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Microorganisms Associated with Pearl Millet Cultivars at Various Malting Stages

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Microbiological analysis of samples taken at various stages of malting pearl millet cultivars was investigated. The results indicated that total bacterial count, mould count, staphylococcal count, coliform count and yeast count ranged from 4.08 to 5.28 $\log_{10}$ CFU/g, 2.50 to 3.71, 1.78 to 4.20, 2.65 to 3.65, and 2.54 to 3.70, respectively. The predominant microorganism in unmalted grain was *Bacillus subtilis*, while green malt had *Rhizopus arrhizus* and *Proteus vulgaris* as the predominant microorganisms. *Rhizopus arrhizus* occurred at a higher frequency (75.0%) in dried and polished malts. Most of the microorganisms were widely distributed, particularly *Torulopsis glabrata*, *Lactobacillus plantarum* and *Streptococcus lactis*. The population of microorganisms isolated from malting stages was not high enough to produce effective dose. However, there is the need to adopt strict hygiene practices to prevent malt from being sources of contamination to malt based foods.

Pearl millet is an important food for millions of people inhabiting the semi-arid tropics and is a major source of calories and vital component of food security in the semi-arid areas in the developing world (FAO and ICRISAT, 1996). The grain is processed in so many ways for preparation of various food products. Some of the products include cooked whole grain, thin and thick porridges, steam cooked grits (couscous, burabosko), kunun zaki, tuwo and fura(Nkama and Ikwelle, 1997; Jideani et al., 1999, 2001, 2002). Pearl millet can also be malted to produce alcoholic beverages and weaning food (Desikachar, 1980; Mosha and Svanberg, 1983; AHRTAG, 1990). There are a lot of investigations carried out on physicochemical and malting properties of pearl millet in recent times. The physicochemical and water absorption characteristics of pearl millet cultivars have been published by Badau *et al*. (2002, 2005a). Steep-out moisture, malting loss, diastatic power (Badau et al., 2006a), sugars (Badau et al., 2005b), amylase activity (Badau *et al*., 2006b), phytic acid content and hydrochloric extractability of minerals in pearl millet as affected by germination time and cultivar have also been published (Badau *et al*., 2005c). Information with regard to microbial load of weaning foods or other food products from malted cereals produced by traditional technologies (such as malting) are not very adequate in this region. The incidence of diarrheal disease is high in children during the weaning stage which could be due to microbial contamination of food and water (Mathar and Reddy, 1983). Badau *et al*. (2006c) have documented the effect of addition of malt flour from pearl millet cultivars and sorghum on production, acceptability and microbial evaluation of weaning food formulations. However, information on the microorganisms associated with the various malting stages of these pearl millet cultivars is still inadequate in this area. Therefore, the objectives of this study were to determine the microbial load, isolate and identify microorganisms at various malting stages of pearl millet cultivars.

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MATERIALS AND METHODS

Three pearl millet cultivars; SOSAT C-88, ZANGO and EX-BORNO were obtained from Lake Chad Research Institute, Maiduguri and International Crops Research Institute for the Semi-Arid Tropics Experimental station at Bagauda, Kano, Nigeria. These pearl millet cultivars were used because they have negligible tannin content (Badau *et al.*, 2002), high hydrochloric acid extractability of minerals (Badau *et al*., 2005c), high sugars content (Badau *et al.,* 2005 b) and high amylase activities (Badau *et al*., 2006b) and generally they have good malting properties(Badau *et al.*, 2006a). Culture media used were nutrient agar (International Diagnostics group, PLC, Plancashire BC9 6AU, U.K.) for total bacterial aerobic plate count, mannitol salt agar (International Diagnostics group, PLC, Plancashire BC9 6AU, U.K.) for staphylococcal count, sabouraud dextrose agar (Biotech Laboratories Ltd, 38 Anson Road Marthesham Heat Ipswich, Suffolk IP5 3RG, U.K.) for yeast count, MacConkey (International Diagnostics group, PLC, Plancashire BC9 6AU, U.K.) for coliform count. Others were *Salmonella/Shigella* (International Diagnostics group, PLC, Plancashire BC9 6AU, U.K.) for

