.3 (0.2)

The standard deviation is given in brackets.

glutenin extracts (49%) occurred in equivalent proportions in the SDS media (Goesaert et al., 2005; Belitz et al., 2009). Reverse phase – high performance liquid chromatography (RP-HPLC) profile showed that higher gliadin peak of gliadin fractions than gliadin peak of gluten explains that gliadins are more extractable than

Table 4. The lysine, LAN and cystine contents of gluten, gliadin and glutenin proteins (mole/10^5 g of protein) thermo-molded at 130 and 150 °C for 5 and 25 min.

Sample		Treatment				
		Control	130°C - 5 min	130°C - 25 min	150°C - 5 min	150°C - 25 min
Gluten	Lysine	19.4 (0.1)	14.2 (0.1)	13.9 (0.2)	13.0 (0.2)	11.7 (0.2)
	LAN	nd	nd	nd	0.1 (0.0)	0.6 (0.0)
	Cystine	5.6 (0.1)	5.4 (0.1)	5.4 (0.2)	5.5 (0.1)	4.7 (0.1)
Glutenin	Lysine	23.5 (3.3)	22.6 (1.6)	19.3 (0.5)	16.1 (0.6)	16.0 (0.6)
	LAN	nd	nd	0.3 (0.0)	0.5 (0.1)	1.5 (0.0)
	Cystine	4.7 (0.0)	4.8 (0.3)	4.6 (0.0)	3.5 (0.1)	3.1 (0.0)
Gliadin	Lysine	8.3 (0.3)	7.1(0.4)	5.8 (0.3)	4.9 (0.4)	2.3 (0.1)
	LAN	nd	nd	nd	0.1 (0.1)	0.5 (0.0)
	Cystine	9.1 (0.1)	9.4 (0.3)	9.4 (0.4)	8.6 (1.1)	8.4 (0.0)

The standard deviation is given in brackets
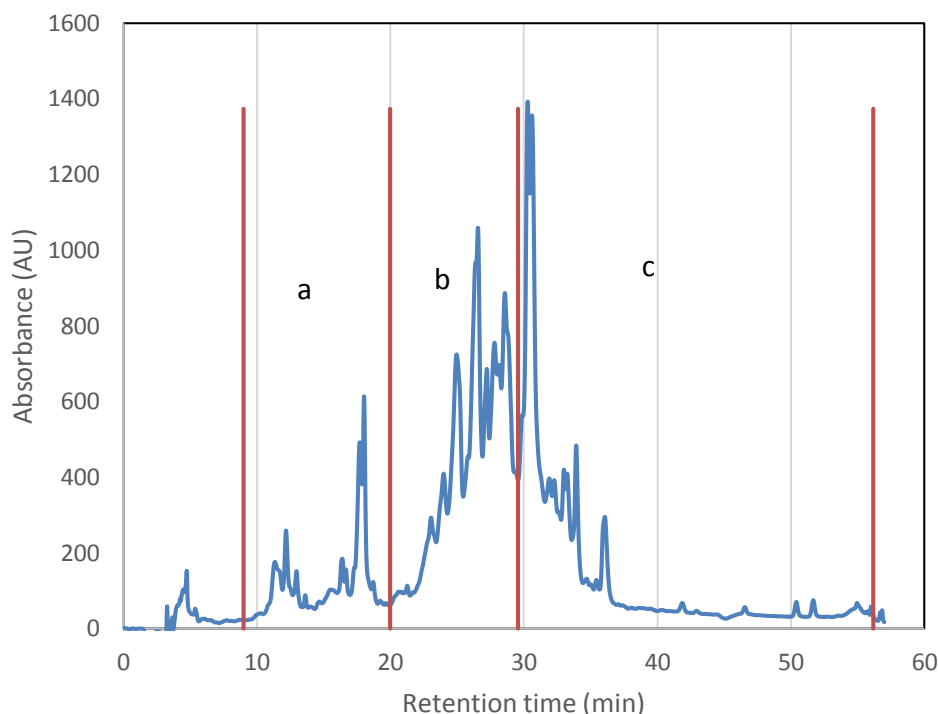
Figure 1. Typical reverse phase –High performance liquid chromatography (RP-HPLC) profiles of the Gliadin extracts (a) ω-gliadin, (b) α-gliadin and (c) γ-gliadin.

non-gliadin fractions (Abrehet, 2015). Indeed, all the gliadins are soluble in the SDS buffer, but not all glutenins are soluble. Small gliadin peak as observed in the glutenin fractions shows that pure fractions of neither gliadin nor glutenin was obtained with ethanol extraction (Abrehet, 2015). The three gliadin types exist in a variable proportion; α-gliadin (21%), γ-gliadin (17%) and ω-gliadin (6%) (Figure 1). The glutenin subunits; B/C-

LMW-GS (36%), HMW-GS (11%) and D-LMW-GS (3%) were obtained from gluten protein (Delcour and Hoseney, 2010; Wieser, 2007; Veraverbeke and Delcour, 2002) (Figure 2). There was a reduction in the extractability of α-gliadin, γ-gliadin and ω-gliadin, HMW-GS and D-LMW-GS with increased molding condition thus explaining the occurrence of polymerization reaction due to the SH-disulfide interchange reactions (Sun et al. 2008; Kieffer et
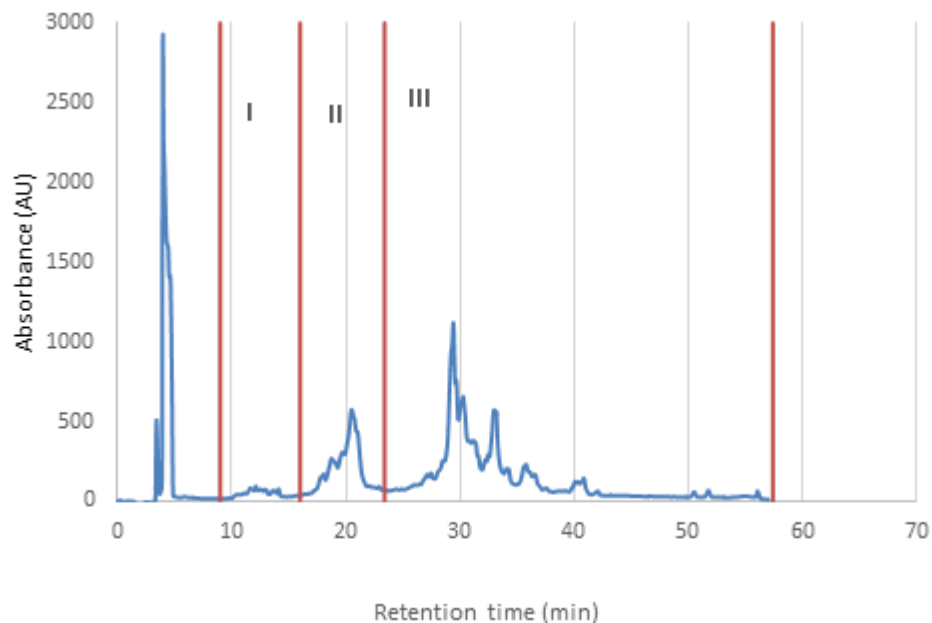
Figure 2. Typical reverse phase –High performance liquid chromatography (RP-HPLC) profiles of the glutenin subunit extracts; (I) D-LMW-GS. (II) HMW-GS and (III) B/C-LMW-GS.

al., 2007; Wieser, 2007; Lagrain et al., 2005; Singh and MacRitchie, 2004; Gallstedt et al., 2004; Domenek et al., 2004; Pommet et al., 2003;) while there was an increase in the extractability of B/C-LMW-GS with increased molding conditions thus explaining the oxidation and protein degradation (Veraverbeke and Delcour, 2002).

Glutenin composition and extractability

Upon extraction with 60% ethanol, the glutenin rich fractions contained 15% gliadins. The proportion of gliadin in the glutenin fractions indicates that the alcohol strength for the fractionation of gluten was not enough to separate the pure fractions of the gliadin and glutenins (Abrehet, 2015). The extractability of the GS decreases with increased molding condition which explains the occurrence of cross links (Abrehet, 2015). The distribution of ω- α- and γ-gliadins in the 15% gliadin of the glutenin enriched fractions was 2, 7 and 5% (Wieser, 2007), respectively, which further decreased with thermo-molding at 130 and 150°C in 5 and 25 min. GS extractability in the glutenin fraction was found to be 4, 23 and 57% for the D-LMW-GS, HMW-GS and B/C-LMW-GS, respectively (Delcour and Hoseney, 2010; Wieser, 2007; Veraverbeke and Delcour, 2002). With thermo-molding at 130 and 150°C in 5 and 25 min was a reduction and distribution in D-LMW-GS and HMW-GS as protein polymerization occurred due to the repetitive sequence of cysteine residue (Kieffer et al., 2007; Lagrain et al., 2005; Singh and MacRitchie, 2004; Weegels et al., 1994). There was increased extractabililty

of B/C-LMW-GS with thermo-molding as the repetitive amino acid chains were broken down due to the thermal effects.

Gliadin composition and extractability

The gliadin fractions composed of 2.5% glutenin with higher proportion of B/C-LMW-GS while the D-LMW-GS and HMW-GS excites at lower concentration. The GS (B/C-LMW-GS, D-LMW-GS and HMW-GS) extractability was significantly affected with increased thermo-molding due to its hydrophobicity property similar to ω-gliadins in the gliadin enriched fractions. 97% of the gliadin fraction contained α-gliadin (46%) and γ-gliadin (37%) susceptible to covalent incorporation in the glutenins network formation as they are rich sources of cysteine residues than ω- gliadin (14.5%). Extractability was significantly reduced by more than 50% after molding at 130°C for 5 min due to the incorporation of gliadins in network formation and occurrence of non-disulfide bonds (Wieser, 2007; Veraverbeke and Delcour, 2002). The increased molding condition diminishes the extraction even though ω-gliadin exists in lesser amount and is unextractable with the application of thermo-molding than α- and γ-gliadins.

Amino acid composition

The three amino acids (cysteine, lysine and lanthionine (LAN)) were significantly affected by thermo-molding at

130 and 150°C for 5 and 25 min (Table 4). The reduction in cysteine and lysine amino acids with increased molding condition was due to sulfhydryl-disulphide interchange, oxidation and β-elimination reactions. LAN was undetectable after thermomolding at 130°C for 5 and 25 min for gluten and gliadin fraction and was detectable during thermomolding at 130°C for 25 min due to the occurence of repetitive sequence of cysteine due to oprotein agrigation. LAN formation increased with increased molding condition due to the sulfhydryl-disulfide interchange reaction and β-elimination reaction occurence. While cysteine and lysine amino acids were observed to be relatively similar with a slight reduction. The reduction in lysine with increased molding condition was due to oxidation and Maillard reactions (darkening of thermo molded plastic plates). Cysteine was converted to dehydroalanine (DHA) and atomic sulphur. Dehydroalanine was convereted in to lysinoalanine (LAL) (not detected) and lanthionine (LAN). The level of LAN formation is an indication of improved network formation.

