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RESEARCH ARTICLE

ISOLATION AND IDENTIFICATION OF THE MICRO FLORA OF TRADITIONALLY PREPARED PEARL MILLET STARTERS

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ABSTRACT

Food fermentation has a long tradition of improving the safety, shelf life and acceptability of foods. However, some notable outbreaks of food borne illness associated with fermented foods have occurred. The efficiency of one of the most common Sudanese fermentation methods of millet flour, using Dura (Sorghum) starter, in a preparation of hygienic millet starters for the production of indigenous foods was investigated. Two millet starters, Wad Ashana (pH 3.96 at 35.2°C) and Sudan II (pH 4 at 35.2 °C) were traditionally prepared. Millet flour was mixed with water extract of dura starter (pH=3.32 at 36.3 °C) in a 1:2 (wt/vol) ratio and left at room temperature (35 to 38°C) for about 24 h. Then, the starters were dried using a laboratory air oven adjusted at 47°C for overnight. The total bacterial count and yeast of Wad Ashana were 4.5×10^7 cfu/g and 5.5×10^4 cfu/g respectively and those of Sudan II were 8.6×10^7 and 6.6×10^5 cfu/g respectively. The microorganisms involved were *Lactobacillus plantarum, Streptococcus lactis, Streptococcus cremoris,* and *Pediococcus pentosaceus*. *Saccharomyces cerevisiae* was also found to contribute in millet fermentation process. With the only exception of *Streptococcus cremoris,* the starters showed the same microbial content after 17 months storage period at 6-8°C.

INTRODUCTION

Pearl millet production in Sudan is estimated as >750,000 tons/year. It is a multi-purpose crop which provides food and feed. It is grown as a staple food in the western region, Dar-fur and Kordofan states. Fermentation is an inexpensive and manageable food preservation technique. It decreases the level of anti-nutritional factors in food, besides fermented foods having low pH are microbiologically safe and could be stored for a long period of time. Fermentation serves as a major source of nourishment for large rural populations and contributes significantly to food security by increasing the range of raw materials which can be used in the production of edible products (FAO, 1998). Sudan probably has the greatest number of fermented foods and the greatest diversity of them in Africa and the Middle East. More than 80 different fermented foods and beverages were originated in the Sudan (Dirar, 1993). Sorghum and millet foods occupy special place among the fermented foods of the Sudan (Dirar, 1991). Sudanese women created various methods of fermentation to make their food. For the preparation of some, important non-alcoholic indigenous fermented millet foods and beverages like Aceda, kisra, Nasha, Hulu-mur …etc., they are accustomed to ferment millet flour using Dura starter more than spontaneous fermentation. Millet grains are usually fermented as damirga. Micro-organisms necessary in food fermentation may be added as pure or mixed cultures or, in some instances, the desired micro-organisms may be present in sufficient numbers in the original raw materials (Frazier and Westhoff, 1978). The development of starter cultures is one of the prerequisites for the establishment of such a small-scale industry in Africa (Holzapfel, 2002). Starter cultures are important for the control and optimization of the fermentation process in order to alleviate the problems of variations in organoleptic quality and microbiological stability observed in African indigenous fermented foods (Dirar, 1993). The primary consideration before introducing starter cultures for traditional small-scale fermentation should be whether these would significantly contribute to an improvement of processing conditions and product quality. Therefore, a thorough understanding of the fermentation process is required. This in turn implies the presence of starters in case of millet since spontaneous fermentation is not practically favored. And hence, the first goal of this study was to prepare millet starters initially by using Dura starter. Then, these would be allowed to become pure millet starters by consecutive use. The second important goal of the present study was to use the traditionally prepared millet starters to isolate and identify the general micro flora responsible for millet fermentation. This would lay a foundation for the production of starter cultures in the future to be utilized for scientific research follow up and for the small-scale processing of traditional millet foods.

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MATERIALS AND METHODS

Two millet genotypes (Sudan II and Wad Ashana) were kindly donated by the agro breeder Adam Mohamed Ali from Wad Medani Agricultural Research Station, El Gezira State, Sudan. The grains of the two samples were milled to fine flour and kept in tightly closed plastic containers at -16 °C (to avoid the development of millet flour bitterness).

Dura starter preparation

Wet Dura starter was obtained from a previous fermentation prepared by a typical Sudanese Kisra seller (sorghum fermented with small amount of wheat flour).The starter was centrifuged at 3000 rpm for fifteen minutes. Then, the Dura starter water extract (supernatant) was directly used for the preparation of millet starters.