Salmonella/Shigella count, potatoes dextrose agar (BDH Chemicals L–Poole, England) for mould count. Irish potatoes obtained in Maiduguri market, Nigeria, agar (Vickers laboratories Ltd, Burley–IN Wharfedale West Yorks, England), dextrose (Oxoid, Ltd, Wade Road, Basingstoke, Hampshire RG 24 v, England) were used to prepare potatoes dextrose agar in the laboratory of Department of Food Science and Technology, University of Maiduguri, Nigeria. Crystal violet, Gram's iodine solution, 95% ethyl alcohol, safranin, bibulos paper and oil immersion were used to separate bacteria into Gram positive and Gram negative. Vasiline was used for motility test by hanging drop method to identify motility of bacteria. Dextrose, xylose, VP, gelatin, citrate and starch were used to identify Gram positive aerobic spore bearing rods, *Bacillus* species. Lactose, sucrose, mannitol, xylose, arginine and NaCl were used to identify Gram positive non–motile, non–sporing rods, *Lactobacillus* species. MacConkey, aesculin, arginine, 65% NaCl, mannitol were used to identify Gram–positive cocci, *Streptococcus* species. Hugh and Leifson, coagulase, phosphotase, VP, lactose, maltose and mannitol were used to identify Gram positive, oxidative negative, catalase positive, fermentative, *Staphylococcus* species.

MR, VP, urease, H_2S, citrate, KCN, phenylalanine were used to identify Gram negative non–sporing rods, *Escherichia* species. Indole, MR, VP, citrate, gelatin, KCN, urease, malonate and gluconate were used to identify *Klebsiella* species. Crystal violet, Gram's iodine solution, 95% ethyl alcohol, safranin, bibulos paper, oil immersion, glucose, sucrose, lactose, galactose, maltose, nitrate, citrate, starch, fructose, catalase, gelatin, VP, mannose. Mannitol, xylose, arabinose and urea were used to identify yeasts upto species level. Lactophenol cotton blue was for identification of moulds. These chemicals/reagents used for the biochemical tests to identify microorganisms up to species level were manufactured by one or some of these manufacturers. These manufacturers included BDH laboratory reagents/chemicals Ltd Poole, England, Mayer and Baker Nigeria Ltd, Biotech laboratories, U.K., May and Baker Ltd, Dagenham, England, Hopkin and William, Chadwell Heat, Essex, England, Harris reagent, Philip Harries Ltd, Shenstone, England and Park Scientific Ltd, Northampton, U.K.

Sample preparation. The pearl millet cultivars were thoroughly cleaned manually using screen and unwanted materials were discarded. Broken grains as well as seeds, sand, bit of metal were removed. Experimental samples were taken using quarterly procedures of Lees (1975).

Malting. The pearl millet cultivars were malted as described by Morall *et al*. (1986), Aniche (1989), Aniche and Palmer (1990), Obizoba and Atii (1994), Gomez *et al*. (1997) and Badau *et al*. (2005c).