Conclusion

Gluten proteins, the storage proteins of wheat and co-product of starch industry display interesting features for non-food applications. Understanding the composition and processing of gluten on network formation is important for biodegradable, renewable organic material formation. The fractionation of gluten with 60% ethanol yielded a glutenin- (49%) and a gliadin-rich (51%) fractions. Among the different amino acids; cystine, lysine and LAN were significantly affected by thermo-molding; the level of cysteine and lysine amino acids decreased with increased modling condition due to protein polymerization reactions. While the LAN increased with molding at 150°C. The reduction of extrability with increased thermo-molding condition improves protein polymerization and mechanical properties.

Conflict of Interests

The author has not declared any conflict of interests.

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Full Length Research Paper

Impact of *Blighia sapida* (k. Koenig) aril powder level in diet on the growth and the well-being of albino Wistar rats

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The aim of the present investigation was to evaluate the impact of *Blighia sapida* aril level in diet on the growth of albino Wistar rats and their welfare. A quantity of *B. sapida* arils was cooked and lyophilized. Thirty six young albinos Wistar rats teams up in six with six homogeneous groups were used for a four weeks research. Young rats group were fed *ad libitum* with six different diets which can be distinguished by the level of *B. sapida* arils powder. There were control diet (0% *B. sapida* arils powder), diet Bs_1 (6.25% *B. sapida* arils powder), diet Bs_2 (12.5% *B. sapida* arils powder), diet Bs_3 (25% *B. sapida* arils powder), diet Bs_4 (50% *B. sapida* arils powder) and diet Bs_5 (75% *B. sapida* arils powder). Body weight was measured weekly and the animals were observed for signs of abnormalities throughout the study. At the end of the experimentation, blood sample was collected and analyzed. Results show that the growth of rats fed with diets Bs_1, Bs_2 and Bs_3 were normal as compared to the growth of rats fed with the control diet. On the other hand, growth retardation was noticed on rats fed with diets Bs_4 and Bs_5. However, rats coat fed with diets Bs_4 and Bs_5 were also normal. Again, for rats fed with diets Bs_4 and Bs_5, a level (p<0.05) of total cholesterol and LDL-cholesterol was noticed as compared to values obtained in rats of the other groups. In the case of natural disaster, the consumption of *B. sapida* aril could be used as nutritional base at a degree neighboring 50% of the nutritional need of the organism. Outside this value, growth retardation and a level of total cholesterol and LDL-cholesterol can be noticed.

Key words: *Blighia sapida* aril, diet, rats, growth, welfare.

INTRODUCTION

The tree called fisanier in French is scientifically known as *Blighia sapida* L. (sapindaceae). It originates from west and Central tropical Africa (Hill, 1952; Bressler et al., 1969). It has been spread by cultivation in the tropical region (Arbonnier, 2002). It is available in West Africa,

Central Africa and also in Caribbean and Jamaica. *B. sapida* is grown because it has several uses. In fact, the tree is grown in towns for its shade. His wood, because it is termite hardy, is used as building material and for cooking (Arbonnier, 2002). The leaves are used as herbal

medicine to treat amygalite, conjuntivitis, wound, icterus, vertigo and epilepsy (Arbonnier, 2002; Orwa et al., 2009). When the unripe fruits are crushed, they produce moss which is used as soap and also as fishing poison (Arbonnier, 2002).

The main reason why *B. sapida* is cultivated is for its arils, the freshly cream which colours the pulp of the fruit. These arils can be consumed freshly or when they are made into sauce or fried in oil (Arbonnier, 2002; Ekué et al., 2010). The consumption of *B. sapida* arils is worldwide. In fact, in Jamaica, they are industrially transformed and put into cans and are sold in America and Great Britain (Orane et al., 2005). Several research results on the beneficial uses of *B. sapida* arils show that it is an essential element that should not to be neglected in diet in many regions due to it lipids (45%), proteins (11%), ash (4%) and vitamin C (65 mg/kg) content (Morton, 1987; Akintayo et al., 2002; Ouattara et al., 2010). Nevertheless, some molecules such as hypoglycine A, saponin, saccharides and fructoligosaccharides (Jimoh et al., 2007; Benkeblia and Lopez, 2015) have negative effects on the organism function (Moyal, 1985, 1986; Chase et al., 1990; CDC, 1992; Bouree et al., 2002; OPS, 2002; Katibi et al., 2015). These negative effects can end up with death caused by hypoglycemia or hemolytic syndrome (Moyal, 1985, 1986; CDC, 1992; Bouree et al., 2002; OPS, 2002; Katibi et al., 2015). Despite everything, potentialities of *B. sapida* arils have been exploited in Côte d'Ivoire and in Jamaica when there were natural disasters in these countries. In Côte d'Ivoire, in the year 1984, a drought caused a diminution of cereals production. Then, consumption of *B. sapida* arils prevented a big famine. Also, the consumption of *B. sapida* aril prevented a big famine in Jamaica. This famine was due to flood which caused lack of cereals. According to an epidemiological study, mortality was caused either by the action of the toxic molecule call hypoglycin A and the high consumption of arils which do not satisfied the nutritional need of the organism (OPS, 2002). For a better understanding of this situation, some questions may be answered. Can *B. sapida* aril be a palliative to the lack of food? If not, to what extent can it be safely consumed?

Then, the aim of this study was to evaluate the effect of *B. sapida* arils consumption in accordance with the level of arils in diet on Albino Wistar rats. In this work, the results of growth, visual observation and blood sample parameters such as lipids parameters, glucose, protein and urea are shown.

MATERIALS AND METHODS

Plant material

B. sapida is planted in the North and the Centre of Côte d'Ivoire. In these regions, the arils of the plant are very much consumed. Arils used for the experimentation were brought from Katiola, a town in the North Central of Côte d'Ivoire, in the months of March, April and

May. In these months, arils are more available.

At Katiola, arils were spread on polythene paper and exposed to sunlight for two weeks, six hours a day, to get dry. In the night, they were kept on the plastic and put in a house at room temperature (25 - 30°C). After the drying process of the fresh arils, the dried arils were kept in plastic bags and sent to Abidjan in the South of Côte d'Ivoire.

Animals

Thirty six young albino *Wistar* rats weighing between 49 and 75 g were used. They were bred in the animal house of Ufr Biosciences of the University Félix HOUPHOUËT-BOIGNY of Abidjan (Côte d'Ivoire). During the breeding, rats were fed with food made by a society IVOGRAIN which is specialized in mass production of livestock food. This food is made up of crude protein matter (15%), crude fat matter (3.5%), cellulose matter (12%), mineral matter (9%), calcium (1%), phosphorus (0.9%), sodium (0.3%), vitamin A (15000 UI/kg), vitamin D3 (3000 UI/kg) and vitamin E (10 mg/kg).

Preparation of the arils

In Abidjan, the dry arils were crushed with a laboratory mortar. Each time, 500 g of *B. sapida* arils powder was cooked, using a gas stone, in one liter of water (500 g/l) for two hours. Approximately, 10 kg of *B. sapida* arils powder was cooked. Arils pasta obtains were lyophilized. The freeze-dry aril obtained was used to formulate five kinds of experimental diets. These experimental diets were named diets Bs_1, Bs_2, Bs_3, Bs_4 and Bs_5. Previously, a control diet was prepared. The composition of the control diet was similar to that recommended by scientists of the American Institute of Nutrition for rapid Growth (AIN-93G) on experiment with animals, particularly, rats (Reeves et al., 1993). Table 1 shows the nutritional composition of the control diet.

Difference between the experimental diets

In the experimental diets, a part of the control diet was substituted by the freeze-dry aril. The degree of substitution was 6.5% for diet Bs_1, 12.5% for diet Bs_2, 25% for diet Bs_3, 50% for diet Bs_4 and 75% for diet Bs_5.

Experimentation

At the beginning of the experimentation, the animals were grouped into six different homogeneous young groups of rats. They were put individually in metabolic cages and maintained under standard laboratory conditions (temperature 25±2°C) with dark and light cycle (12/12 h). In the metabolic cages, rats were acclimatized to this condition and fed with the control diet five days before the beginning of the experiment. After that, the animals were fed *ad libitum* with the different diets (control diet, diets Bs_1, Bs_2, Bs_3, Bs_4 and Bs_5) during 28 days according to the different group they belong to.

Treatment of animals during the experimentation

Body weight of each rat was measured weekly and the animals were observed for signs of abnormalities throughout the study. At the end of the experimentation, blood samples were collected at the vena cava level of all the animals and put in individual vacuum valve. The blood samples were taken in the morning after giving ether anesthesia to the albino rats. Samples were collected with a

Table 1. Nutritional composition of the control diet.

Diet composition Component	g/kg of food
Protein	
Casein	200
Sugar	
Saccharose	100
Starch of maize	550
Fiber	
Agar-agar	32
Fats	
Palm oil	70
Mixture of vitamins	10
Mixture of mineral	35
L-cystine	3

The control diet composition used in the experimentation is approximately equal to that recommended by the American Institute of Nutrition for rapid Growth (AIN-93G) proposed by Reeves et al. (1993).

sterile disposable syringe. Also, at the end of the experimentation, the urine (24 h urine) of each animal was taken, measured and analyzed.

Biochemical parameters

The blood samples were centrifuged at 8000 r.p.m for 15 min to harvest the plasma which was used for the various analyses. Glucose was assayed by glucose oxidase-peroxidase method using a kit (Sigma Diagnostics, Sigma kit #315). Total cholesterol and triglycerides were measured as described by Richmond (1973) and Trinder (1969b), respectively. Urea was estimated by the diacetyl method of Wybenga et al. (1971). Total protein was estimated using assay kits (Sigma Diagnostics, St. Louis, MO, USA). Biochemical analysis of serum samples was performed using an automatic chemistry analyzer (Hitachi model 902, Roche).

Statistical analysis

The experimental results were expressed as the mean±S.E.M. Data were assessed by the method of analysis of ANOVA followed by Dunnett test (Ostle, 1966; Woolson, 1987). p value of < 0.05 was considered as statistically significant.

