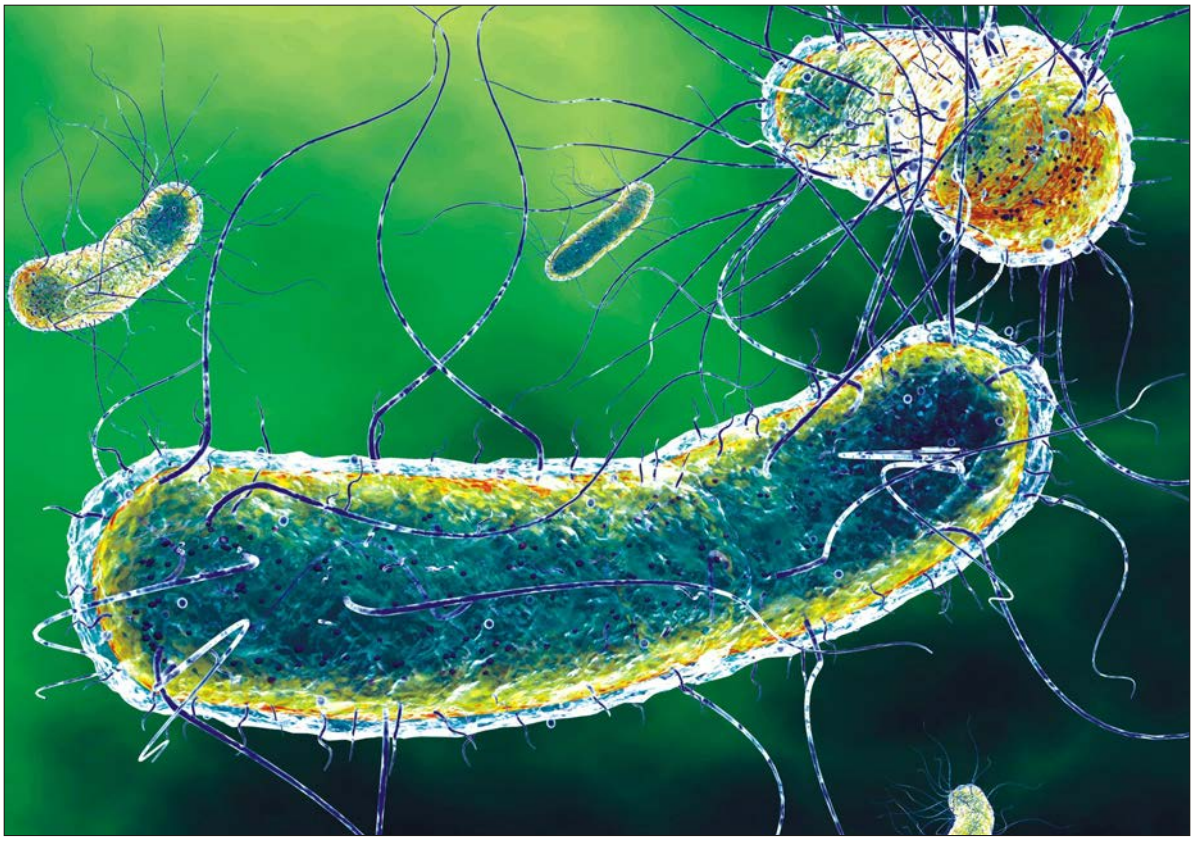

Current Research in Bacteriology

ISSN 1994-5426

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Current Research in Bacteriology 3 (3): 138-147, 2010
ISSN 1994-5426

Characterization and Distribution of Microorganisms Associated with Kisra Bread Preparation from Three Sorghum Varieties in Sudan

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Abstract: The aim of the present study is to identify and quantify the microorganisms involved in the traditional fermentation of sorghum (*Sorghum bicolor*) flour of three local cultivars (Fetarita, Safra and Ahmer) used for kisra bread production. Fermented dough was prepared in the traditional way used by Sudanese housewives. Biochemical changes in the three sorghum varieties showed a drop in pH from 5.9 to 3.80 while, titratable acidity (% lactic acid) increased from 0.22 to 1.4% after 24 h fermentation. The microbiological analysis revealed that lactic acid bacteria dominated over the Enterobacteriaceae group, yeasts and molds, indicating that sorghum fermentation was mainly lactic acid fermentation. The identification and distribution tests of the microorganisms showed that *Pediococcus* was the dominant lactic acid bacterium. Enterobacteriaceae groups were not detected after 16 h fermentation. Similar to *Pediococcus*, *Aspergillus niger* and *Klebsiella pneumoniae* were dominated over the other molds and Enterobacteriaceae group, respectively