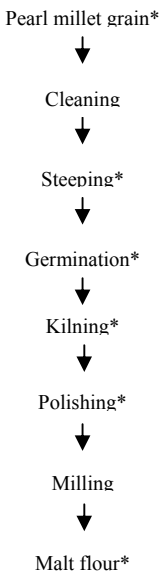

Fig. 1. Flow chart for malting of Pearl Millet grain. *Sampling points

The grains were steeped at room temperature (32 ± 2°C) for 12 h. The steep liquor was changed after 6 h. One air rest period of 1 h was applied after 6 h of steeping. After steeping, the grains were washed and drained. The grains were immersed in a 0.1% (vol/vol) solution of a commercial bleach (parazone) containing 3.5% sodium hypochlorite (diluted to 0.1% by taking 5 ml of parazone and made up to 175 ml with distilled water) for 20 min as reported by Morall *et al*. (1986). After sterilization, the grain was wrapped in wet pieces of cotton cloth and placed on wet jute bag. Another wet jute bag was used to cover the grains wrapped in the wet cotton cloth (Obizoba and Atii, 1994). The pearl millet grains were allowed to germinate at room temperature (32 ± 2°C) for 72 h. During germination, small quantity of water (15 ml) was sprayed on the germinating grains using an atomizer spray and they were "turned" (by moving a clean wooden rod inside the germinating grains) at the same time (Aniche, 1989). At the end of germination, the germinated grains were dried to moisture content of 5.00 ± 0.5% in a fan fitted oven (Gallenkamp, England) for 24 h. The dried germinated grains were polished by removing roots and rootlets. Rootlets and shoots of the grains were separated from kernels by rubbing between the palms in a sieve (Endecotts Ltd, London, England) of 0.6 mm mesh size. The sieve allowed the rootlets and shoots to escape but retained the kernels (Morall *et al.,* 1986; Aniche and Palmer, 1989). The polished malt was milled into flour with hammer mill (Gibbon Electric, Essex, U. K.) to pass through 1 mm mesh screen, packed in plastic containers and stored in iron cupboard shortly before use. Sampling of the grains at each stage of malting was done following similar procedures

reported for other foods (Badau *et al*., 2001a). Samples of the grains were taken from unmalted grain, steeped grain, green malt, dried malt, polished malt and malt flour.

Microbiological analysis. The microbiological analysis of samples from the various malting stages of pearl millet cultivars was determined using aseptic technique as described by Collins and Lyne (1970), Harrigan and McCance (1976), Nkama *et al*. (1994), Badau *et al*. (1999), Badau *et al*. (2001b), Badau *et al*. (2001c) and Badau *et al.* (2005d).

Preparation of samples for microbial studies. Samples at each stage of malting were taken for each of the pearl millet cultivars. The samples were milled and placed in sterile sampling containers and stored in refrigerator prior to microbiological analysis.

Preparation of culture media. The culture media prepared were potato–dextrose agar (BDH Chemicals L–Poole, England), nutrient agar (International Diagnostics group, PLC, Plancashire BC9 6AU, U.K.), mannitol salt agar (International Diagnostics group, PLC, Plancashire BC9 6AU, U.K.), sabourand dextrose agar (Biotech Laboratories Ltd, 38 Anson Road Marthesham Heat Ipswich, Suffolk IP5 3RG, U.K.), MacConkey (International Diagnostics group, PLC, Plancashire BC9 6AU, U.K.). S*almonella/Shigella* (International Diagnostics group, PLC, Plancashire BC9 6AU, U.K.). These were prepared following manufacturer instructions and as described by Collins and Lyne (1970), Harrigan and McCance (1976) and Nkama *et al*. (1994). Potatoes dextrose agar and Czapek–dox–agar were also prepared in the laboratory of Department of Food Science and Technology, University of Maiduguri, Nigeria as reported by Badau *et al*. (1999).

