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Full Length Research Paper

Utilization of mouldy sorghum and *Cassia tora* through fermentation for feed purposes

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Microbial fermentation of mouldy grains brought about by lactic acid bacteria is gaining much significance owing to their ability to inhibit mould growth and detoxify mycotoxins while improving the nutritive value and safety of the product. In the present study the potential of developing a probiotic feed ingredient from a combination of mouldy sorghum and *Cassia tora* seeds, using spontaneous fermentation was explored. The effect of fermentation at 0, 24 and 36 h on the microflora, ergosterol, mycotoxins and nutritive value, of mouldy sorghum was assessed individually and in combination with *C. tora* seeds. A reduction in mould counts upto 58 and 96% was observed at 24 and 36 h of fermenting mouldy sorghum. Total plate count increased by 2 fold and *Lactobacillus* count increased by 4 fold when mouldy sorghum was fermented singly or with *C. tora* seeds. Fermentation decreased ergosterol by 76%, aflatoxin to non-detectable levels at 36 h of fermentation and fumonisin B1 to non-detectable levels at 24 and 36 h of fermentation of mouldy sorghum. Fermentation resulted in marginal improvement in nutritive value of mouldy sorghum when estimated in terms of proximate principles and mineral elements. Addition of *C. tora* resulted in considerable increase in nutritive value particularly with respect to protein and mineral elements like iron and calcium in mouldy sorghum.

Key words: Microbial fermentation, lactobacillus, mycotoxins, mould.

INTRODUCTION

Mouldy sorghum is underutilized for food and feed purposes, because of its poor quality and acceptability and safety concerns. Mould damage has been recognized as a major cause for decreased yield and quality of sorghum grain and nutritive value (Bandyopadhyay et al., 2000). Sorghum is an excellent substrate conducive for fungal contamination and elaboration of mycotoxins, which are the secondary metabolites of various species of fungi like *Aspergillus and Fusarium* (Williams and McDonald, 1983; Vasanthi and Bhat, 1998). Various mycotoxins such as T2-toxin, aflatoxin and fumonisin have been detected in sorghum (Rukmini and Bhat, 1978; Shetty and Bhat, 1997; Bhat et al., 2000). Consumption of mouldy sorghum containing fumonisin resulted in a self-limiting mycotoxicosis disease outbreak in India (Bhat et al., 1997).

Cassia tora (*Cassia obstusifolia* L) is a common herbaceous annual occurring as a weed throughout India and belongs to the family of leguminasoae. Studies have shown methanolic extracts of *C. tora* improved microbiological safety by detoxifying aflatoxin B_1 (Choi et al., 1998). The seeds of *C. tora* have been shown to contain high amounts of protein and essential amino acids (Anonymous, 1992). Currently *C. tora* seeds are being used as a source of galactomannan gums in the food industry. However because of the presence of various antinutritional factors like trypsin inhibitors, polyphenols, saponins and haemagglutinins (Anonymous, 1992) their

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utilization, as a protein source particularly in animal feed has not been explored so far. Various detoxification procedures attempted such as autoclaving, acid treatment, alcohol extraction showed limited success in removing these factors (Anonymous, 1992).

Microbial fermentation of mouldy grains is gaining much significance owing to their detoxification potential of mould contamination while improving the nutritive value and safety of the product (Bol and Knol, 1992; Laitila et al., 2002; Shetty and Jespersen, 2006). Various studies have shown that fermentation involving lactic acid bacteria have improved the quality and safety of animal feeds through production of antimicrobial substances like bacteriocins or metabolites such as phenyllactic acid (Strom et al., 2002; Savadogo et al., 2006; Chen and Hoover, 2003).

In the present study an attempt has been made to assess the potential of developing a probiotic feed ingredient from a combination of mouldy sorghum, *C. tora* seeds and spontaneous fermentation. Effect of fermentation on the nutritive value, microflora and mycotoxins in mouldy sorghum singly and in combination with *C. tora* seeds was assessed.

MATERIALS AND METHODS

Collection of samples

Mouldy sorghum samples were obtained from Mahboobnagar district, which is a major sorghum growing area of Andhra Pradesh, India. Samples were selected based on visual presence of mould on the grains. *C. tora* seeds were obtained from a local co-operative society. All the samples were finely powdered in an electric grinder and stored at -20°C until analysis.

Fermentation protocol

Powdered mouldy sorghum and *C. tora* seeds samples were subjected to spontaneous fermentation either singly or in combination. Fermentation was carried out in duplicates at 0, 24 and 36 h at room temperature (28 +/-2°C). A ratio of 3:1 of sorghum to *C. tora* seeds was used based on the levels used for traditional fermentation of legumes and cereals in india (Pushpamma and Chittemma, 1981). After fermentation samples were lyophilized, powdered and stored at -20°C until analysis. Microbiological and chemical analysis was performed before and after fermentation of powdered sorghum samples in replicates of two.

Analysis for microflora

Analysis was carried out to assess total mould count, total plate count and *Lactobacillus* counts in the samples by standard protocols (Booth, 1971). Potato Dextrose Agar (PDA) was used for enumerating mycoflora, nutrient agar for total plate count and *Lactobacillus* MRS agar for *Lactobacillus* count. Bacteria and fungi were identified on the basis of characteristics given in Bergy's Manual (1986), Onions et al. (1981) and Navi et al. (1999). Catalase test and Gram stain were used to confirm presence of *Lactobacillus*.