RESULTS

Observation and growth

All the rats used in this work had, after the

experimentation, generally, either normal coat or normal appearance. At the growth level, some differences were noticed. There were no significant difference ($p > 0.05$) of growth between the control group and group of rats fed with diets Bs_1, Bs_2 and Bs_3. But, growth of rats fed with diets Bs_4 and Bs_5 was relatively low ($p < 0.05$) as compared to that of the control group. The growth of rats fed with diets Bs_4 and Bs_5 was also low ($p < 0.05$) when compared with that of rats fed with the diets Bs_1, Bs_2 and Bs_2. Lower growth was observed in the animals fed with diet Bs_5 than those which consumed diet Bs_4. The percentage of the difference of growth between animals of the control group and those which consumed diet Bs_4 was 28.75% ($p < 0.05$) while this difference between the control group and those which consumed diet Bs_5 was 61.53% ($p < 0.001$). Table 2 shows the curve of rats' growth according to *B. sapida* aril powder level in diet.

Effect on the urinary volume

The urinary volumes obtained from rats fed with diets Bs_1, Bs_2 and Bs_3 were not significantly different ($p > 0.05$) from that obtained from rats fed with the control diet. But, in rats fed with diets Bs_4 and Bs_5, urinary volumes were respectively high ($p < 0.05$) and very high ($p < 0.001$) when compared with urinary volume obtained from rats fed with the control diet.

Urinary volumes obtained was 6.78±1.04 ml/24 h for rats fed with the control diet, 6.57±2.25 ml/24 h for rats fed with diet Bs_1, 6.55±1.10 ml/24 h for rats fed with diet Bs_2, 6.60±1.34 ml/24 h for rats fed with diet Bs_3, 3.11±0.73 ml/24 h for rats fed with diet Bs_4 and 1.90±1.11 ml/24 h for rats fed with diet Bs_5. These results are shown on Figure 1.

Effect of *B. sapida* aril powder level in diet on plasmatic triglycerides

Triglycerides level observed were 1.60±0.24 g/l for rats fed with diet Bs_1, 1.86±0.17 g/l for rats fed with diet Bs_2, 1.42±0.30 g/l for rats fed with diet Bs_3, 1.47±0.20 g/l for rats fed with diet Bs_4 and 1.37±0.19 g/l for rats fed with diet Bs_5. These values were not significantly ($p > 0.05$) different from that obtained in rats fed with the control diet (1.66±0.11 g/l). These results are shown in Figure 2.

### Effect of *B. sapida* aril powder level in diet on plasmatic total-cholesterol, LDL-cholesterol and HDL-cholesterol

Total-cholesterol and LDL-cholesterol values obtained in rats fed with diet Bs_5 were 0.98±0.07 g/l and 0.51±0.07 g/l, respectively. These values were significantly high ($p < 0.05$) as compared to that obtained in rats of the control group. The total-cholesterol and LDL-cholesterol found in

Table 2. Rat growth according to *B. sapida* aril powder level in diet.

	Body weight (g)					
	Control diet	Diet Bs$_1$	Diet Bs$_2$	Diet Bs$_3$	Diet Bs$_4$	Diet Bs$_5$
Week 0	57.9± 5.41[a]	54.67±5.51[a]	54.65±5.45[a]	57.73±5.30[a]	54.33±5.48[a]	54.62±5.02[a]
Week 1	76.55±5.47[a]	75.04±5.25[a]	73.54±5.57[a]	76.92±5.27[a]	58.23±5.41[b]	59.23±4.97[b]
Week 2	108.26±5.32[a]	105.05±5.67[a]	107.82±5.49[a]	110.56±5.37[a]	85.44±5.53[b]	50.96±5.10[c]
Week 3	120.03±5.01[a]	127.41±5.54[a]	128.96±5.51[a]	135.96±5.32[a]	97.32±5.56[b]	52.25±4.98[c]
Week 4	131.4±5.49[a]	143.32±5.60[a]	150.42±5.58[a]	154.97±5.29[a]	93.72±5.54[b]	60.55±4.89[c]

Means with same letter are not significantly different; a, b and b, c indicate mean which are significantly different ($p < 0.05$); a, c are mean which are very significantly different ($p < 0.001$). Growth evolution of animals fed with the diets Bs$_1$, Bs$_2$ and Bs$_3$ were respectively similar to those of the control group ($p>0.05$) while growth of rats fed with the diets Bs$_4$ and Bs$_5$ was relatively low as compare to that of the control group.
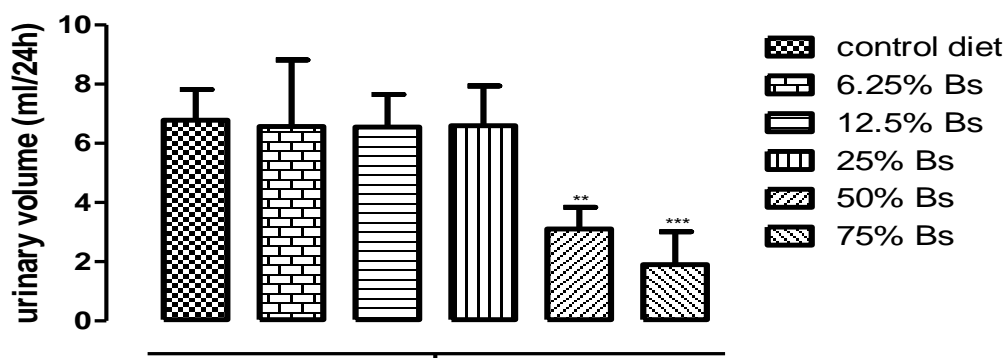
Figure 1. Urine volume in relation to the diet consumption. The urine volumes obtained from rats fed with diet Bs$_1$, Bs$_2$ and Bs$_3$ were not significantly different ($p>0.05$) from that obtained from rats fed with control diet. But iiin rats fed with Bs$_4$ and Bs$_5$ urine volumes were respectively high ($p<0.05$) and very high ($p<0.001$) when compared with that obtained from rats fed with the control diet. (**): high variation; (***): very high variation.
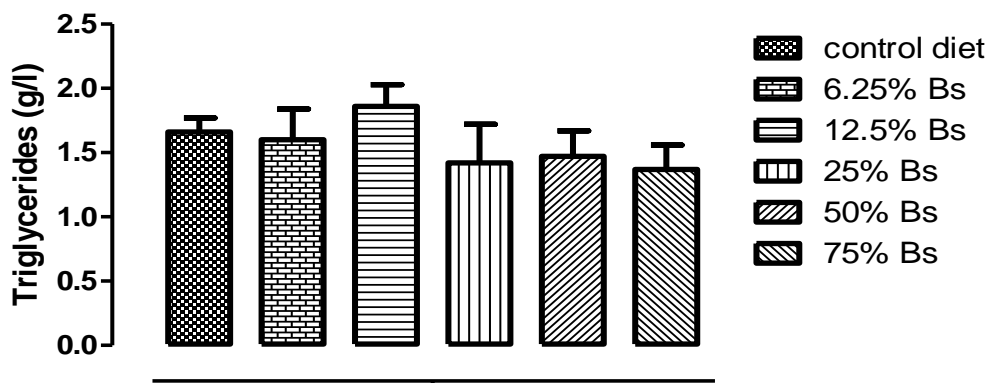
Figure 2. Effect of *B. sapida* aril powder level on plasmatic triglycerides. Plasmatic triglycerides values were not significantly ($p>0.05$) different from that obtained in rats fed with the control diet.

rats of the control group were 0.88±0.07 and 0.40±0.00 g/l, respectively.

The values of total-cholesterol found in rats fed with diets Bs$_1$, Bs$_2$, Bs$_3$, Bs$_4$ and Bs$_5$ were 0.82±0.11, 0.84±0.05, 0.80±0.03, 0.78±0.05 and 0.98±0.07 g/l, respectively. These values were not significantly different ($p > 0.05$) when compared with that of the control group. The variation of LDL-cholesterol in the experimental group was not significantly different ($p > 0.05$) from those obtained in the control group. These values were

Figure 3. Effect of *B. sapida* aril powder level on plasmatic total-cholesterol, LDL-cholesterol and HDL-cholesterol. Significant augmentation ($p<0.05$) of total-cholesterol, LDL-cholesterol and HDL-cholesterol in rats fed with diet Bs_5 as compared to that obtained in rats of the control group was observed. (*): Significant augmentation.

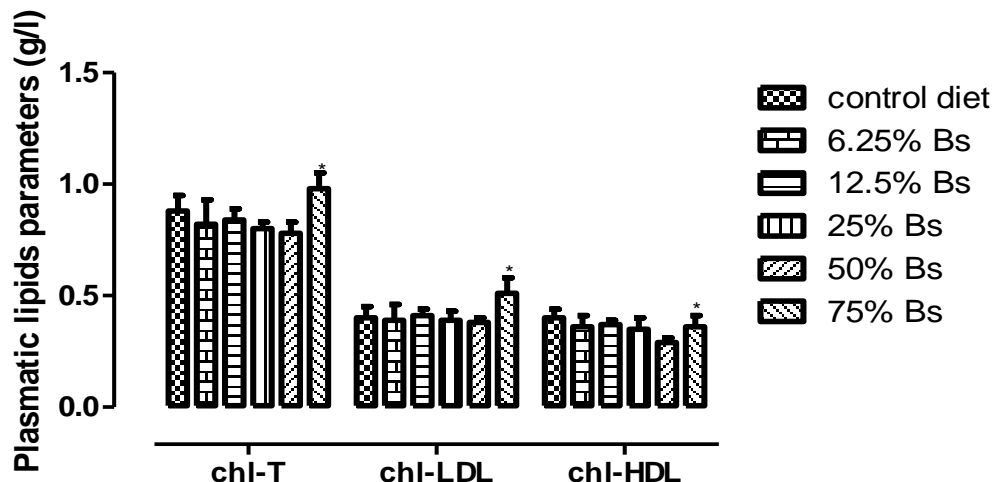
Figure 4. Effect of *B. sapida* aril powder level on plasmatic glucose. Plasmatic glucose values were not significantly ($p>0.05$) different from that obtained in rats fed with the control diet.

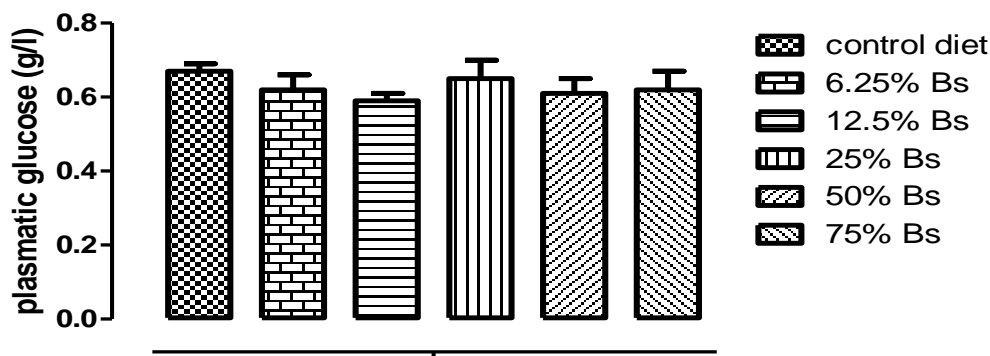0.39 ± 0.07 g/l for diet Bs_1, 0.41 ± 0.03 g/l for diet Bs_2, 0.39 ± 0.04 g/l for diet Bs_3, 0.38 ± 0.02 g/l for diet Bs_4 and 0.51 ± 0.07 g/l for diet Bs_5.