Isolation and identification of bacteria, mould and yeast. Ten gram of each stage of malting for the three pearl millet cultivars was suspended into 90 ml of sterile water to give 10^{-1} dilution. This was done up to the 10^{-6} dilution. Each diluent of samples was plated out in duplicate using the pour plating technique as described by Harrigan and McCance (1976), Nkama *at al*. (1994), Badau *et al*. (1999), Badau *et al*. (2001a, b, C) and Tou *et al*. (2006). Incubation of bacteria was in an aerobic incubator for 24 and 48 h at 37°C. Plates for fungal growth were incubated at room temperature (32°C) for 72 h. After incubation period for bacteria and fungi, their colonies appearing on the countable agar plates ($30 \leq$ Colony Forming Units on Incubated Plate ≤ 300) (Pelczar *et al*., 1977) were counted using a digital colony counter (Gallenkamp, England). The average colony obtained from the countable plates, were expressed as colony forming unit per gram (CFU/g). All pure cultures of bacteria, moulds and yeast were maintained on nutrient agar, potato–dextrose agar and sabourand dextrose agar slants, respectively. These isolates were kept in the refrigerator prior to identification. The cultural characteristics of discrete colonies such as color, shape and pigmentation of the colonies were observed and noted. It was followed by characterization of the isolates using microscopic examination for cell morphology. Microscopic examinations for detailed cell morphology and Gram reaction were carried out on 24 h old bacteria culturing (Harrigan and McCance, 1976). Biochemical tests were conducted on each isolate for the tests. The isolate was inoculated in peptone water and left for 24 h at 37°C. Biochemical tests carried out on all the bacteria isolates in each sample were starch hydrolysis, catalase test and utilization of sugars. The sugars used were glucose, sucrose, mannose, mannitol, xylose, arabinose, lactose, galactose, starch, maltose and fructose. Voges Proskauer, nitrate reduction, indole and motility tests were carried out using the methods described by Collins and Lyne (1970), Harrigan and McCance (1976). Results obtained from these tests were compared with literature standards using diagnostic tables showing the biochemical reactions identifying many genera and species of bacteria (Cowan and Steel, 1961). Fungal isolates were identified based on cultural and morphological characteristics. It was prepared by taking an inoculum from the edge of an isolated colony, placing it on a slide and mounting fluid (Lactophenol cotton blue) added. This was covered with a cover slip and heated on a flame to expel air bubble. The prepared slide was mounted on a microscope, morphological characteristics and types of spores noted. The moulds were identified as reported by Gilman (1957), Gaffa and Jideani (2001), and Singh *et al*. (1991). Frequency of the microorganisms was calculated based on percentage occurrence on 216 plated materials (Lee and Lim, 1985; Owhe–Ureghe *et al*., 1993).

Statistical analysis. Statistical analysis was carried out as described by Gomez and Gomez (1983), Mead *et al*.(1993) and with computer program (Statistix, Version 4.1 U.S.A),. Analysis of variance was used to determine differences among microbial count. The means were separated using Duncan multiple range test (Gomez and Gomez, 1983).

RESULTS AND DISCUSSION

The $\log_{10}$ CFU/g of total bacterial plate count, yeast, mould, coliform and staphylococcal counts of the various malting stages of pearl millet cultivars are presented in Figure 2. Green malt had the highest microbial count, followed by dry malt and polished malt. Malt flour and unmalted grain had the least. Steeped grain did not show growth. Total bacterial count, mould count, staphylococcal count and coliform count, ranged from 4.08 to 5.28 $\log_{10}$ CFU/g, 2.50 to 3.71, 1.78 to 4.20 and 2.65 to 3.65, respectively. Malting which involves steeping, germination and kilning of cereal grains expose the grains to contamination. Sterilization of the steeped grains eliminated

Table 1. Distribution of Microorganisms and their percentage frequency of occurrence at various stages of malting of pearl millet[1]

Microorganisms	Malting Stages					
	Unmalted grain	Steeped grain	Green malt	Dried malt	Polished malt	Malt flour
Candida utilis	12.5	-[2]	25.0	25.0	37.5	37.5
Candida albicans	8.30	-	20.2	12.5	33.3	41.7
Candida tropicalis	25.0	-	29.2	25.0	-	45.8
Candida pseudotropicalis	20.8	-	29.2	-	-	-
Staphylococcus aureus	62.5	-	37.5	62.5	41.7	20.8
Bacillus subtilis	70.8	-	29.2	33.3	37.5	45.8
Bacillus coagulans	54.2	-	41.1	41.7	50.0	41.7
Proteus vulgaris	58.3	-	50.0	45.8	33.3	-
Lactobacillus delbruekii	41.7	-	33.3	-	-	-
Esherichia coli	12.6	-	8.33	8.33	4.2	-
Klebsiella aerogenes	37.5	-	29.2	25.0	29.2	33.3
Saccharomyces cerevisiae	-	-	-	20.8	8.3	20.8
Torulopsis glabrata	-	-	8.3	-	-	-
Lactobacillus plantarum	-	-	37.5	-	-	-
Streptococcus lactis	-	-	20.8	-	-	-
Bacillus cereus	-	-	-	-	45.8	33.3
Aspergillus niger	25.0	-	29.2	66.7	41.7	20.8
Aspergillus nidulans	29.2	-	45.8	-	12.5	-
Rhizopus arrhizus	62.5	-	50.0	75.0	75.0	33.3
Aspergillus fumigatus	-	-	25.0	58.3	-	12.5

[1] Frequency calculations was based on percentage occurrence on 216 plated materials.
[2] -: Not detected.

the initial microorganisms. The green malt had the highest microbial count which could be due the exposure of the germinating grains to various sources of contamination.