Key words: Sorghum, fermentation, Kisra, lactic acid bacteria, Enterobacteriaceae, yeast, molds

INTRODUCTION

Grain sorghum (*sorghum bicolor* L. Moench) is staple food for the people of the arid and semi-arid region of Africa and Asia. It is widely grown in the semiarid regions because of its drought tolerance. Grain sorghum is considerd the fifth most extensively grown in the world and the third largest crop harvest in the USA. In Africa, sorghum is the major contributors of protein and calories for economic and cultural reasons. The food preparation methods of sorghum are generally simple, the basic diet in most Africa being a porridge or stiff paste prepared by adding pounded flour to hot water. Different traditional fermented food and beverage are prepared from sorghum in different part of the world. Fermentation of the

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sorghum flour has been reported to decrease the levels of the antinutritional factors and increase protein availability, *in vitro* digestibility and nutritive value (Osman, 2004; Ibrahim *et al*., 2005; Elkhalifa *et al*., 2005; Wedad *et al*., 2008; Mohammed *et al*., 2010). Kisra, is a fermented sorghum bread which constitutes the staple diet in Sudan. Traditionally the bread is made by utilizing the natural microflora in sorghum flour. The fermented dough known as Ajean, is prepared traditionally by mixing sorghum flour with water in a ratio of 1:2 in a round earthenware container called a khumara. A small amount of the previously fermented dough is then added to the mixture to act as a starter. Mohammed *et al.* (1991) and Hamad *et al.* (1992, 1997) studied changes in the population of microorganisms during Kisra fermentation. They found that the bacterial population increased with fermentation time and reach platue after 9 h and lactic acid bacteria was dominante miro-organism. In order to commercialize the Kisra production, it is necessary to understand the fermentation process and identify the micro-organism involved.

MATERIALS AND METHODS

Sudanese sorghum (*Sorghum bicolor*) of the cultivars locally known as Fetarita, Safra and Ahmer were purchased from local grain markets in Omdurman city. All samples were carefully cleaned and freed from dirt, stones, chips and other extraneous grains or grits. Sorghum grains were milled at the local grain market to fine flour using a Diamant Mill, model 500 mm (Denmark). The flour was transferred to the laboratory and stored at 25°C until used.

Preparation of Fermented Dough

Fermented dough was prepared in the traditional way used by Sudanese housewives. In the laboratory, sorghum flour was mixed with sterile distilled water in a 1:2 (w/v) ratio. A small amount of the previously fermented dough was then added to the mixture of flour and water to act as a starter (about 5%). This mixture was incubated at 30°C for 24 h in a sterile covered flask (2 kg flour + 4 L water). Each fermentation was performed in duplicate and sampled every 4 h during the fermentation period (24 h). Samples of 50 g fermented dough were placed in a sterile stomacher bag and mixed with 450 mL sterile 0.1% peptone water (Oxoid CM9) using a stomacher lab-blender 400 (Seward Medical, London, UK) for 2 min. After a further serial dilution in 90 mL 0.1% peptone water, the samples were plated on different selective agar media for enumeration and identification of microorganisms.

Enumeration and Identification of Enterobacteriaceae

According to Mehlaman (1984) in the Compendium of Method for the Microbiological Examination of Foods, Enterobacteriaceae were enumerated in duplicate pour plates of Violet Red Bile Glucose Agar (VRBG, Oxoid Cm 485), medium and the plates were over laid after solidification with 3 to 4 mL of additional VRBG agar. All plates were incubated in an inverted position at 35°C±2 for 18-24 h in an electrical incubator (Memmert Gm 6H-Co KG-Germany). The plates were then counted using a colony counter (Model 3327-American Optical) and the results were reported as standard plate count per gram dough. Representative single colonies showing variation in appearance were picked from the plates used for viable counts at each sampling time (every 4 h during 24 h). Isolates were purified then further characterization was carried out using the API 20E system (API 20E system B Biomerieux, Marcyl Etaile, France). The percentage of every genus was reported at each sampling time from the total count.