The values of HDL-cholesterol found in rats fed with diets Bs_1, Bs_2, Bs_3, Bs_4 and Bs_5 were 0.36 ± 0.05, 0.37 ± 0.02, 0.35 ± 0.05, 0.29 ± 0.02 and 0.36 ± 0.05 g/l, respectively. These values were not significantly different ($p > 0.05$) when compared with that of the control group. The effect of *B. sapida* aril powder level on plasmatic total-cholesterol, LDL-cholesterol and HDL-cholesterol is shown on Figure 3.

Effect of *B. sapida* aril powder level diet on plasmatic glucose

Plasmatic glucoses level obtained were 0.62 ± 0.04 g/l for

rats fed with diet Bs_1, 0.59 ± 0.02 for rats fed with diet Bs_2 g/l, 0.65 ± 0.05 g/l for rats fed with diet Bs_3, 0.60 ± 0.04 g/l for rats fed with Bs_4 and 0.62 ± 0.05 g/l for rats fed with diet Bs_5. These values were not significantly different ($p > 0.05$) from that obtained in rats of the control group which was 0.67 ± 0.02 g/l (Figure 4).

Effect of *B. sapida* aril powder level in diet on plasmatic protein

Plasmatic protein obtained in rats fed with diets Bs_1, Bs_2, Bs_3, Bs_4 and Bs_5 were not significantly different ($p > 0.05$) from that obtained in rats fed with the control diet. These values were 68.50 ± 2.74 g/l for rats fed with control diet, 77.00 ± 5.40 g/l for rats fed with diet Bs_1, 72.17 ± 4.26 g/l for rats fed with diet Bs_2, 71.00 ± 1.79 g/l for rats fed with diet
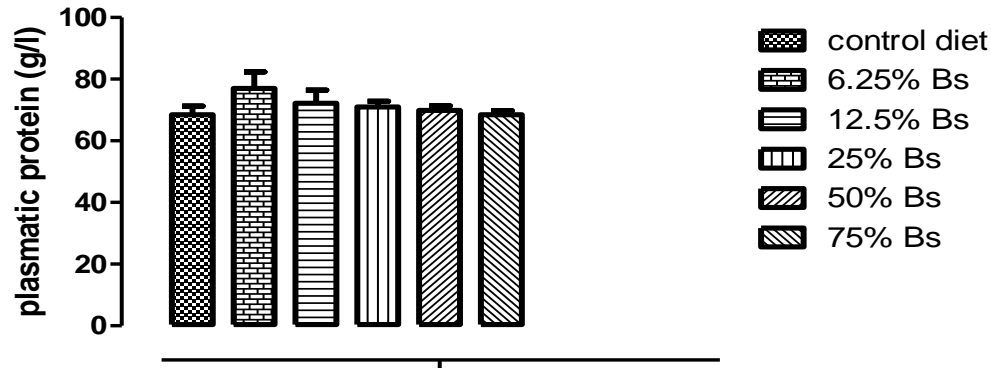
Figure 5. Effect of *B. sapida* aril powder level on plasmatic protein. Plasmatic protein values were not significantly (p>0.05) different from that obtained in rats fed with control diet.

Figure 6. Effect of *B. sapida* aril powder level on plasmatic urea. Plasmatic urea values were not significantly (p>0.05) different from that obtained in rats fed with the control diet.

Bs_3, 69.83±1.47 g/l for rats fed with diet Bs_4 and 68.50±1.89 g/l for rats fed with diet Bs_5 (Figure 5).

Effect of *B. sapida* aril powder level in diet on plasmatic urea

Plasmatic urea obtained in rats fed with diets Bs_1, Bs_2, Bs_3, Bs_4 and Bs_5 were not significantly different (p > 0.05) from that obtained in rats fed with the control diet. These values were 0.13±0.01 g/l for rat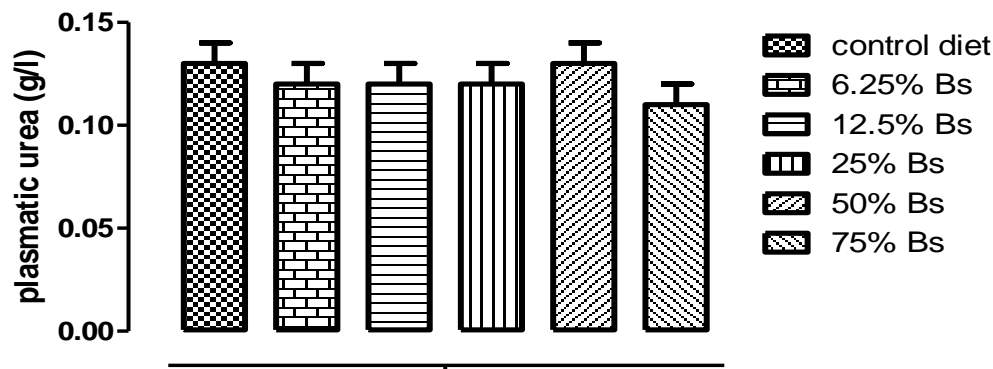s fed with control diet, 0.12±0.01 g/l for rats fed with diet Bs_1, 0.12±0.01 g/l for rats fed with diet Bs_2, 0.11±0.01 g/l for rats fed with diet Bs_3, 0.13±0.01 g/l for rats fed with Bs_4 and 0.11±0.01 g/l for rats fed with Bs_5 (Figure 6).

DISCUSSION

The absence of growth difference in rats fed with experimental diets (diets Bs_1, Bs_2, Bs_3, Bs_4 and Bs_5) in comparison with growth of rats fed with the control diet could be explained by the fact that these diets are also nutritiously rich. In fact, the different diets may contain satisfactory nutriment elements such as carbohydrate, lipid, vitamin and mineral which are favorable for good growth (Basiotis et al., 2002; American Dietetic Association, 2007; Cindy et al., 2013).

As for rats fed with diets Bs_4 and Bs_5, growth retardation was noticed when compared with the growth of rats fed with the control diet. This growth retardation was higher among rats fed with diet Bs_5 than those fed with diet Bs_4. However, the coat of all rats was natural. Growth retardation in rats fed with diets Bs_4 and Bs_5 may be the result of an inadequate alimentation due to a deficiency of some nutritional elements (IRD, 1996; Katona and Mokhda, 1998; Lefere, 2000). It may be a deficiency of protein (FAO/WHO, 2007). In fact, according to an analysis of the nutritional composition of dry *B. sapida* aril made by Ouattara et al. (2010) the protein content was 11.99±1.12 g/100 g. This protein content is lower than the one recommended by the Food Agriculture Organization (FAO, 2003) which must be between 12 and 15% in diet. The mixtures of control diet with freeze-dry aril correspond to a diminution of protein in diet. So, when the substitution of the control diet by the

freeze-dry aril is important, the diminution of protein content in the diet obtained is higher. This clearly explains the best growth of rats which consumed the diet Bs_4 than those which consumed the diet Bs_5. Protein restriction in diets Bs_4 and Bs_5 was very important than protein content of the other diets (control diet, diets Bs_1, Bs_2 and Bs_3) so that it has caused malnutrition due to the deficiency of protein or some amino acids (Drewnowski, 2005). The deficiency of protein or some amino acids justify the growth retardation observed in rats fed with these diets (diets Bs_4 and Bs_5) than the growth of rats which consumed the other diets (control diet, diets Bs_1, Bs_2 and Bs_3). But, no death was observed in the groups of rats fed with diets Bs_4 and Bs_5: this suggests that *B. sapida* aril powder contain adequate quantity of protein and nutritionals elements easy to use by the rats fed with these diets (diets Bs_4 and Bs_5) even if they do not particularly have sufficient protein.

The absence of lipids level in rats fed with diets Bs_1, Bs_2, Bs_3 and $Bs_{4\ as}$ compared to that obtained in rats fed with the control diet suggested that lipids have been used as source of energy in order to satisfy the energy need of the anamal instead of the decrease of carbohydrate in diets. Nevertheless, lipids level obtained in rats fed with diet Bs_5 is higher when compare with that obtained in rats of the control diet. This situation is due to the lipids abundance in the diet Bs_5 and to the fact that they have not been all utilized as source of energy: they may be accumulated in the rats fed with this diet and then they increased the level of total-cholesterol and LDL-cholesterol. Since no death was noticed, the freeze-dry aril can be used as a palliative to prevent untimely death in the case of lack of food.

Conclusion

Consumption of *B. sapida* aril powder does not affect the growth of rats until the degree was approximately 50%. At a degree near the value of 50% and above, body weight and growth are affected. At these values of consumption, a rate of total-cholesterol and LDL-cholesterol is noticed. This rate is due to the high content of the diet on fat based on aril. So, *B. sapida* aril should be consumed with moderation to improve body wellbeing. Therefore, it cannot absolutely be utilized as a palliative to the lack of food but it can contribute to avoiing untimely death in the case of lack of food.

Conflict of interest

The authors did not declare any conflict of interest.

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Full Length Research Paper

Comparison of metal content in seeds of *Moringa ovalifolia* and *Moringa oleifera*

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The study aimed at determining the minor and major elements present in seeds of *Moringa ovalifolia* and *Moringa oleifera*. The seeds were air-dried after collection, then ground to a fine powder and digested with acids in a microwave system prior to analysis by inductively coupled plasma optical emission spectroscopy (ICP-OES). The concentration of different elements in *M. ovalifolia* seeds such as Ca, K, Fe, Ni, Cu, Mn, Co and Cd varied ($P<0.05$) amongst the 3 sites were sampled. However, no significant difference ($P>0.05$) in the concentrations of elements such Mg, Li, Ba, Si, Al, Na and Zn was observed between sites. Potasium (K) was found to be present in highest amounts at all sites with average concentration of 7938 mg/kg while Li (10.78 mg/kg) had the lowest concentration. This study showed that the content of some metals in *M. ovalifolia* seeds varied depending on geographical sites. Both *M. ovalifolia* and *M. oleifera* seeds are a good source of important minerals and need to be explored further for use as supplements and ready source of dietary minerals in animal and human food.