During germination, grains could be exposed to contamination during sprinkling of water, personnel and equipment used in polishing and milling. Microorganisms isolated at the various stages of malting are shown in Table 1. The predominant microorganism in unmalted grain was *Bacillus subtilis*, while green malt had *Rhizopus arrhizus* and *Proteus vulgaris* as the predominant microorganisms. *Rhizopus arrhizus* occurred at a higher frequency (75.0%) in dried and polished malt. *Bacillus subtilis* occurred most frequently in malt flour. Most of the microorganisms were widely distributed (except few) particularly *Torulopsis glabrata*, *Lactobacillus plantarum* and *Streptococcus lactis*.

Yeast, mould and bacteria were isolated at various malting stages. *Bacillus subtilis* was isolated from unmalted grains at high frequency (70.0%). *Bacillus subtilis* is a spore bearer from soil and can easily come in contact with the grain. Almost all the microorganisms encountered in this study were isolated from green malt with Proteus vulgaris and *Rhizopus arrhizus* predominating. *Proteus vulgaris* is common in soil and vegetation and can easily contaminate food (Collins and Lyne, 1970). *Rhizopus arrhizus* was predominant in dried and polished malt because it is a common fungus in the air and can settle on the grains during sprinkling of water that exposes the grains to contamination. *Candida tropicalis* and *Bacillus subtilis* predominating malt flour. Never the less, the microorganisms could be eliminated at cooking temperature and microbial counts were not high enough to produce effective dose to cause food spoilage and food poisoning.

Bacillus cereus, *Bacillus coagulans* and *Bacillus subtilis* isolated from the various stages of malting are Gram positive spore forming rods and have been implicated in food spoilage and food poisoning. *Bacillus subtilis* and Bacillus *coagulans* are non pathogens, widely distributed and can spoil many foods held above refrigerator temperatures (Jay, 1987). On the hand, *Bacillus cereus* is a pathogenic microorganism and can cause food poisoning in food contaminated with the organism at level ranging from 10^7 to 10^9 CFU/g (Hauge, 1955; Midura *et al*., 1970; Gilbert, 1979). Gram positive non spore forming rods encountered at the various malting stages were *Lactobacillus delbruekii* and *Lactobacillus plantarum*. *Lactobacillus* spp. have not been implicated in food poisoning. However, development of off-odor was recorded in food that had total count of 10^7 to 10^8 CFU/cm^2 on the surface of food (Hossetine *et al.,* 1969). The Gram negative rods encountered at the various stages of malting were *Escherichia coli, Klebsiella aerogenes* and *Proteus vulgaris*. *Escherichia coli* is the most important member of the coliform group and it's presence in large numbers in foods is generally taken to indicate faecal contamination (Jay, 1987). *Escherichia coli* food poisoning was noticed when volunteers were feed with foods contaminated at levels of 10^6–10^8 organisms/g or ml (Wentworth *et al*., 1956). *Klebsiella* spp. have been implicated in histamine associated poisoning and *Proteus vulgaris* causes custard rot (Jay, 1987).

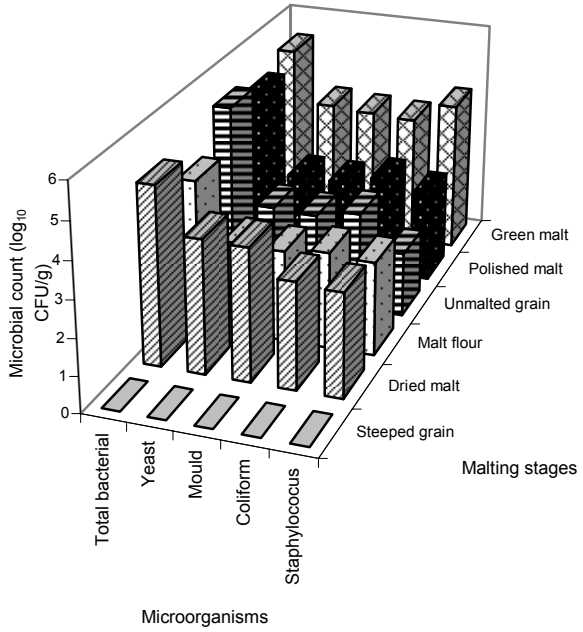

Fig. 2. Microbial count of pearl millet cultivars at various malting stages.