Enumeration and Identification of Lactic Acid Bacteria

Lactic acid bacteria were enumerated in pour plates of de Man, Rogosa and Sharp (MRS) medium (Oxoid CM 359) according to Gilliland *et al.* (1984) in the Compendium of Methods for the Microbiological Examination of Foods. The plates were incubated at 37EC for 48 h under anaerobic conditions using Gas Pak (H_2+Co_2) anaerobic systems (BBL, Microbiology Systems, Div. Becton Dickinson and Co. Cockeysville. Med.). After incubation, the plates were counted and reported as SPC per gram dough. Representative single colonies were picked from plates used for viable count, at each sampling time according to colony morphology. Isolated colonies were purified and further characterized using API 50 CHL [Biomerieux Sa 6928 Morcy I=E-Toile-France]. The percentage of every genus was reported at each sampling time from the total count.

Counts of Yeasts and Molds and Identification

Yeasts and molds were enumerated on acidified potato dextrose agar, which was acidified by the addition of the proper amount of sterile 10% tartaric acid (Fluka B AG B Buchs. SG). The acidification of the medium is necessary to provide for suppression of bacterial growth by adjusting the medium to pH 3.5±0.1. Pour plates were inverted and incubated at 25°C±1 for 3-7 days (Koburger and Marth, 1984). Results were reported as SPC per gram dough (FDA, 1984). Molds were classified according to Frazier and Westhoff (1988) and Barnett and Hunter (1972). The count of every genus was reported as a percentage of total count every 4 h.

RESULTS

Titratable Acidity and pH

The initial pH of the dough for the three varieties at start of fermentation was 5.8 (Fetarita), 5.9 (Safra) and 6.0 (Ahmer). With the progress of fermentation total acidity continued to increase and this coincided with the pH decrease. At the end of fermentation the pH decreased to 3.8, 3.8 and 3.9 for Fetarita, Safra and Ahmer, respectively. After 24 h fermentation, the maximum titrable acidity was 1.3, 1.36 and 1.31% for Faterita, Safra and Ahmer, respectively. In fact their is a sharp drop in pH to 4.0 while total acidity increased to 1.1% in the 3 varieties at 16 h of fermentation. After that, the pH slowly decreased until it reached 3.8 and the total acidity increased to 1.4 after 24 h of fermentation (Fig. 1-3).