Millet starter preparation

To identify the micro flora responsible for millet fermentation, a starter from each millet genotype was prepared traditionally. Twenty grams of millet flour were mixed with 40 ml Dura starter water extract (pH=3.32) and left at room temperature (35-38°C) for about 24 hrs. Then, the starters of Wad Ashana (pH=3.96) and Sudan II (pH=4) were dried using a laboratory air oven adjusted at 47°C for overnight. The dried millet starters were kept in air tight polyethylene bags and stored in a refrigerator (6-8°C).

Activation of millet starters for microbial analysis

Prior to microbial analysis the cold stored dry preparations of the two millet starters were activated using different ratios of the starter: the corresponding millet flour: the tap water. Fermentation was allowed for 22-24 h. at room temperature. The raw flours and the activated stored starters of Wad Ashana (pH=3.83) and Sudan II (pH= 3.45) millet genotypes were subjected to microbial analysis. After 17 months the micro flora of the reactivated stored Wad Ashana (pH=3.75) and Sudan II (pH= 3.81) millet starters were investigated once more.

Isolation and Identification of the microflora responsible for fermentation in Wad Ashana and Sudan 11 millet starters

Preparation of media and serial dilutions

Media were prepared following the standard methods described in Harrigan (1998). These media were NA (Nutrient agar), MRS (De Man Rogosa and Sharp, 1960), plate count agar (PCA) and potato dextrose agar (PDA). They were sterilized at 121^{o}C under pressure (15 pound/inch^{2}) for 15 minutes. Serial dilutions from 10^{-1} to 10^{-6} were prepared according to the methods described in Harrigan and McCance (1976).

Isolation of microorganisms

Total viable count of bacteria

Total viable count of bacteria was determined according to the method described by Hayes (1998) using the pour plate method. In this method two sets of sterilized Petri dishes were inoculated with 1 ml aliquots from the different sample dilutions. Fifteen mills of sterile plate count agar were added to each Petri dish, mixed well with the aliquot and left to solidify. The colonies were counted after incubation at 37°C for 48 hours using a colony counter and the number of viable cells was calculated as cfu/gm.

Lactic acid bacteria

Lactic acid bacteria were counted using surface spread plate method. Fifteen mills of sterile MRS agar were poured in each Petri dish and left to solidify. Using sterile pipettes, 0.1 ml aliquots from the different dilutions were transferred to MRS plates and evenly spread over the whole surface using sterile L- shaped glass rods (hockey stick). Plates were incubated at 37°C for 2-3 days under anaerobic system. The colonies were counted and considered lactic acid bacteria. Representative colonies were isolated and sub-cultured in MRS medium for identification (Hayes, 1998).

Yeasts and moulds

Yeasts were enumerated using spread plate method. From suitable dilutions, 0.1 ml samples were aseptically transferred and spread onto solidified PDA containing 0.1g chloramphenicol per one litre of medium to inhibit bacterial growth. The PDA plates were incubated at 28^{o}C for 2-3 days after which the colonies of yeasts in every Petri dish were counted to find out the total viable count of yeast. Representative colonies were isolated and sub-cultured in PDA medium for identification (Hayes, 1998).

Identification of microorganisms

Lactic acid bacteria genera

Morphological and Biochemical characteristics were considered for identification of lactic acid bacteria genera. The Biochemical tests were: gram stains, catalase test, acid from glucose test as well as sporulation test which were carried out according to Harrigan and MaCance (1976). The other tests were: anaerobic growth (Kiss, 1984), Motility test (Barrow and Feltham, 1993), oxidase test (Steel, 1961) and fermentation/ oxidation test (Hugh and Leifson, 1953).

Lactic acid bacteria species

Lactobacillus

The additional biochemical criteria used for identification of lactobacilli were: production of ammonia from arginine (Barrow and Feltham, 1993), Growth at 15 and 45°C and fermentation of carbohydrates tests which were done according to Harrigan and MaCance (1976). *Lactobacillus spp.* was taxonomically identified according to the scheme outlined by Rogosa (1975).

Cocci group

The media used were prepared according to Harrigan and MaCance (1976). All tests for identification of cocci were carried out under anaerobic conditions. They were the same tests used for identification of lactobacilli in addition to the following tests: growth at 45°C and 10°C, growth in 6.5 and 10% sodium chloride and growth at pH 7 and pH 4.5 which

were all carried out as described in Harrigan and MaCance (1976). Aesculine hydrolysis was also examined (Barrow and Feltham, 1993). *Streptococcus and pediococcus spp.* were further identified taxonomically according to the scheme outlined by Deibel and Seeley (1975) and Kitahara (1975) respectively.

