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Microbial Profile of Maize-Pigeon Pea Biscuit in Storage

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Abstract

The production of biscuit from a blend of maize (Zea mays) and pigeon pea (Cajanus cajan) flour had earlier been reported in the literature. However, there is no information on the shelf stability of the biscuit. This study therefore investigated the microbial profile of the biscuit stored for 30 days at 29 °C and 70 % relative humidity. The proximate and microbiological qualities of the biscuit were determined using standard methods. Biscuit had 5.19 % moisture, 1.90 % ash, 21.59 % fat, 0.62 % crude fibre, 15.83 % protein and 54.86 % carbohydrate. The microbial load of the biscuit was below the standard limit of National Food and Drug Administration and Control (NAFDAC) on the day of production but increased above the permissible level during storage. *Staphylococcus*, *Enterobacter* and *Bacillus species* were found in the biscuit during storage. The public health importance of these microorganisms was discussed.

Key words: *biscuit, maize, pigeon pea, microorganisms*

Introduction

The observed increase in snack consumption among Nigerians may be attributed to socio-economic development and urbanisation. Snacks generally tend to be high in calories and fat, but low in proteins, vitamins and other nutrients (Rampersad *et al.*, 2003). Fortification of cereal flour with low-cost legume flours such as pigeon pea in the production of snacks would help to alleviate the perennial problem of protein malnutrition, in Nigeria, especially among the school-age children, who are the major snack consumers. Food formulations combining cereals and legumes have been recommended for the alleviation of protein malnutrition in developing countries since the amino acid profiles of the two

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food groups are complemented by their combination (Saxena *et al.*, 2010). Biscuit is among the most commonly consumed baked snacks in Nigeria (Ogunjobi and Ogunwolu, 2010). Biscuit possesses several attractive features such as wider consumption base, relatively long shelf-life, more convenience and good eating quality which make it attractive for protein fortification and other nutritional improvements (Zaker *et al.*, 2012). Biscuit is predominantly based on refined wheat flour (Zaker *et al.*, 2012). However, acceptable biscuits have been produced from composites of wheat and non-wheat flours (Hussain *et al.*, 2006; Zaker *et al.*, 2012) or solely from a variety of non-wheat flours (Olaoye *et al.*, 2007; Hussein *et al.*, 2011).

Pigeon pea, also locally known as 'otili' in south west of Nigeria is well tolerated by the soil and climatic conditions of western region of Nigeria (Adebowale and Maliki, 2011). It is a rich source of protein, carbohydrate and certain minerals (Akande *et al.*, 2010). Pigeon pea protein, though deficient in the sulfur-containing amino acids, methionine and cysteine, is a rich source of lysine (Rampersad *et al.*, 2003). It thus supplements the essential amino acids of cereals (Hassan *et al.* 2011). The use of pigeon pea in the supplementation of cereals has been widely reported (Adeola *et al.*, 2011; Hassan *et al.*, 2011; Okpala and Okoli, 2011).

Previous study by Echendu *et al.* (2004) revealed that it was possible to fortify maize with pigeon pea to produce acceptable biscuit. The study recommended a ratio of 60:40 (maize: pigeon pea) for biscuit production. However, the study did not report on the microbiological profile of the biscuit during storage. Information on the microbiological profile is important from food safety standpoint and also to determine appropriate processing parameters needed to arrest the proliferation of microorganisms.

It is therefore the aim of this study to determine the microorganisms that are associated with the storage of biscuit produced from composite flour of 60 % maize and 40 % pigeon pea.

Materials

Maize, pigeon pea, sugar, margarine, baking powder (sodium bicarbonate) and eggs were obtained from Odo-ori market, Iwo, Osun State.

Methods

Production of maize flour

This was done according to Houssou and Ayemor (2002). The processing was carried out in a local Mill. Maize grains were cleaned and tempered (sprinkling of water). The tempered maize grains were thereafter poured in a locally fabricated machine which dehulled and degermed the grains. The maize grits were then winnowed, milled and sieved using a 500 μm mesh (Fig. 1).