Gram positive cocci isolated at the various malting stages were *Staphylococcus aureus* and *Streptococcus lactis*. The presence of *Staphylococcus aureus* in large numbers in foods is undesirable (Jay, 1987). The minimum number of cells of *Staphylococcus aureus* required to produce the minimum level of enterotoxin considered necessary to cause the gastroenteritis in man (1 ng/g) differs for substrates and for particular enterotoxin, but the range is from 5×10^6 to 10^{10} CFU/g or ml (Noleton and Bergdoll, 1980; 1982; Bennet and Amos, 1983; Silverman *et al*., 1983; Notermans and Van Otterdijk, 1985). The presence of *Streptococcus lactis* in foods in large numbers may indicate faecal contamination (Jay, 1987). It has been implicated in food spoilage such as souring.

Moulds isolated from the malting stages were *Aspergillus spp.* and *Rhizopus spp. Aspergillus* spp. isolated and identified were *Aspergillus fumigatus, Aspergillus nidulans* and A*spergillus niger*. Some *Aspergillus spp.* are pathogenic. *Aspergillus fumigatus* was among fungi isolated in cereal grains. Farmers suffer from the inhalation of the spores that cause lung damage and commonly referred to as farmer's lung disease (Hough, 1991). *Rhizopus spp.* can cause food spoilage and occasional causes of serious (and often fatal) infections in humans (Larone, 1995). The yeasts isolated from the various malting stages were *Candida spp.* and these species of yeast have been implicated in food spoilage especially refrigerated food and disease of humans (Jay, 1987). *Torulopsis glabrata* was isolated from the malting stages and it is widespread in nature. The yeast can grow on refrigerated foods of many types and cause spoilage (Gardner, 1971). The most widely distributed microorganisms at various stages of malting were *Candida utilis, C. albicans, Staphylococcus aureus, Bacillus subtilis, Bacillus coagulans, Klebsiella aerogenes, Aspergillus niger* and *Rhizopus arrhizus*. Most of the microorganisms encountered in this study have been isolated in weaning food formulations by Badau *et al*. (2006 c).

The more widely distributed the organisms, the higher is the chances of the organisms to be associated with the samples rather than contamination from analysis. Therefore, there were high chances that these microorganisms were associated with the grains at the various stages of malting. The predominating organisms at the various stages of malting were *Bacillus subtilis, Rhizopus arrhizus, Rhizopus arrhizus* in unmalted grain, dried malt and polished malt, respectively. On the other hand, *Proteus vulgaris* and *Rhizopus arrhizus* were the predominating microorganisms in green malt and malt flour had *Candida tropicalis* and *Bacillus subtilis* as the predominating microorganisms. The predominating microorganisms are the likely ones to cause food spoilage and food poisoning if favorable conditions are provided. The microbial count encountered at the various malting stages was not high enough to produce effective dose and the malt flour was safe for human consumption.

CONCLUSION

The various malting stages had demonstrated that they could be a source of microbial contamination if strict hygiene practices are not adopted. Strict hygiene practice is necessary to prevent the transfer of microorganisms from malt to malt based foods. The predominating organisms at the various stages of malting were *Bacillus subtilis*, *Rhizopus arrhizus* and *Rhizopus arrhizus* in unmalted grain, dried malt and polished malt, respectively. Green malt had *Proteus vulgaris* and *Rhizopus arrhizus* as the predominant microorganisms, while *Candida tropicalis* and *Bacillus subtilis* occurred most frequently in malt flour. *Torulopsis glabrata*, *Lactobacillus plantarum* and *Streptococcus lactis* were widely distributed.

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