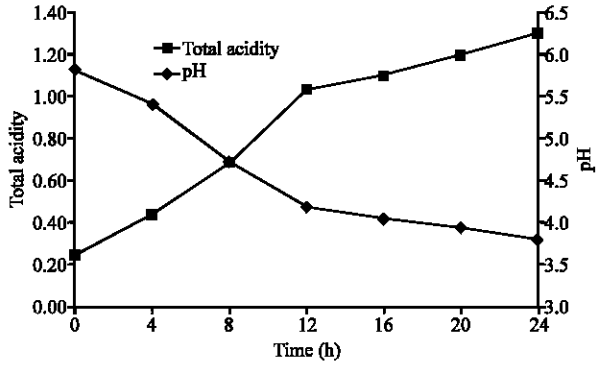

Fig. 1: Total acidity and pH during fermentation of Faterita variety

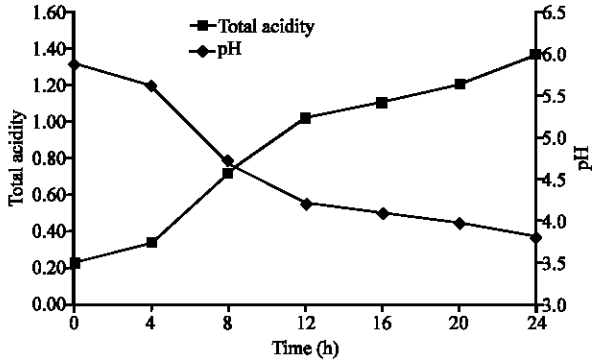

Fig. 2: Total acidity and pH during fermentation of Safra variety

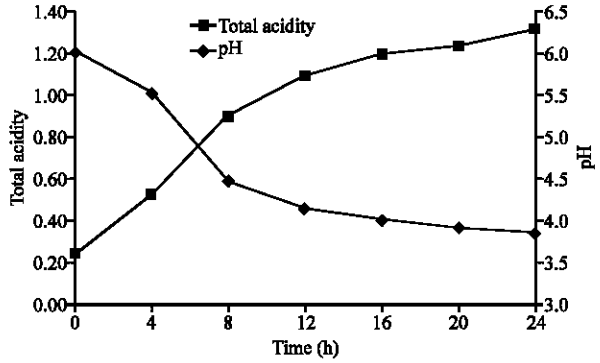

Fig. 3: Total acidity and pH during fermentation of Ahmer variety

The Main Microorganisms in Kisra Fermentation

Lactic acid bacteria were dominant microflora and their number increased during fermentation of the three sorghum varieties. The initial numbers of lactic acid bacteria, Enterobacteriaceae group, yeasts and molds for the three varieties ranged from 6.7 to 7.7 cfu g^{-1}, from 3.2 to 4.8 cfu g^{-1}, 1.9 to 4.4 cfu g^{-1} and 2.0 to 3.4 cfu g^{-1}, respectively. At the end of the fermentation process (24 h) the number ranged from 8.9 to 9.5 cfu g^{-1} for lactic acid bacteria, from 1.0 to 1.7 cfu g^{-1} for yeasts and from 1.0 to 1.3 cfu g^{-1} for molds. The Enterobacteriaceae were almost not detected after 16 h. In the three fermentations, the numbers of lactic acid bacteria increased with fermentation time until they reached a stationary phase at 12 h (log 9.4 cfu g^{-1}). The counts of the Enterobacteriaceae increased in the first stages of fermentation then they started to decrease and they were not detected after 16 h of fermentation. The numbers of yeasts and molds increased in a steady rate during fermentation time up to 8 h, after that there was a slight decrease till the end of fermentation (Fig. 4-6).

Distribution of Microorganisms During Fermentation

The lactic acid bacteria isolated from the three varieties were identical and they included *Lactobacillus coprophilus*, *Lactobacillus cellobiosus*, *Lactobacillus brevis* and *Pediococcus pentosaceus*, For Fetarita variety at zero time, *Pediococcus pentosaceus* predominated (42% of the isolates) over *Latobacillus coprophilus* (28%),

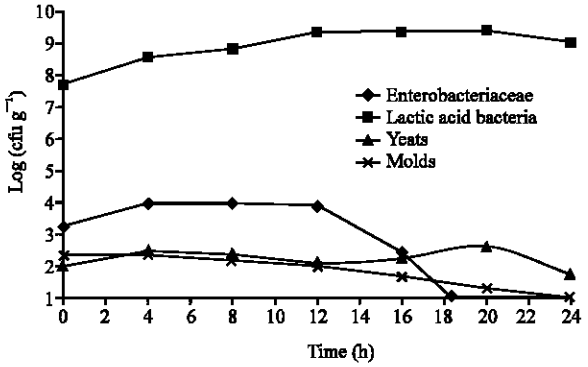

Fig. 4: Microbial counts during the fermentation of Fetarita variety

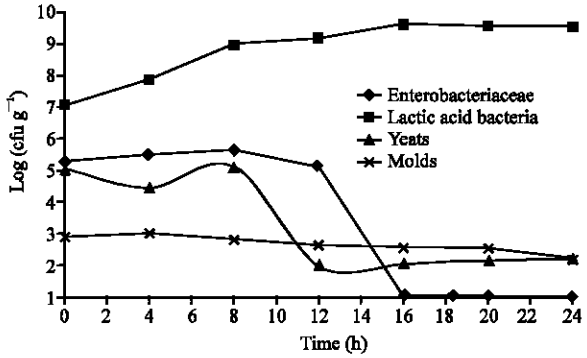

Fig. 5: Microbial counts during the fermentation of Safra variety

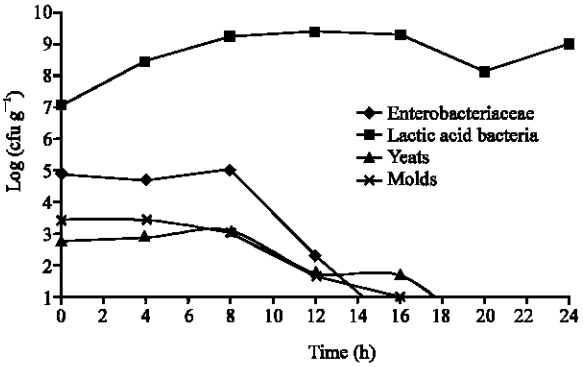

Fig. 6: Microbial counts during the fermentation of Ahmer variety

Lactobacillus cellobiosus (19%) and *Lactobacillus brevis* (11%). The distribution ratio between the strains throughout the fermentation progress was constant. At the end of fermentation *Pediococcus* dominated (47%) over *L. coprophilus* (34%), *L. cellobiosus* (17%) and *L. brevis* (2%). Similar distribution patterns were observed at the beginning and at the end of the fermentation of Safra and Ahmer varieties (Table 1).