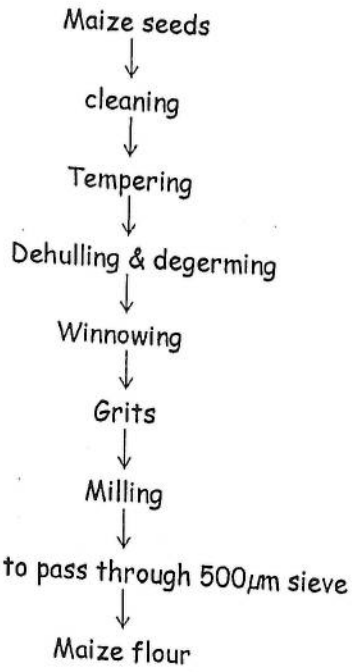

Figure 1: Production of Maize flour
Source: Modified Housou and Ayemor (2002)

Production of pigeon pea

The method of Mueses *et al.* (1993) was modified to produce pigeon pea flour. Pigeon pea seeds were cleaned, sorted and subjected to

steaming in a retort at 120 °C for 5 min. Thereafter, the seeds were manually dehulled, cooked for 5 min using a pressure pot, dried in a cabinet dryer at 65 °C for 2 days, milled and sieved using a 500 μm mesh (Fig. 2).

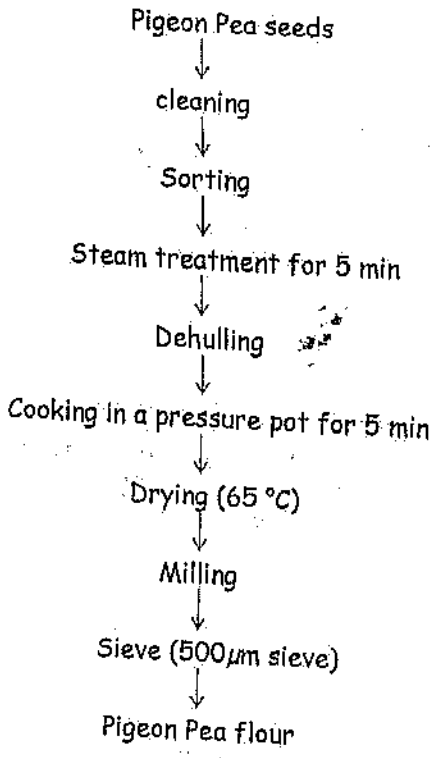

Figure 2: Production of Pigeon pea flour
Source: Modified Mueses *et al.* (1993)

Production of biscuit

The method of Akpapunam and Darbe (1994) was modified. Margarine (90 g), 20 ml whole egg and 12 ml of invert glucose syrup were thoroughly mixed in a Kenwood Chef Mixer (running at speed 4 for 10 minutes) until a smooth gel was obtained. Exactly 30 percent of maize-pigeon flour blend (60 g), 40 g of granulated sugar, 1.50 g of salt, 1.25 g of baking powder and 15 g of edible starch, were dry mixed thoroughly in a kitchen blender running at maximum speed, then poured into a 500 ml- beaker and pre-gelatinized with 100 ml boiling water. The pre-gelatinized mixture was then poured on the fat mixture (margarine, whole egg and invert glucose syrup) in the Kenwood Chef Mixer bowl. Mixing of the ingredients was done for 5 minutes, with the remaining flour (70%; 140 g) added without pre-gelatinization, and mixed for another 5 min to form a firm and consistent dough. The dough was kneaded, rolled into flat sheets and cut into shapes before being baked in an oven at 180 °C for 45 min.

Storage

Biscuit samples were packaged in low-density polyethylene and stored for 30 days at 29 °C and 70 % relative humidity.

Proximate analysis

The proximate composition of the flours and biscuit was done according to the methods of A.O.A.C. (2005).

Microbiological analysis

The media used were Nutrient agar (NA), Potato Dextrose agar (PDA), Salmonella Shigella agar (SSA) and Eosin Methylene Blue (EMB). The media were prepared according to manufacturer's instructions. NA was prepared by weighing 7 g of the agar into a 500 ml conical flask and dissolving it with 250 ml distilled water. The conical flask was covered with non-absorbent cotton wool plug wrapped with aluminum foil and warmed on a heating mantle for the mixture to homogenise. Thereafter, the content of the flask was sterilised in an autoclave at 121 °C for 15 min. Preparation of PDA, SSA and EMB were done in this way as described for NA after weighing 9.75 g, 15 g and 9.43 g respectively into conical flasks. Chloramphenicol was aseptically added to molten PDA at 45 °C in order to discourage bacterial growth. The microorganisms were isolated using the pour plate method. The serial dilution method of Meynell and Meynell (1970) was used. About 1 g of each sample was added into test tubes containing 10 ml sterile water and these served as stock solutions. About 1 ml was aseptically removed from each of the stock