Yeasts

Young growing cultures of yeasts were prepared and the pure isolates were identified according to Harrigan (1998). These cultures were examined microscopically; the shape of yeasts' cells, the form of budding, the presence of pseudo hyphae and ascospore formation were observed and registered. In addition, growth at 37 and 42 °C, growth on corn meal agar and starch hydrolysis were investigated. The other tests carried out were: assimilation of carbohydrates (Kiss, 1984), nitrate reduction (Barrow and Feltham, 1993), cycloheximide resistance and urea hydrolysis (Barrnet *et al.,* 1990).

RESULTS AND DISCUSSION

As it was shown in table 1, the Lactic acid bacterial count of Sudan 11 millet flour (4.5×10^4) increased to 1.78×10^7 cfu/g after fermentation and that of Wad Ashana flour although it was undetectable prior to fermentation, but it reached up to 3.48×10^7 cfu/g after fermentation. On the other hand, the yeast counts of Wad Ashana (8.5×10^2) and Sudan 11 ($1.oo\times10^2$) increased to 5.5×10^4 and 6.6×10^5 respectively after fermentation. It is worthy to mention that, no moulds were recorded. Consistently, Mohammed et al (1991) found that lactic acid bacteria constituted very low proportion of the population of the natural flora in sorghum flour prior to fermentation. However, the microbial population of Dabar sorghum fermented dough was dominated by lactic acid bacteria, with yeast counts remaining low. Also, the results of this study concerning the raw flour agreed with those of Hussein (2008) who recorded the values of 3.0×10^4 cfu/ml and 5.95×10^3 cfu/ml for the *Lactobacillus* and yeast counts respectively in raw Pearl millet flour. Furthermore, they were in line with those of Farahat (1998) and Hamad (1997) who reported yeast counts in Dabar dough of less than 10^5 and 5×10^4 cfu/g respectively. The pH recorded previously for fermented dough was about 3.4 to 4 (Hamad *et al.*, 1992, Mohammed *et al*. (1991) and Badi, 1987), and that complied with the range obtained by this study 3.45 to 4. Fermentation of millet had involved the presence of *Lactobacillus spp*. and *Saccharomyces spp*. (Tables 2-5). Evidently, Hamad and Fields (1979) and Zamora and Fields (1979) in their studies of fermentation of cereals concluded that, although the micro-flora may differ from crop to another, the lactic acid bacteria will be part of the flora. The Study of Hussein (2008) also revealed the presence of Lactic acid bacteria and *Saccharomyces spp*. during the fermentation of millet. Gobbetti *et al.* (1994) and Steinkraus (1996) reported that a co – metabolism between yeast and lactic acid bacteria has been suggested, whereby the bacteria provided the acid environment, which favored the growth of yeast and this consequently provide vitamins and other growth factors to the bacteria. The results of the present study (Tables 2, 3, 4 and 6) further revealed that, the fermentation of millet flour for 24 hours under normal conditions using dura starter was highly contributed by the presence of the gram positive, catalase-negative, rod–shaped and spherical bacteria namely *Lactobacillus plantarum, Streptococcus lactis, Streptococcus cremoris and Pediococcus pentosaceus.* Furthermore, *Saccharomyces cerevisiae* was also found to contribute in millet fermentation process, but not Moulds. Evidently, in ogi, a fermented porridge prepared from sorghum in West Africa, the main microorganisms involved were *Lactobacillus spp.* particularly *Lactobacillus plantarum* and two types of yeast (Odunfa and Adeyele, 1985). Similarly, in ogi prepared from maize the part of the microorganisms involved were *Lactobacillus plantarum* and *Saccharomyces cerevisiae* (Omemu, 2011). Badi (1986) stated that, kisra (a Sudanese pancake- like bread prepared from fermented sorghum or millet flour) fermentation involved members of the genera *Lactobacillus* and *Streptococcus* among bacteria, and *Candida* and *Saccharomyces* among the yeast. In togwa, a Tanzanian fermented food prepared from sorghum, maize, millet and maize – sorghum, the microorganisms were tentatively identified as *Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus fermentum, Pediococcus pentosaceus* and yeast *Issatchenkia orientalis, Saccharomyces cerevisiae* and *Candida tropicalis* (Mugula *et al*., 2003). Mustafa (2002) and Ali (2009) isolated *S. cerevisiae* from fermented sorghum dough.