Key words: Elemental analysis, Inductively Coupled Plasma Optical Emission Spectroscopy, macronutrients,

INTRODUCTION

In many developing countries, the supply of minerals is inadequate to meet the mineral requirements of farm animals and rapidly growing population (Anjorin et al.,

2010). Minerals are essential in animal feed and human nutrition. Minerals cannot be synthesized by animals and humans but are provided from plants or mineral- rich

water (Mosha et al., 1995).

There are 13 known species in the Moringaceae family and *M. oleifera* is the most widely studied and cultivated as a multi-purpose tree (Ramachandran et al., 1980). The only endemic southern Africa Moringa species is *Moringa ovalifolia* which is distributed from central-southern Namibia to south-western Angola (Dyer, 1975). Moringa seeds have a number of benefits. For instance, they contain iron and amino acids in addition to anti-inflammatory and antiseptic properties (Padayachee and Bijnath, 2012).

Determination of minerals and trace elements is important in enhancing production efficiency in plants and foods (Rodriguez et al., 2011). Some of the trace elements, which include iron, manganese, zinc and copper, are essential micronutrients with a variety of biochemical functions in all living organisms (Hicsonmez et al., 2012). Different elements have many functions in plant growth and development. Metal ions, including iron, zinc and copper, are required for catalytic and structural properties of many proteins and are therefore essential for growth and development of all organisms. Essential elements also play a major role in nerve transmission, blood circulation, cellular integrity, energy production and muscle contraction (Belay and Kiros, 2014). However, excessive amounts of these metals, or of non-essential metals such as cadmium (Cd) and lead, are toxic and inhibit plant growth (Guo et al., 2008).

The macronutrients are distinguished between two sub groups, major ones and secondary ones. The nutrients like nitrogen (N), phosphorus (P) and potassium (K) are referred to as the major macro-elements, and calcium (Ca), magnesium (Mg), and sulfur (S) are the secondary ones. The micronutrients, which are needed only in trace amounts, are iron (Fe), manganese (Mn), boron (B), zinc (Zn), copper (Cu), molybdenum (Mo), chloride (Cl), sodium (Na), nickel (Ni), silicon (Si), cobalt (Co) and selenium (Se) (Ronen, 2007; Pakade et al., 2013). Micro-nutrients are important just like macro-elements. Plant performance is crucially dependent on adequate supply of all elements including those that are demanded in relatively small quantities (Ronen, 2007).

The aim of the study was to compare the mineral content present in *M. ovalifolia* and *M. oleifera* seeds. Elements found in the seeds were divided in two categories, namely major (Ca, K, Mg and Na) and minor (Ba, Cd, Co, Li, Mn, Si, Al, Zn, Cu, Ni and Fe) elements. To the best of the authors' knowledge, no such comparative study of the two species has been done before and the elemental composition of *M. ovalifolia* seeds is yet to be reported.

MATERIALS AND METHODS

Chemicals and reagents

Nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) were purchased

from Sigma Aldrich (Johannesburg, South Africa). Ultra-pure water from a Milli-Q Millipore purification system (MA, USA) was used in the experiment.

Instruments

Metal content were analyzed using inductively coupled plasma optical emission spectroscopy (ICP-OES) (Spectro Genesis, Spectro, Germany). Before metal analysis, samples were digested using the microwave instrument (Anton Par, Switzerland).

Sample collection

M. oleifera seeds were collected from one of the biggest and oldest Moringa farms in Limpopo Province, South Africa. *M. ovalifolia* seeds were collected in Namibia from the following areas: Moringa Farm (designated as area A, which is 40 km west of Okahandja, Otjozondjupa region), Halali (designated as area B, situated in Etosha National Park, Kunene region), and in Tsumeb (designated as area C, Oshikoto region). A map of Namibia showing the collection areas is provided in Figure 1. Samples were collected in paper bags.

Preparation of samples

Dry seeds were deshelled and the resulting kernels were ground into fine powder using a pestle and mortar. Samples were kept in a cool dry place until use.

Microwave digestion of seeds for metal analysis

The method by Pakade et al. (2013) was used. A mass of 0.1 g ground seed powder was placed in a microwave digestion vessel and concentrated nitric acid (8 mL) and hydrogen peroxide (2 mL) were added. Digestion was carried out for about 30 min in the microwave. After digestion, the samples were transferred to a 25 mL volumetric flask and made up to volume with deionised water. The solutions were directly analysed for metal content using ICP-OES and results were expressed as mg/kg of dry weight.

Data analysis

The experiments were carried out in triplicates and result presented as mean ± standard deviation (SD). Normality of the data was tested using Kolomongorov-Smirnov test and the normally distributed data was analyzed by ANOVA and means were compared with a post-hoc Scheffe multiple comparison test, using SPSS™ for windows® version 18. All analyses were done at confidence interval (CI) = 95%, $\alpha = 0.05$.

RESULTS AND DISCUSSION

Comparison of metal content in M. ovalifolia and M. oleifera seeds from different sites

Both minor elements: (Ba, Cd, Co, Li, Mn, Si, Al, Zn, Cu, Ni, Fe) and major elements (Ca, K, Mg and Na) were present in seeds of M. ovalifolia. The concentrations of
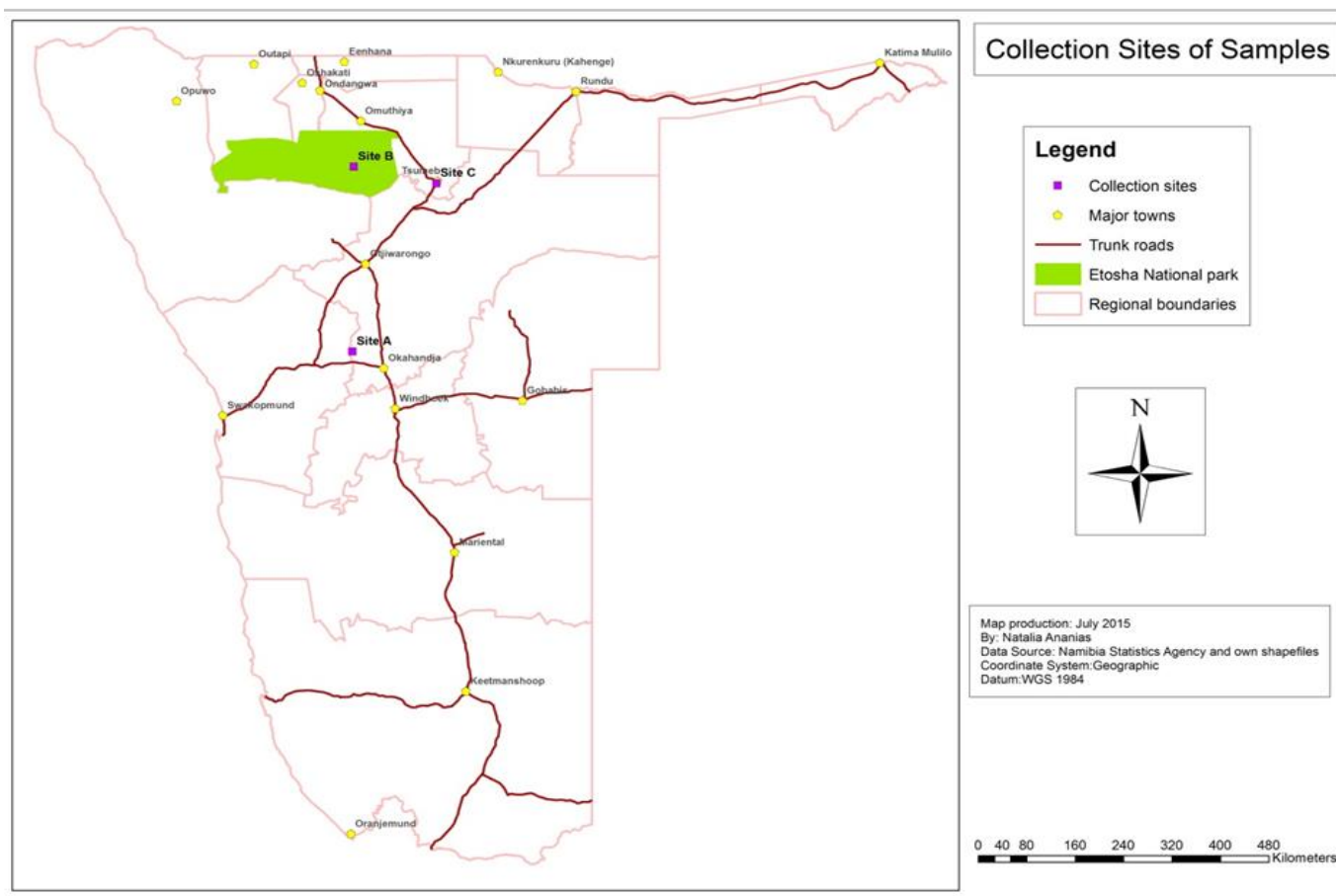
Figure 1. Map of Namibia showing different collection sites for *M. ovalifolia.*

minor elements in the two Moringa species are shown in Table 1, while that of major elements are shown in Figure 1. Similar types of elements were detected in *M. oleifera.* Among the micro nutrients, barium (Ba) had the lowest concentration (1.5-2.1 mg/kg) at all three sites of *M. ovalifolia*. This was followed by Co and Cd. Fe had the highest concentration among the micronutrients (62.7-84.7 mg/kg) and this is followed by the Zn (59.1-63.9 mg/kg) and Al (23.2-28.4 mg/kg). There was no significant difference (P>0.05) in site concentrations of Ba, Li, Si, Al, and Zn, whereas there was a significant difference (P<0.05) in Fe, Ni, Cd, Mn, Co and Cu concentrations at different sites. Cd concentration at site A (6.2 mg/kg) was significantly higher (P<0.05) to sites B (3.9 mg/kg) and C (3.9 mg/kg). There was a lower concentration of Co from site C (1.92 mg/kg) compared to sites A (3.4 mg/kg) and B (3.8 mg/kg). Site C had a higher concentration of Mn (26.5 mg/kg) compared to site A (17.7 mg/kg) and B (18.8 mg/kg). This illustrates that the concentration of some of these individual micro nutrients varied according to the site.

The concentration of K was highest in all sites compared to other elements (average of 6399.9 mg/kg) among the macro nutrients (Figure 2). This might be because potassium is generally found in the highest concentration in plants and is the third most abundant mineral in the human body. Except for N, the plants require more potassium than any other nutrient. Thus, its mobility in the plant allows it to influence almost all aspects of plant growth. Potassium increases crop yield and improves quality, it increases root growth and improves drought resistance (Armstrong et al., 1998). Ajayi (2008) reported that seeds were good sources of mineral elements. The study by Ajayi (2008) revealed that potassium as the most prevalent mineral element with concentration of 2470 and 1680 ppm in *Artocarpus heterophyllus* and *Treculia africana*, respectively. Mg is the second highest macronutrient in the seeds followed by Ca and Na.