added to another set of test tubes containing 9 ml sterile water each to make 10^{-1} dilution. Similar transfers were repeated to make 10^{-4} decimal dilution. Thereafter, about 0.5 ml of the 10^{-4} decimal dilution was introduced into sterile Petri dishes and sterile molten agar was poured into the plates. The inoculated plates were allowed to set and incubated in an inverted position in order to avoid condensed water vapour on the plate cover from dropping on the culture. The NA, SSA and EMB plates (for bacterial culture) were incubated at 37 °C for 24 hr while the PDA plates (for fungal culture) were incubated at 25 °C for 72 hr. Thereafter, the number of colonies found on each media after incubation were counted. The streak plate method was used in as much as the colonies on the PDA were in unicellular forms. Sterile molten agar was poured into Petri dishes and allowed to solidify. Distinct colonies were picked from the mixed cultured plates and separately streaked in quadrant streak-plate method to obtain pure culture isolates. The streaked plates were incubated at 37 °C for 24 hr for bacterial growth and 25 °C for fungal growth. This process was repeated until pure cultures were obtained. In obtaining pure cultures, PDA and NA were prepared in McCartney bottles and sterilized in an autoclave. The media were allowed to set in an incline position to make agar slants. The pure bacterial isolates were transferred into sterilised NA slant and incubated at 37 °C for 24 hr while the PDA slants containing pure fungal isolates were incubated at 25 °C for 48 hr.

Fungal identification was done by observing the cultural (colour, shape, elevation and surface) and morphological characteristics of the isolates. The bacterial isolates were confirmed by their morphological (gram staining), cultural (configuration, margin, elevation, optical characteristics, consistency and pigmentation) and biochemical (indole, motility, catalyse, coagulase, oxidase, starch hydrolysis, sugar utilisation, urease and citrate utilisation tests).

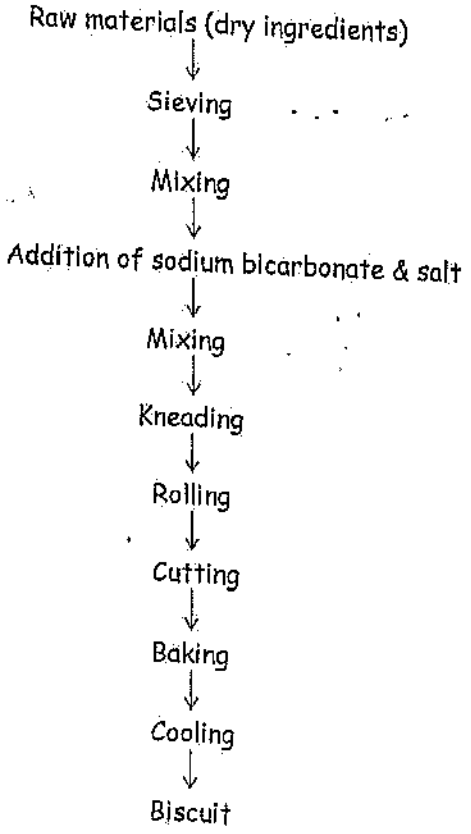

Figure 3: Production of Biscuit
Source: Modified Akpapunam and Darbe (1994)

Data analysis

The data obtained for proximate composition were subjected to descriptive analysis. Means having significant differences were separated using Duncan Multiple Range Test at 5 % level.

Results and discussion

Table 1 shows the proximate composition of the maize, pigeon pea and maize-pigeon pea flours, and biscuit. The flour samples and biscuit varied significantly ($p < 0.05$) with respect to the proximate composition. Maize flour and biscuit recorded the highest (10.36 %) and lowest

Table 1: Proximate composition of flour and biscuit

Sample	Moisture, %	Ash, %	Fat, %	Crude fibre, %	Protein, %	Carbohydrate, %
Maize flour	10.36±0.08^{d}	1.03±0.02^{a}	1.75±0.02^{a}	0.33±0.01^{a}	8.72±0.01^{a}	77.80±0.07^{d}
Pigeon pea flour	6.16±0.04^{b}	2.24±0.03^{d}	2.34±0.03^{c}	4.40±0.01^{d}	28.35±0.24^{d}	56.51±0.28^{b}
Maize-pigeon pea flour	8.25±0.02^{c}	1.41±0.03^{b}	2.22±0.03^{b}	1.73±0.03^{c}	20.11±0.04^{c}	66.29±0.05^{c}
Biscuit	5.19±0.09^{a}	1.90±0.03^{c}	21.59±0.03^{d}	0.62±0.02^{b}	15.83±0.09^{b}	54.86±0.04^{a}