The finding of only a limited number of lactobacillus strains in the fermented millet dough understudy is in line with the presence of single strains of *L. Sanfrancisco* and *L. brevis* in European rye sour dough preparations (Böcker et al., 1990). On the other hand, *Streptococcus lactis* was found to be the main micro-organism involved in the fermentation of Mahewu, a maize meal fermented by the Bantus of South Africa for 24 hours using small amount of wheat flour as the source of inoculum (Odunfa, 1985). Moreover, Mohammed *et al*. (1991) found that when Dabar sorghum flour from the Sudan was fermented using an inoculum from a previous batch of fermented dough, at the constant temperature of 30°C, the only important microbial species that was always found in very large counts was *Pediococcus pentosaceus*. Other important lactic acid bacteria were *Lactobacillus confusus, L. brevis* and *Enterococcus faecium*. Lei and Jakobsen (2004) found differences in the dominance of pediococcus and lactobacillus at different stages in Koko (millet porridge) fermentation and explained that as indication of ability of *Pediococcus spp.* to survive throughout the Koko production. The *Lactobacillus spp.* appeared in the second fermentation and not in the first one due to the complex nutritional requirements (amino acids and vitamins) of lactobacillus which was just available in the second fermentation (Orla-Jensen, 1919). *L. fermentas* and *L. plantarum* were found to give the best aroma as final flavour of "ting" of southern Africa a sort of porridge made from maize or sorghum. Yeast was also thought to participate in the fermentation process of this food (Moss *et al*., 1984).

Generally, the few microbiological studies made on spontaneously fermented millet products have reported findings of *Lactobacillus salivarius, Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus jensenii, Lactobacillus cellobiosus, Lactobacillus plantarum, Pediococcus spp.* and *Leuconostoc spp.* (Oyeyiola, 1991; Antony and Chandra, 1997; Olasupo *et al.,* 1997 and Nwachukwu *et al.,* 2010). Although the Wad Ashana and Um Garfa millet starters were initially prepared traditionally under non aseptic conditions, but no contaminants were recorded. Furthermore, the same species (*Lactobacillus plantarum, Streptococcus lactis, Pediococcus pentosaceus* and *Saccharomyces cerevisiae)* were isolated from the same starters after 17 months storage as dry preparations at 6-8 °C.

Table 1. Average counts for total bacteria, lactic acid bacteria and yeasts

Sample	Total viable count of bacteria Cfu/g	Lactic acid bacteria Cfu/g	Yeasts Cfu/g
Wad Ashana raw flour	4.50×10^{5}		8.5×10^{2}
Sudan 11 raw flour	4.3×10^{5}	$4.5\bar{\times}10^{4}$	$1.oo\times10^{2}$
Wad Ashana starter	4.5×10^{7}	3.48×10^{7}	5.5×10^{4}
Sudan 11 starter	8.6×10^{7}	1.78×10^{7}	6.6×10^{5}

Table 2. Biochemical tests for identification of Lactic acid genera from two starters of millet (Genotype Wad Ashana and Sudan 11)

Sample	Gram staining	Shape	Endospore Staining	Motility	Growth in air	Catalase Test	Oxidase Test	O/F Test	Glucose Test (acid)	Genus
Wad Ashana	+	Rod	-	-	+	-	-	F	+	*Lactobacillus*
Wad Ashana	+	Spherical	-	-	+	-	-	F	+	*Streptococcus*
Wad Ashana	+	Spherical	-	-	+	-	-	F	+	*Pediococcus*
Sudan 11	+	Rod	-	-	+	-	-	F	+	*Lactobacillus*
Sudan 11	+	Spherical	-	-	+	-	-	F	+	*Streptococcus*
Sudan 11	+	Spherical	-	-	+	-	-	F	+	*Pediococcus*
Wad Ashana (after 17 months storage)	+	Rod	-	-	+	-	-	F	+	*Lactobacillus*
Wad Ashana (after 17 months storage)	+	Spherical	-	-	+	-	-	F	+	*Streptococcus*
Sudan 11 (after 17 months storage)	+	Rod	-	-	+	-	-	F	+	*Lactobacillus*
Sudan 11 (after 17 months storage)	+	Spherical	-	-	+	-	-	F	+	*Streptococcus*
Sudan 11 (after 17 months storage)	+	Spherical	-	-	+	-	-	F	+	*Pediococcus*

Table 3. Biochemical tests for identification of the genus *Lactiobacillus* from Wad Ashana and Sudan 11 millet starters

Sample	Growth at 15	Growth at 45°C	NH3 from arginine	Sugars 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	Species
Wad Ashana	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	*L- plantarum*
Sudan 11	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	*L- plantarum*
Wad Ashana (after 17 months storage)	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	*L- plantarum*
Sudan 11 (after 17 months storage)	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	*L- plantarum*