Table 1: Changes in the percentage distribution of different groups of isolated lactic acid bacteria during fermentation of sorghum varieties

Sorghum varieties	Time (h)	Total count of lactic acid bacteria (cfu g^{-1})	*Lactobacillus coprophilus*	*Lactobacillus celliobiosus*	*Lactobacillus brevis*	*Pediococcus pentosaceus*
			(%)			
Fetarieta	0	43	28	19	11	42
	4	36	31	19	11	39
	8	56	36	18	9	37
	12	97	37	15	5	43
	16	83	27	19	5	49
	20	69	28	20	3	49
	24	59	34	17	2	47
Safra	0	38	26	21	11	42
	4	27	22	15	11	52
	8	53	32	15	8	45
	12	85	24	15	5	56
	16	73	23	14	5	58
	20	63	29	13	5	53
	24	47	32	17	4	47
Ahmer	0	43	30	14	19	37
	4	32	31	19	6	44
	8	58	36	19	5	40
	12	99	31	14	4	51
	16	89	29	18	6	47
	20	76	34	17	4	40
	24	58	38	16	3	43

The Enterobacteriaceae isolated from Fetarita and Safra included *Klebsiella pneumoniae*, *Enterobacter sakazakii* and *Enterobacter agglonerans*, whereas *Kleb. pneumoniae*, *Enterobacter agglonerans* and *Serratia* were found in Ahmer variety. For Fetarita, at zero time *Kleb. pneumoniae* represent 56% of the isolates, followed by *Enterobacter sakazakii* (28%) and *Enterobacter agglonerans* (16%). After 12 h of fermentation the percentage changed to 50, 38 and 12% for the three strains, respectively. At zero time the distribution ratio of the three microorganisms in Safra was similar to that of Fetarita. After 12 h of fermentation *Klebsiella pneumoniae* dominated with 78% followed by *Enterobacter agglonerans* (22%) while *Enterobacter sakazakii* was not detected. After 16 h fermentation the growth of these two strains was also inhibited. At the beginning of the fermentation of Ahmer *Klebsiella pneumoniae* formed 53% of the isolates followed by *Enterobacter agglonerans* (26%) and *Serratia ficaria* (21%). After 12 h *Klebsiella pneumoniae* increased up to 72% while *Ent. agglonerans* and *Serratia* decreased to 17 and 11%, respectively. Similar to Safra and Fetarita, all the strains were inhibited after 16 h of fermentation (Table 2).

Table 3 shows the changes in percent distribution of molds during fermentation of Fetarita, Safar and Ahmer. At zero time *Aspergillus niger* constituted the majority of isolated strains (72%), whereas *Fusarium* and *Alternaria* were 17 and 8%, respectively. As the fermentation progressed *Aspergillus niger* dominated reaching 100% after 12 h, while the other strains were inhibited after 8 h of fermentation. In Ahmer variety in addition to the three molds found in Fetarita, *Penicillium* was also isolated at zero time but not detected after 4 h fermentation. Similar to Ahmer variety in Safra 4, *Aspergillus niger*, *Fusarium*, *Alternaria* and *Pencillium* were isolated at zero time with *Aspergillus niger* forming the majority. After 8 h *Alternaria* and *pencillium* were not detected. Whereas, *Fusarium* growth was inhibited after 12 h. As with the other varieties *Asergillus niger* dominated till the end of the fermentation.

Table 2: Changes in the percentage distribution of different groups of isolated enterobacteriaceae bacteria during fermentation of sorghum varieties

Sorghum varieties	Time (h)	Total count of Enterobacteriaceae bacteria (cfu g^{-1})	*Klebsiella pneumoniae*	*Enterobacter agglonerans*	*Serratia ficaria*	*Enterobacter sakazakii*
			(%)			
Fetarieta	0	18	56	16	0	28
	4	34	58	18	0	24
	8	29	51	14	0	35
	12	8	50	12	0	38
	16	15	47	20	0	33
	20	0	0	0	0	0
	24	0	0	0	0	0
Safra	0	58	57	24	0	19
	4	101	66	19	0	15
	8	147	53	27	0	20
	12	37	78	22	0	0
	16	0	0	0	0	0
	20	0	0	0	0	0
	24	0	0	0	0	0
Ahmer	0	58	53	26	21	0
	4	101	55	31	24	0
	8	148	58	24	18	0
	12	36	72	17	11	0
	16	0	0	0	0	0
	20	0	0	0	0	0
	24	0	0	0	0	0

Table 3: Changes in the percentage of distribution of different groups of isolated molds during fermentation of Sorghum varieties