Values represent means ± Standard Error of mean for 3 replicates. Means with different letters in the same column are not significantly different at $p < 0.05$

(5.19 %) moisture contents, respectively. Echendu *et al.* (2004) reported higher moisture content for biscuit (5.70 %) and lower moisture for maize (4.90 %) than the values obtained in this study. The difference may be due to the different processing methods adopted by the two reports and difference in the maize varieties. However, the moisture contents of the flours and biscuit are within the limit recommended for safe storage of cereal flours and biscuit. Microorganisms have an absolute demand for water; no microbial growth can occur without water (Frazier and Westhoff, 1978). Hence, the lower the moisture content, the lower the microbial activity. The pigeon pea flour had the highest ash (2.24 %), fat (2.34 %), crude fibre (4.40 %) and protein (28.35 %) contents. The maize-pigeon pea flour blend recorded higher ash (1.41 %), fat (2.22 %), crude fibre (1.73 %) and protein (20.11 %) contents than maize flour which had 1.03 % ash, 1.75 % fat, 0.33 % crude fibre and 8.72 % protein. The carbohydrate contents of the samples were 77.80 %, 56.51 %, 66.29 % and 54.86 % respectively for maize, pigeon pea and maize-pigeon pea flours and biscuit respectively. The fat content of biscuit was much higher than those found in the flours, may be as a result of the margarine incorporated into the biscuit. The high fat content may be undesirable, from the standpoint of storage stability, due to increased tendency to rancidity. The lower protein content of biscuit, when compared with the composite flour, may be due to Maillard reaction, involving carbohydrate and protein, during biscuit processing. There were differences in the values of the other components of the proximate composition obtained for both the flour and biscuit samples in this study and that of Echendu *et al.* (2004). Furthermore, the observation by Echendu *et al.* (2004) that maize had higher fat content than pigeon pea contradicts the findings of this study. However, this study agrees with Echendu *et al.* (2004) that pigeon pea flour had higher ash, fibre and protein contents than maize

Table 2: Microbial load (cfu/g) of flour and biscuit (Day of production)

Sample	Total bacterial count	Bacillus	Lactose fermenters	Non-lactose fermenters	Shigella	Fungi
Maize flour	3.32×10^{5}	2.2×10^{5}	1.7×10^{5}	1.2×10^{5}	No growth	2.0×10^{5}
Pigeon pea flour	5.4×10^{5}	1.8×10^{5}	2.8×10^{5}	No growth	No growth	No growth
Maize-pigeon pea	2.9×10^{5}	3.5×10^{5}	2.0×10^{5}	1.6×10^{5}	No growth	2.6×10^{5}
Biscuit	4.5×10^{5}	2.5×10^{5}	2.0×10^{5}	2.3×10^{5}	No growth	2.5×10^{5}

flour and that addition of pigeon pea flour to maize flour resulted in increase in these nutrients. The observation that maize flour had higher carbohydrate content than pigeon pea flour was also similar to that of Echendu *et al.* (2004). Hassan *et al.* (2011) reported 8.50 %, 3.73 %, 23.23 % and 2.13 % for moisture, ash, protein and fat, respectively. Thus, the fat content reported by this study is similar to that of Hassan *et al.* (2011).