The variation observed in some metal concentration of *M. ovalifolia* seeds may have been due to either different genetic makeup of the plants or more probably due to

Table 1. Concentration (mg/kg; dry weight) of minor elements in *M. ovalifolia* and *M. oleifera* seeds from different sites (Mean ± SD of 3 replicates are presented).

Elements	*M. ovalifolia* Site A (Moringa Farm)	*M. ovalifolia* Site B (Halali)	*M. ovalifolia* Site C (Tsumeb)	*M. oleifera* Limpopo
Ba	2.1±0.1	1.5±0.1	1.5±0.1	1.9±0.4
Cd	6.2±0.3	4.0±0.9	4.0±0.1	3.7±0.1
Co	3.4±0.2	3.8±0.1	1.9±0.4	2.8±0.3
Li	10.9±0.1	10.8±0.1	10.8±0.1	10.8±0.1
Mn	17.7±0.2	18.8±0.1	26.5±1.0	21.2±0.2
Si	12.6±0.1	13.9±0.4	13.3±0.6	16.6±0.7
Al	28.4±2.6	25.8±1.5	23.2±0.8	16.0±0.9
Zn	59.1±0.9	63.9±0.4	65.5±0.3	64.0±1.2
Cu	29.8±0.4	29.0±0.2	12.5±0.3	7.5±0.4
Ni	27.4±1.0	11.1±1.4	19.2±1.2	38.8±6.4
Fe	84.7±2.2	79.1±0.2	62.7±0.7	93.9±5.1
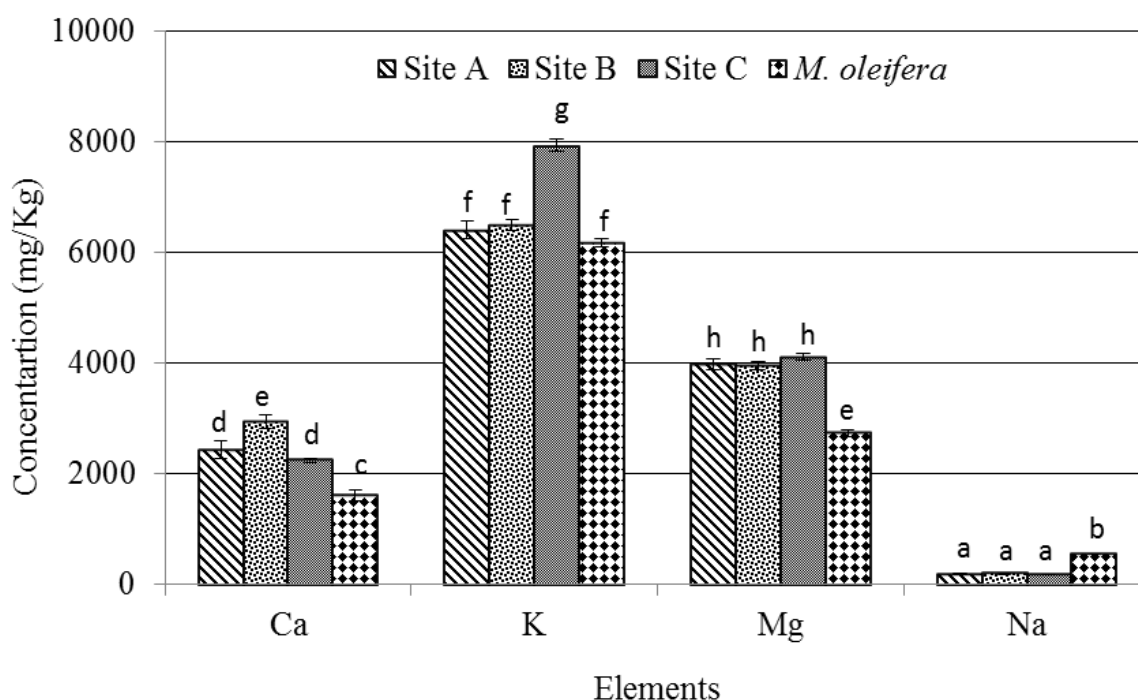
Figure 2. Comparison of concentration of major elements in seeds of *M. ovalifolia* from different sites in Namibia (Site A, B and C) and *M. oleifera* (from Limpopo, South Africa). Means (±SE) of three replicates are presented. Mean with the same letters were not significantly different from each other (p<0.05).

the environmental effects. It is reasonable to expect the concentration of seed elements of plants from Etosha to be different from others as these have been exposed to drought conditions since there is a low rainfall in that region unlike other sites. Moreover, there is a possibility that Moringa plants from Tsumeb may have accumulated some heavy metals, as that region has Cu mining.

Comparison of the metal content in the seeds of the two Moringa species

Among the micronutrients, there were no significant differences in the concentrations of Ba, Li, Zn, Si and Co in the seeds of the two species (Table 1). There were significant difference in the concentrations of Cd, Mn, Cu,
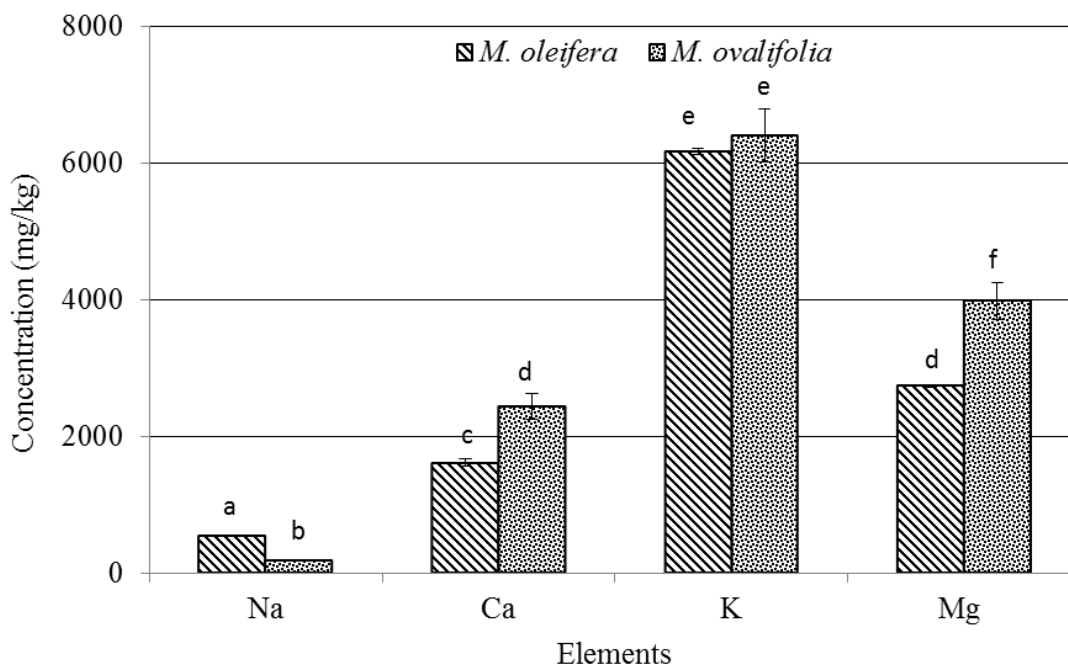
Figure 3. Comparison of macronutrients in the seeds of *M. oleifera* and *ovalifolia.* Means (±SE) of three replicates are presented. Mean with the same letters were not significantly different from each other (p<0.05).

Table 2. Concentrations of trace elements in plants (dry weight) to the deficient, normal and phytotoxic levels (Pugh et al., 2002).

Level	Concentration (mg/kg)			
	Cd	Cu	Pb	Zn
Deficient	NA	<1.5	-	<10
Normal	0.05-2	3-30	0.5-10	10-150
Phytotoxic	5-700	20-100	30-300	>100
This study	3.7-6.2	7.5-29.8	NA	59.1-65.5

NA = not analyzed.

ovalifolia. Among the macronutrients, there was no significant difference in the concentration of K of the two species (Figure 3) but there were significant differences in the concentration of Ca, Mg and Na. However, the general trends of the micro- and macro-nutrients elements in the seeds of the two Moringa species were the same. Elements that are highest in *M. ovalifolia* were also highest in *M. oleifeira* and vice versa. *M. ovalifolia* seeds had higher concentrations of the following elements: Ca (2428 mg/kg), K (6400 mg/kg), Mg (3975 mg/kg), Cu (29.8 mg/kg) and Al (28.4 mg/kg) than that of *M. olefeira*. *M. oleifera* seeds have higher concentrations of Si (16.5 mg/kg), Fe (93.9 mg/kg), Na (545.2 mg/kg) and Ni (38.8 mg/kg) compared to *M. ovalifolia*. A study by Anjorin et al. (2010) reported that Ca and Mg were relatively higher in

M. oleifera leaves and seeds from Nigeria. However, this study also showed high content of Ca and Mg in *M. oleifera* (South Africa) and *M. ovalifolia* (Namibia) seeds. This indicates that Moringa seeds are good source of Ca and Mg, and hence they can be used in farm animals or humans as food. Some people cook and eat flesh Moringa pods, especially *M. oleifera*, that contain seeds as a source of food. This practice should thus be encouraged as these may prove to be a good source of much needed micro- and macro- nutrients.

Comparison of metal content with normal levels

The Cd, Cu, and Zn concentrations in the seeds were compared to the deficient, normal and phytotoxic levels (Table 2). The concentrations of these metals in *M. ovalifolia* and *M. oleifera* seeds is within the normal levels. Therefore, according to this analysis, the *Moringa seeds* in this study are fit for human consumption. Although levels of trace elements in the soil are much higher (Pakade et al., 2013), there is little accumulation in the biomass to reach phytotoxic levels. This is important for human consumption of Moringa seeds as food.

Conclusion

This study showed that some metal content of *M.*

ovalifolia seeds varies depending on geographical location (sites). Both *M. ovalifolia* and *M. oleifera* seeds are a good source of important minerals and needs to be explored further for use as a supplement and ready source of dietary minerals in animal and human food. There was a significant variation in micronutrients and macroelements in *M. oleifera* and *M. ovalifolia* seeds for some elements but also some did not show significant differences. This might be attributed to the variable uptake of minerals by the plants and variable agro-ecologies of the different regions. *M. oleifera* is the most well-known of the genus and its given recognition as a multipurpose tree. However, more studies needs to be done on *M. ovalifolia* for its potential as food and medicinal plant.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Indicator microorganisms, *Salmonella*, *Listeria monocytogenes*, Staphylococcal enterotoxin, and physicochemical parameters in requeson cheese

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Requeson cheese is an artisanal dairy product widely consumed in Latin America. However, no data is available on its microbiological and physicochemical quality. Eighty-four samples were collected. Identification and enumeration of indicator microorganisms and pathogens were done by conventional culture methods, meanwhile staphylococcal enterotoxin by immunoassay. Bromatological parameters were measure collection day. Indicator and pathogenic microorganisms per samples were aerobic mesophilic bacteria (n = 100), coliform bacteria (n = 100), molds-yeasts (n = 70), *Staphylococcus aureus* (n = 74), and *Salmonella* (n = 3). Neither *Listeria monocytogenes* nor Staphylococcal enterotoxin was detected. Temperature ranged from 3 to 19°C, pH from 5.1 to 6.9, acidity from 0.1 to 0.7%, and sodium from 290 to 3970 mg/100 g. Protein content ranged from 2.1 to 13% and lipids from 4.1 to 13%. Starch as adulterant was present in 56 samples.