1= Amygdalin 5= Galactose 8= Gluconate 12= Mannose 16= Rhamnose 20= Sucrose
2= Arabinose 6= Glucose (acid) 9= Lactose 13= Melezitose 17= Ribose 21= Trehalose
3= Cellobiose 7= Glucose (gas) 10= Maltose 14= Melibiose 18= Salicin 22= Xylose
4= Fructose 11= Mannitol 15= Raffinose 19= Sorbitol 23= Esculin

Table 4. Genus *Streptococcus*

Sample	Growth at 10	45 °C	Growth at 6.5% Na Cl	Arginine hydrolysis	Esculin hydrolysis	Fermentation of carbohydrates 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Fermentation	Species
Wad Ashana	+	-	-	+	-	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	Homo	*Streptococcus lactis*
Sudan 11	+	-	-	+	-	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	Homo	*Streptococcus lactis*
Sudan 11	+	-	-	-	+	+	-	+	-	+	-	-	-	+	+	+	+	-	-	-	Homo	*Streptococcus cremoris*
Wad Ashana (after 17 months storage)	+	-	-	+	-	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	Homo	*Streptococcus lactis*
Sudan 11 (after 17 months storage)	+	-	-	+	-	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	Homo	*Streptococcus lactis*

1=Lactose 6= Arabinose 11= Salicin
2= Galactose 7= Xylose 12= Raffinos
3= Glucose 8= Sucrose 13= Inulin
4= Maltose 9= Trehalose 14= Glycerol
5= Salicin 10= Mannitol 15= Sorbitol

Table 5. Genus *Pediococcus*

Sample	Growth at 45°C	Growth at pH 7 / 4.5		Arginine hydrolysis	Growth at 10% Na Cl	Fermentation of carbohydrates																Fermentation	Species
						1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
Wad Ashana	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	Homo	*Pediococcus pentosaceus*
Sudan 11	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	Homo	*Pediococcus pentosaceus*
Sudan 11 (after 17 months storage)	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	Homo	*Pediococcus pentosaceus*

1= Arabinose 6=Maltose 11=Dextrin 15=Sorbitol
2= Ribose 7=Melezitose 12=Starch 16=Arbutin
3= Xylose 8=Sucrose 13=Glycerol
4= Rhamnose 9=Trehalose 14=Mannitol
5= Lactose 10=Maltoriose

Table 6. Yeast Isolates

Sample	Colony shape	Cell			Sporulation	Growth at 37C	Growth at 42C	Growth on corn meal agar	Nitrate reduction	Starch hydrolysis
		Shape	Vegetative growth	Pseudo hyphae						
Wad Ashana	Creamy, smooth, circular and small	Ovoid	Budding	-	+	+	+	+	-	+
Sudan 11	Creamy, smooth, circular and small	Ovoid	Budding	-	+	+	+	+	-	+
Wad Ashana (after 17 months storage)	Creamy, smooth, Circular and small	Ovoid	Budding	-	+	+	+	+	-	+
Sudan 11 (after 17 months storage)	Creamy, smooth, circular and small	Ovoid	Budding	-	+	+	+	+	-	+

Table No.5 (continuation)
Yeast isolates

Sample	Urea hydrolysis	Resistance to cycloheximide	Assimilation of carbohydrates						Species
			Glucose	Sucrose	Lactose	Melibioses	Raffinose	Galactose	
Wad Ashana	+	-	+	-	+	-	+	-	*Saccaromyces cerevisiae*
Sudan 11	+	-	+	-	+	-	+	-	*Saccaromyces cerevisiae*
Wad Ashana (after 17 month storage)	+	-	+	-	+	-	+	-	*Saccaromyces cerevisiae*
Sudan 11 (after 17 months storage)	+	-	+	-	+	-	+	-	*Saccaromyces cerevisiae*

And again no moulds were recorded. The flora appeared to be stable within one dough (Hamad *et al.*, 1997), especially when it was activated using its corresponding millet flour. And, this in turn may indicate the predictable nature of such a dough or starter upon consecutive use Evidently, Böcker and others (1990) showed for rye sour dough starter preparations that non-sterile, but well-defined manufacturing conditions exert a selective pressure.These result in the continuity over years of the microbial flora in consecutive preparations which further indicate their adaptability to that environment and reliability on each other's metabolic properties e.g starch hydrolysis

Conclusion

Microbiologically safe fermented millet flour could be traditionally produced using well prepared millet starters. And the latter could also be utilized for the development of defined starter preparations or starter cultures containing both lactic acid bacteria and yeast to ensure accelerated acidification, an improved and more predictable fermentation process and desirable sensory attributes when establish small-scale industry of traditional Sudanese foods. Studies to verify the potential of these isolated mico organisms as starter cultures are in progress.

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