Table 2 shows the microbial load of the freshly prepared flour and biscuit samples. The total bacterial count ranged from 2.9×10^5 cfu/g in maize-pigeon pea flour blend to 5.4×10^5 cfu/g in pigeon pea flour. The fact that the microbial count of the biscuit sample was relatively high indicated that there were some heat-resistant microorganisms associated with flour blend dough. *Bacillus* species was found to be present at a level of 1.8×10^5 cfu/g in pigeon pea flour to 3.5×10^5 cfu/g in the flour blend. *Bacillus sp.* possesses the ability to form endospores, which are known to be resistant to heat, ultraviolet light and desiccation (Frazier and Westhoff, 1978). Except for the biscuit sample, lactose fermenters were present at a higher level than non-lactose fermenters. Lactose-fermenters have the ability to utilize lactose sugar and include *Klebseilla* and *Enterbacter* species. Non-lactose fermenters, on the hand, are unable to utilise lactose and they include *Salmonella*, *Pseudomonas*, *Proteus* and *Shigella* species. Some strains of lactose-fermenting bacteria have been implicated in food poisoning (Dube, 1983; McDonough, 2000; Eze *et al.*, 2011). According to Eze *et al.* (2011), the National Food and Drug Administration and Control (NAFDAC) recommended a viable count of $5.0 \times 10^5 - 1.0 \times 10^6$ cfu/ml for *E. coli*, *Lactobacillus spp.* and *S. aureus* as the official public health standard. The microbial loads of the flour and biscuit samples increased during storage (Table 3), indicating that the storage condition was favourable to microbial growth. Butt *et al.* (2004), Seevaratnam *et al.* (2012) and Nagi *et al.* (2012) also observed an increase in microbial loads of wheat flour and biscuits during storage.

Table 3: Microbial load (cfu/g) of flour and biscuit (A month after production)

Sample	Bacillus	Total bacterial count	Lactose fermenters	Non-lactose fermenters	Shigella	Fungi
Maize flour	11.6×10^5	25.6×10^5	17.2×10^5	9.0×10^5	No growth	42.0×10^5
Pigeon pea flour	10.4×10^5	57.0×10^5	53.5×10^5	No growth	No growth	No growth
Maize-pigeon pea	18.8×10^5	32.0×10^5	38.0×10^5	11.0×10^3	No growth	24.8×10^5
Biscuit	16.4×10^5	47.0×10^5	16.0×10^5	30.8×10^5	No growth	12.8×10^5

Table 4: Morphological and Biochemical characteristics of Bacterial isolates from Biscuit

Isolate	Catalase test	Coagulase test	Starch hydrolysis	Motility	Gram staining	Indole test	Citrate test	Glucose test	Lactose test	Sucrose test	Shape	Probab organi
1	+	-	+	+	+	-	+	A	A	-	C	*Staph*
2	+	+	-	-	+	-	+	AG	AG	AG	C	*Staph*
3	+	-	+	+	+	-	-	-	A	-	B	*Bacillu*
4	+	+	+	-	-	+	-	A	AG	A	B	*Enterc*
5	+	-	-	+	-	+	+	A	AG	A	B	*Enterc*

(+) - Presence, (-) - Absence, A - Acid production; G - Gas production; C - cylindrical; B - Bacillus, *Staphy.- Staphyloccus sp., Bacillus - Bacillus sp., Entero. - Enterobacter sp.*

Table 4 confirms the presence of *Staphylococcus sp.*, *Enterobacter sp.* and *Bacillus sp.* Saranraj and Geetha (2012) reported that the incidence of these microorganisms have been implicated in food poisoning outbreaks, resulting from consumption of baked products. *Bacillus sp.* is associated with ropiness in baked products. Ropiness can develop very rapidly under warm and humid conditions (Saranraj and Geetha, 2012). Hence, it is a common problem in the warm climates of Mediterranean countries, Africa and Australia (Saranraj and Geetha, 2012). Ropiness in bakery products is characterised by discoloration from brown to black and the release of a rotten fruit odour (Saranraj and Geetha, 2012), and cause food poisoning symptoms such as vomiting, nausea, diarrhea and headache (Aydin *et al.*, 2009). Preservatives, such as propionate, may be used to eliminate the problem of ropiness (Bailey and Holy, 1993).

Table 5 reveals that *Aspergillus flavus* is associated with the storage of biscuit. According to Saranraj and Geetha (2012) *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp., *Monilia* sp., *Mucor* sp. and *Eurotium* sp. are the most common moulds found in bakery products. Moulds are often found on the surface of the bakery product and can cause undesirable odors (Jarvis, 2001).

Table 5: Morphological and cultural characteristics of Fungal isolate

Colour/Pigmentation of mycelium	Septation	Colour of spores	Types of spores	Appearance of spores head	Probable organism
Cream	Septate	Light green	Conidia	Smooth	*Aspergillus flavus*

Conclusion

Storage of biscuit produced from maize and pigeon flour blends at 29 °C and 70 % relative humidity is associated with some pathogenic microorganisms. It is important to carry out a moisture sorption study in order to determine the appropriate packaging materials and storage conditions to optimize the quality attributes and shelf life of the biscuits.

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