Key words: Indicator microorganism, *Salmonella*, *Listeria monocytogenes*, Staphylococcal enterotoxin, macronutrients, sodium.

INTRODUCTION

Food contamination is a direct consequence of inadequate hygiene practices during manufacture, handling, transport, storage and marketing.

Microorganisms from other sources are transferred to the surface of foods where they have the nutrients needed to proliferate to levels ranging from 10^2 to 10^5 CFU/cm²

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(Quiroz-Santiago et al., 2009). In the dairy industry, for example, both milk and utensils can contribute to microorganism load, including pathogenic microorganisms (Jayarao et al., 2006; Marth and Steeler, 2001; Vázquez-Salinas et al., 2001). Cheeses made from raw milk are especially susceptible, and can contain pathogenic bacteria such as *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Latorre et al., 2009; Schoder et al., 2003; Torres-Vitela et al., 2012; Tunick and Van Hekken, 2010). However, cheeses made from pasteurized milk can also contain microorganisms when handling and marketing conditions are inadequate (Licitra, 2010). In one example, both *Klebsiella* and *Enterobacter* species were identified from fresh cheese made from pasteurized milk (Renye et al., 2008), demonstrating the importance of proper hygiene measures in cheese production installations. Control of refrigeration temperature is also a critical parameter in ensuring the safety of fresh cheese (Bishop and Smukowsi, 2006).

Precise classification of cheese types is difficult due to the diversity of names, country of origin and lack of knowledge of the manufacturing process. In Mexico, a number of fresh cheeses are made, including requeson, ricotta, cottage, *panela*, *adobera*, "feta", and cream cheese. Some of these cheeses are made from whey, and can be solid, semi-solid or soft. These are made using one of two processes: concentration of the whey followed by molding; or thermal coagulation of whey with or without addition of acid, and finished fresh or aged (CODEX, 2011). Requeson cheese is produced via heat precipitation of whey proteins (mainly β-lactoglobulin and α-lactalbumin) in an acid medium. The final product is bland, soft in texture, salty and granulated, with high moisture content, and requires refrigerated storage (NOM-243-SSA, 2010). It has low macronutrient content, but contains micronutrients such as sodium, iron and calcium (Pérez-Lizaur et al., 2014). Requeson cheese is widely consumed in Latin America and Spain, and is considered highly perishable. Despite its popularity, no reports have been published on its safety or the presence of pathogenic microorganisms in requeson cheese (Pintado and Malcata, 2000). Neither are there reports on its marketing or nutritional quality. Requeson cheese is made from the whey discarded from manufacture of other cheese types, meaning its nutrient and mineral contents can vary widely between different producers. These variations can determine the type of microorganisms present in the final product (Pitado et al., 2005).

The present study objective was to identify and quantify the presence of aerobic mesophilic bacteria, coliform bacteria, molds and yeasts, *Staphylococcus aureus*, *Salmonella*, *L. monocytogenes* and Staphylococcal enterotoxin in requesón cheese acquired from independent retailers. Data were also reported as bromatological parameters, the temperature at the time of purchase, pH, acidity, sodium concentration, and starch, protein and lipids contents.

MATERIALS AND METHODS

Sample collection

Requeson cheese samples were acquired from fifty-five small, independent dairy retailers located in five distribution centers in the metropolitan area of Guadalajara, Mexico. Over a six month period, a total of 84 samples were taken of requesón cheese sold in bulk (Marques de Cantú, 1991). Each sample (500 *g*) was immediately placed in a sterile plastic bag, hermetically sealed and transported in a cooler (Igloo, Houston, USA) to the laboratory for analysis within one hour of collection.

Sample preparation

Sub-samples (10 *g*) from each sample were placed in a sterile plastic bag with 90 ml sterile peptone diluent (SPW; Bioxon Mexico) and pummeled in a stomacher for 1 min. Subsamples were analyzed to quantify (APC), Coliform bacteria (CB), *S. aureus,* and Yeasts and Molds (Y-M). Separate sub-samples (25 *g*) were taken for *L. monocytogenes* and *Salmonella* identification, placed in sterile plastic bags containing 225 ml sterile *Listeria* enrichment medium (LEM; DIFCO. Sparks, MD) or sterile lactose broth (LB; Bioxon Mexico), and pummeled in a stomacher for 1 min.

Microbiological analyses

Analysis of APC, CB, Y-M, *S. aureus*, *Salmonella* and *L. monocytogenes* were done following methods described in the U.S. Food and Drug Administration (Bacteriological Analytical Manual [BAM], 2013). Briefly, for APC, CB, Y-M and *S. aureus*, serial dilutions in SPW (Bioxon, Mexico) from the sub-sample homogenates were prepared. Aliquots (1 ml) of each dilution were transferred to petri dishes (three petri dishes per dilution). Plate count agar (PCA), violet red bile agar (VRBA) and potato dextrose agar (PDA) containing 50 mg/L ampicillin (Bayer, Leverkusen, Germany) and 0.6% dichloran rose bengal (Sigma-Aldrich. St. Louis, MO) were poured into the inoculated petri dishes.

Determination of *S. aureus* was done by taking 0.1 ml of each dilution and spreading it onto Baird-Parker medium (BPM) using a sterile bent glass streaking rod. The PCA, VRBA and BPM plates were incubated at 35°C/48 h, while PDA plates were incubated at 25°C/5 days.

Sub-samples for *Salmonella* identification were incubated in LB at 35°C for 18 to 24 h. Enrichment was done in soy Rappaport Vassiliadis broth (RV_B) and tetrathionate broth (T_B) for 24 h at 41.5°C. The RV_B and T_B were streaked onto plates containing bismuth sulfite (BS) agar, xylose lysine desoxycholate (XLD) agar, and Hektoen enteric (HE) agar and incubated for 24 h at 35°C. Three to five typical *Salmonella* colonies were randomly chosen from each selective medium for biochemical identification using urea broth, lysine-iron agar (LIA) and triple sugar iron (TSI). The *Salmonella* spp. genus was identified by serological analysis using somatic antigen polyvalent serum (Antiserum poli A&VI, Bioxon, Mexico).

Subsamples for *L. monocytogenes* quantification were incubated in *Listeria* enrichment medium base containing sodium pyruvate (BD DIFCO, Sparks, MD) at 30°C for 48 h. The medium was then

Table 1. Average, mean, maximum and minimum values for indicator microorganisms and *S. aureus* contents in requeson cheese samples (n=84).

Parameter	Aerobic mesophilic bacteria*	Coliform bacteria*	Molds/Yeasts	*S. aureus**
Average	7.7	2.9	4.9	5.8
Maximum	8.9	6	6.5	7.7
Minimum	5.2	0.5	2.6	3.3
Mean	7.6	2.4	4.8	5.7

*Data expressed as log CFU/g.

streaked onto Oxford medium (OXA) and incubated at 35°C for 48 h. Fresh mobility and sheep blood hemolysis tests were done for the typical colonies and the CAMP and biochemical tests (API Listeria, bioMérieux) were done to confirm dubious reactions. Presence of staphylococcal enterotoxin in both cheese types was determined following AOAC procedure 12095 B-ES-2003/09. Briefly, samples (25 g) were mixed with 25 ml extraction buffer, homogenized in a stomacher (Lab-Blender 400) for 2 min and incubated at 18 to 25°C for 15 min. Two milliliters of supernatant were transferred into a 1.5-ml Eppendorf tube and centrifuged for 15 min at 4,000 *g*. The pH was adjusted to between 7.5 and 8.09 using 1 N NaOH and then 500 ml of solution transferred to a SET2 strip (bioMérieux, Marcy-l'E´toile, France).

Physicochemical parameters

Temperature measurements were taken from the geometric center of each sample using a thermometer with a probe (Cooking Thermo Timer, Cooper Atkins, Middlefield, USA). For each pH measurement, 100 ml deionized water was added to the bag, the contents manually homogenized from outside the bag for 1 min and pH measured with a potentiometer (pH meter 320, Corning, Corning, USA). Acidity was measured as a percentage (%) by manually homogenizing 10 g sample with 25 ml deionized water, adding phenolphthalein and titrating with 0.1 N sodium hydroxide. Sodium was quantified using Mohr titration. Requesón cheese samples (10 g each) were placed in precipitate flasks, 15 ml deionized water at 50°C added and mixed with a glass rod. Another 20 ml hot distilled water were added to disperse the sample, which was transferred to a matrass and completed to 100 ml with deionized water. The sample was run through filter paper, 50 ml of permeate placed in an Erlenmeyer flask and 1 ml potassium chromate added. This solution was titrated with a standard 0.1 M silver nitrate solution until a persistent red-brown color appeared for 30 s (Nielsen, 2010a).

Protein/nitrogen quantification was done following the Kjeldahl method. Digestion was done using a 5 g sample and adding 25 ml concentrated sulphuric acid and a 5 g Missouri tablet as catalyzer (Merck, Darmstadt, Germany). It was then neutralized with sodium thiosulfate and the distillate captured in 50 ml boric acid with methylene blue and methyl red as indicators. This solution was titrated with 0.1 N HCl. Protein content was measured using a 6.38 conversion factor (Nielsen, 2010c, 2010). Lipids were analyzed using the Gerber method. A sample (11 g) was placed in a Gerber bottle, and 10 ml 90% sulfuric acid and 1 ml isoamylic alcohol added. The bottle was closed, shaken and centrifuged for 4 min. It was then placed in a water bath at 60°C for 5 min and lipids content measured (Nielsen, 2010b). Starch was quantified qualitatively by adding an iodine-iodide solution to a 5 g sample; appearance of a blue color indicated the presence of starch.