Sorghum varieties	Time (h)	Total count of Molds (cuf g^{-1})	*Aspergillus niger*	*Fusarium* sp.	*Penicillium* sp.	*Alternaria* sp.
			(%)			
Fetarieta	0	18	72	17	0	11
	4	24	63	21	0	16
	8	13	69	23	0	8
	12	10	100	0	0	0
	16	6	100	0	0	0
	20	2	100	0	0	0
	24	1	100	0	0	0
Safra	0	13	46	31	8	15
	4	19	53	21	10	16
	8	11	73	27	0	0
	12	7	86	14	0	0
	16	6	100	0	0	0
	20	6	100	0	0	0
	24	3	100	0	0	0
Ahmer	0	27	74	0	8	18
	4	28	64	29	0	7
	8	36	72	17	0	11
	12	5	100	0	0	0
	16	1	100	0	0	0
	20	1	100	0	0	0
	24	1	100	0	0	0

DISCUSSION

Lactic acid bacteria were the dominant microflora and their number increased during fermentation of the three sorghum varieties, in the fermentation of the three varieties and at each interval the number of lactic acid bacteria was greater than the number of yeasts and molds and they dominated till the end of fermentation. This fact implied that Kisra fermentation is mainly a lactic acid fermentation. This was in agreement with Sanni *et al.* (2002), who reported that lactic acid bacteria species were the predominant microorganisms

during fermentation of Ghanaian maize. The increase in acid producing bacteria resulted in increased the amount of lactic acid produced with concomitant drop in pH with an increase in fermentation time. similar sequence of changes in lactic acid bacteria with drop in pH an increase in acidity during fermentation were reported by El-Hidai (1978), Mohammed *et al.* (1991) and Hamad *et al.* (1992, 1997) in kisra, Gassem (1999) in khamir Nout (1991) in sorghum based infant food formula, Ijabadeniyi (2007) in ogi produced traditionally and Vieira-Dalodé *et al.* (2007) in gowe. The lactic acid bacteria isolated from the three varieties were identical and they included *Lactobacillus coprophilus*, *Lactobacillus cellobiosus*, *Lactobacillus brevis* and *Pediococcus pentosaceus* and they were similar to those isolated from different fermented foods such as Kisra (El-Hidai, 1978; Abdel-Gadir and Mohamed, 1983; El-Mahdi, 1985; Mohammed *et al.*, 1991; Hamad *et al.*, 1992, 1997), tef (Gashe, 1985); ogi (Adegoke and Babalola, 1988) and khamir (Gassem, 1999). These results show that *Pediococcus pentosaceas* was the dominant lactic acid bacterium throughout the fermentation process for the three sorghum varieties.

The counts of the Enterobacteriaceae increased in the first stages of fermentation then not detected after 16h. The inhibition of Enterobacteriaceae could be due to the growth of lactic acid bacteria, which outnumber the Enterobacteriaceae and result in fast acid production. The Enterobacteriaceae isolated and identified from Fetarita and Safra included *Klebsiella pneumoniae*, *Enterobacter sakazakii* and *Enterobacter agglonerans*, whereas *Kleb. pneumoniae*, *Enterobacter agglonerans* and *Serratia* were found in Ahmer variety. These Enterobacteriaceae members isolated during the fermentation process were common on plants and soils and also reported in the natural fermentation of soya beans (Mulyowidarso *et al.*, 1989), fermented sorghum (Mohammed *et al.*, 1991; Nout, 1991; Gassem, 1999), cassava and maize (Adegoke and Babalola, 1988) and in legumes (Odibo *et al.*, 1992)

The molds isolated from the three varieties were identical and they included *Aspergillus niger*, *Fusarium*, *Alternaria* and *Pencillium*. At the end of fermentation *Asergillus niger* dominated over the other molds till the end of the fermentation.

In conclusion these results show that certain biochemical and microbial changes occur during Kisra fermentation. *Pediococcus pentosaceas* was the dominant lactic acid bacterium throughout the fermentation process for the three sorghum varieties. After 16 h of fermentation of the three varieties all the Enterobacteriaceae group were inhibited possibly due to the low pH that resulted from lactic acid produced by lactic acid bacteria. Similar to *Pediococcus pentosaceus*, *Aspergillus niger* dominated during the fermentation process for the three sorghum varieties, this may be due to its tolerance to high acidity or its presence at high level on raw material. The identified microorganism can be used starter culture for large scale Kisra production.

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