RESULTS AND DISCUSSION

Microbiological analysis

Levels of APC, CB and Y-M were generally high (Table 1). Of the 84 analyzed samples, 27 had APC levels of 9.0 to 8.1 log CFU/g, 46 had levels between 8.0 and 7.1 log CFU of BMA/g, and eleven had levels <7 log CFU of BMA/g. Overall, CB levels ranged from 0.5 to 6 log CFU/g. Thirty-six samples had levels from 1.1 to 3.0 log CFU/g, while 29 samples had levels between 3.1 and 5.0 log CFU/g. All samples were contaminated with Y-M, with 70 having levels ranging from 4.1 to 6.0 log CFU/g (Table 1). Contamination with *S. aureus* was also ubiquitous, with 74 samples having levels between 5.1 and 7.0 log CFU/g (Table 1). Staphylococcal enterotoxin was not detected in any of the analyzed samples. *Salmonella* was isolated in only three samples and *L. monocytogenes* was not detected in any.

Both in Mexico and developed countries such as the US, identity regulations do not include the composition of the cheeses of Latin America; this makes naming and describing them quite difficult (Van Hekken and Farkye, 2003). Requeson cheese is a fresh cheese consumed widely in Mexico. It consists of a soft granular mass that will not hold a mold, and is produced only from the whey discharged after cheese production. Generally made on a small scale, often using artisanal techniques, it is most frequently marketed in bulk in independent dairy stores, where it can be exposed to temperature abuse.

Requeson cheese manufacture involves heating to 85 to 95°C, which substantially reduces or inactivates most non-sporulated bacteria in the whey. However, it can easily be recontaminated under the poor safety conditions with which it is normally handled and marketed in Mexico. Its high nutrient and moisture contents, and near-neutral pH (Hough et al., 1999; Pintado et al., 2001), allow microorganisms to quickly become active if it is not adequately refrigerated. In response to the lack of requeson cheese safety and microbiology data in Mexico, the present microbiological analysis was limited to identifying indicator microorganisms commonly used to evaluate food safety and microbiological quality. Analyses were also done of the presence of two

pathogenic bacteria frequently reported in fresh cheeses, and Staphylococcal enterotoxin.

The generally high indicator microorganism frequencies observed here may have resulted from both direct and cross-contamination (Table 1). Levels this high can be produced in two ways. The first scenario is that initialcontamination level was low but microbial growth became extremely active, quickly reaching maximum concentrations. The second is that contamination level was high during preparation, with no later microorganism growth; which is the most likely scenario in the present case. However, substandard food safety conditions during requeson cheese preparation and marketing can greatly increase the likelihood of both possibilities.

Approximately 50% of the samples had CB counts >3 log CFU/g. This microbial group may contribute to cheese flavor and aroma (Menendez et al., 2001), but in requeson cheese its presence is linked to inadequate post-thermal treatment hygiene practices (Renye et al., 2008). Although no previous studies have been done of requeson cheese microbiology, the present results can be compared to reports on similar types of cheese. For example, high CB and Y-M levels have been reported for other fresh cheeses (Renye et al., 2008; Williams and Withers, 2010).

The main pathogenic microorganisms involved in cheese contamination are *L. monocytogenes*, *S. aureus*, *Salmonella* and *E. coli* pathotypes (Williams and Withers, 2010). The present analyses focused on *Salmonella*, *L. monocytogenes* and *S. aureus*, because *Salmonella* (Franco et al., 2010; Heredia et al., 2001; Soto-Beltran et al., 2015), *S. aureus* (Aguilar et al., 1983; Parrilla-Cerrilo et al., 1993) and *L. monocytogenes* (Latorre et al., 2009; Moreno-Enriquez et al., 2007; Rodas-Suárez et al., 2006) have been isolated from different foods in Mexico. From a public health perspective, *Salmonella* infections, and *S. aureus* food poisoning are endemic in Mexico; indeed, from 2009 to 2011, 381,320 salmonellosis cases, 139,000 typhoid fever cases and 122,200 food poisoning cases were reported (Secretaría De Salud, 2011). *Salmonella* can be found in cheeses made from raw or pasteurized milk. Of the different cheese types, fresh cheeses are most frequently involved in disease outbreaks traced to cheese consumption (Michanie et al., 2001). In the present data, 3.5% of the requesón cheese samples were *Salmonella*-positive, a low frequency near that reported previously. For example, in a study of Requiejǎo (a semi-hard Brazilian cheese) none of 25 samples were found to have *Salmonella* (Viana et al., 2009). In a study of fresh cheeses, *Salmonella* was not identified in any of the samples, although *L. monocytogenes* was identified (Menendez et al., 2001). The latter microbe is common in fresh cheeses and frequently implicated in outbreaks from fresh cheese consumption (CDC, 2012; Jackson et al., 2010; MacDonald et al., 2005; Torres-Vitela et al., 2012; Vázquez-Salinas et al., 2001). However, *L. monocytogenes*

was not detected in the present requeson cheese samples.

In contrast, *S. aureus* levels surpassed 5 log CFU/g in 94% of the samples and coagulase-positive strains were detected. Staphylococcal enterotoxin was not detected in the analyzed samples, but the high frequency of *S. aureus* and requeson cheese is near neutral pH and high nutrient content could still provoke staphylococcal enterotoxin synthesis (Williams and Withers, 2010).

Most of the samples (77%) were stored or marketed under conditions of temperature abuse (>5°C), which favors growth of microbes, including pathogenic bacteria (FDA, 2011; Food Code, 2009). In addition, the samples near-neutral pH and low acidity can promote bacterial development and survival (Pintado et al., 2001). The *Salmonella*-positive samples were found to have temperature/pH/acidity values of 6°C/pH 6.1/0.14 acidity and 15°C/pH 6.2/0.27 acidity, values that may have favored this pathogen's survival in the requeson cheese. This is supported by a study of the relation between physicochemical parameters and *Listeria* presence in fresh cheese (Soto-Beltran et al., 2014). In this study, pH was found to be significantly ($P < 0.05$) related to *Listeria* presence and negatively ($P > 0.05$) linked to salinity. Other studies have confirmed that changes in fresh cheese physicochemical parameters can favor microbial activity. In a study of ricotta cheese, storage at 17°C was found to negatively affect organoleptic and microbiological characteristics and lower shelf life to 12.5 days compared to refrigerated storage (Hough et al., 1999).

Physicochemical parameters

Temperature varied widely in the samples at the moment of sampling (Table 2). Nineteen were <5°C and the remaining 65 were >5°C; of the latter, eleven were between 17 and 19°C. Values for pH ranged from 5.1 to 6.9 with an average of 6.1 (Table 2). Titratable acid, expressed as lactic acid, averaged 0.3% in the samples with a range of 0.1 to 0.7%. Sodium was >466 mg/100 g in 76 of the samples.

Composition analysis showed most of the samples (61) to have protein content between 5.1 and 7.0 g/100 g, while casein ranged between 2.2 and 7 g/100 g of cheese (Table 3). Lipids content ranged from 4.1 to 13 g/100 g with 44 samples having values between 9.1 and 11 g/100 g. Starch was detected in 56 of the samples.

The significant differences ($P<0.05$) observed here in sample physicochemical parameters may be due to variations in whey characteristics caused by its origin, type and source process. Requeson cheese nutrient content will also be affected by the composition of the whey used to make it. Whey contains substantial amounts of protein, lipids, lactose and calcium (Miranda-Miranda et al., 2009). Whey protein is essentially albumin

Table 2. Average, mean, maximum and minimum values for sodium content, acidity, pH and temperature in requeson cheese samples (n=84).

Parameter	Sodium (mg/100)	Acidity (%)	pH	Temperature (°C)
Average	1660	0.3	6.10	11
Maximum	3970	0.7	6.90	19
Minimum	290	0.13	5.10	3
Mean	1360	2.80	6.1	9.6

Table 3. Distribution of requesón cheese samples based on total protein, casein and lipids contents*.

Sample	Total protein	Casein	Lipids
1.1 a 2.0	-	-	-
2.1 a 3	3	12	-
3.1 a 4	8	12	-
4.1 a 5	4	52	4
5.1 a 6	29	0	4
6.1 a 7	32	8	4
7.1 a 8	0	-	16
8.1 a 9	4	-	4
9.1 a 10	4	-	28
10.1 a 11	-	-	16
11.1 a 12	-	-	4
12.1 a 13	-	-	4

*Data expressed as g/100 g requesón cheese.

and globulin because any casein is removed during cheese curdling (Franchi, 2010; Vázquez-Puente et al., 2010). Requeson cheese protein content is influenced by whey acidity and processing temperature. In one study, protein content doubled when the whey was acidified to 0.45% and heated to 93°C (Raftari et al., 2009). This contrasts with the present results in which samples with the highest protein content (8.4 and 9.6%) had low acidity (0.1 to 0.2%).

According to the Mexican Food Equivalents System 2014 (Sistema Mexicano de Alimentos Equivalentes - SMAE), requeson cheese is a very low-fat (6.38 g/100 g) animal product (Pérez-Lizaur et al., 1993). However, of all samples in the present study, 76 samples surpassed the very low-fat cut-off level. In the (CODEX, 2014), dairy products are classified as non-fat or skim when they contain ≤10% lipids. In the present study, only 38% of the samples fell within this classification while the remaining 62% had 10 to 13% lipids, which would classify them as low-fat products.

For the sodium content, SMAE indicate 466 mg/100 g as average contents for this element in the requeson cheese. However, in the study, only eight samples presented 290 to 380 mg of sodium/100 g, meanwhile 76 samples were >466 mg of sodium/100 g. Sodium chloride improves cheese flavor and lengthens its shelf life by reducing water activity, thereby inhibiting bacterial development (Hutton, 2002). The USDA National Nutrient Database for Standard Reference (USDA, 2014), does not include a nutritional description for requesón cheese, but it is generally not recommended to use high sodium levels to decrease pathogen presence in this kind of food (Soto-Beltran et al., 2014).

On the other hand, level of protein in samples was lower than SMAE (11.94 g of protein/100 g of requeson cheese). The objective of determination of starch is because of its use as a stabilizer and anti-agglutinating agent in some fresh cheeses, although its use is not recommended in cheeses produced from whey (CODEX, 2014). This is notable since over half the samples contained starch.

Conclusions

In Mexico, requesón cheese production is essentially artisanal and it is sold largely through independent marketers. This constitutes a serious challenge in the pathogen control and food safety in fresh cheeses in general, and particularly, in requesón cheese. The present results indicate requesón cheese in Mexico to be at high risk of contamination and highlight an acute need for implementing adequate food safety measures throughout the product's trajectory, from production to marketing. Contamination risk was especially high during post-production handling and storage. Sample contamination could have been substantially reduced with use of individual packaging and proper retail storage conditions. As is the case with other highly perishable products, avoiding contamination of requesón cheese requires standardized operating and hygiene procedures during production, storage, transport to retailers and marketing. A good first step towards this goal would be to ensure fulfillment of applicable regulations controlling requesón cheese physicochemical and food safety parameters to ensure product characteristics and hygiene.

Conflict of Interests

The authors have not declared any conflict of interests.

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