Analysis for nutritive value

All the samples were analysed for proximate principles namely, moisture, crude protein, total ash, fat as crude ether extract, crude fiber and carbohydrate and mineral elements by standard methods (Raghuramulu et al., 1983). Carbohydrate was calculated by difference and energy by Atwater factors (Raghuramulu et al., 1983). Mineral elements analysed by atomic absorption spectrophotometry method on Varian Techtron AA-6 model comprised iron, calcium, magnesium and zinc.

Analysis of mycotoxins

Samples were analyzed for mycotoxins namely aflatoxin B1 and fumonisin B1. Fumonisin B1 was analyzed by HPLC and aflatoxin B1 by TLC. Fumonisin analysis was performed as per the method of Shephard et al. (1990) and Stack and Eppley (1992). Briefly, fumonisin B1 was extracted by methanol-water (3:1), purified on Strong Anion Exchange Cartridges (SAX) and HPLC analysis was performed after pre-column derivatization of fumonisin B1 with ortho phthaldialdehyde (OPA) on HP 1100 Series system equipped with G1321A fluorescence detector and G1311 A Quaternary pump (RP C18 column 4.0x 300 mm, 10 µm size, Waters Spherisorb, 335 nm excitation and 440 nm emission wavelengths, mobile phase acetonitrile- water- acetic acid in the ratio of 50:50:1 at a flow rate of 1 ml/min). The detection and quantification of fumonisin was achieved by comparing peak retention time and area of samples with the standard fumonisin (Sigma Co.USA). The limit of detection was 12 ppb. Extraction and cleanup for aflatoxin was carried out according to AOAC method 980.20 using acetone-water (85:15, v/v) as extraction solvent and silica gel column chromatography for clean-up and purification (AOAC, 2000). The purified and dried aflatoxin extracts were spotted on precoated silica gel plates (Merck Silica gel 60 on glass, 20 x 20 cm) along with standard aflatoxin B1 in different concentrations with chloroform-acetone (85:15) as the developing solvent. The resolved aflatoxin spots were detected as blue fluorescence spots under long range UV light and quantified in comparison with fluorescence intensities of standard aflatoxin (Sigma Co.USA) spots. The limit of detection of aflatoxin B1 was 5 ppb.

Analysis of ergosterol

Ergosterol, which is used as a chemical index of fungal infestation, was analyzed by the method of Schwadorf and Muller (1989) with the modifications suggested by Sashidhar et al. (1988). Briefly, samples were saponified with potassium hydroxide and alcohol and the saponified mixture was extracted with n-hexane and evaporated under vacuum at 40°C. The dry residue was dissolved in a known amount of benzene- acetonitrile (98:2) and 10 µl aliquot was spotted along with standard ergosterol of varying concentrations on activated precoated silica gel G plates of 250 µm layer thickness (Merck, Silica gel 60 on glass, 20 x 20 cm). After developing the plates in benzene-acetone (9:1v/v) they were exposed to iodine vapors for 30 s to increase the fluorescent intensity of ergosterol. Development of brown spots indicated the presence and location of ergosterol. The plates were allowed to stand for 20 - 30 min to allow the brown colour to disappear and visualized under long wave UV source. An Rf of 0.75 with greenish blue fluorescence indicated presence of ergosterol. Concentration was estimated visually by comparing the fluorescence intensities of the samples with standard spots.

Table 1. Effect of fermentation on microflora in mouldy sorghum and *C. tora* seeds.

Sample	Fermentation time (h)	Total mould count (cfu/g dry wt	Total plate count (cfu/g dry wt)	Total *Lactobacillus* Count (cfu/g dry wt.)
Mouldy sorghum	0	8.5×10^5	17.5×10^5	16.0×10^4
	24	3.5×10^5	3.0×10^6	Uncountable
	36	3.0×10^4	7.0×10^7	1.0×10^8
***Cassia tora* seeds**	0	6.0×10^4	2.0×10^4	3.7×10^5
	24	2.0×10^4	4.0×10^6	5.3×10^5
	36	1.0×10^4	1.0×10^8	6.0×10^7
Mouldy sorghum + *Cassia tora* seeds	0	2.0×10^5	16.0×10^5	2.0×10^4
	24	1.0×10^4	5.0×10^6	3.0×10^6
	36	1.5×10^4	4.0×10^7	1.0×10^8

*Results are average of 2 replicate analysis.

Table 2. Effect of fermentation on ergosterol and mycotoxins in mouldy sorghum.

Fermentation time (h)	Ergosterol (µg/gm)	Aflatoxin B1 (µg/kg)	Fumonisin B1 (µg/kg)
0	200	5.0	75.0
24	50	5.0	ND
36	50	ND	ND

RESULTS

Effect of fermentation on microflora

The effect of fermentation at different intervals on the mould, bacteria and lactobacilli counts in mouldy sorghum with and without *C. tora* seeds is shown in Table 1. The results indicated a reduction in mould count at 36 h of fermentation in all samples. In mouldy sorghum the mould counts decreased to 58% at 24 h and to 96% at 36 h of fermentation. A similar decrease in mould count was also observed for *C. tora* seeds during fermentation. Mould counts also decreased considerably when mouldy sorghum was fermented with *C. tora* seeds for 24 and 36 h.

The bacterial population as assessed by total plate count increased steadily with increase in fermentation time in all the samples (Table 1). On the basis of colony morphology and microscopic observation, Grams reaction and catalase test, the bacteria were presumptively identified as belonging to lactic acid bacteria group. It was found that rods mostly belonging to *Lactobacilli*, which increased considerably with fermentation, were predominant in the samples. The initial *Lactobacillus* counts were higher in fermented *C. tora* seeds when compared to those in mouldy sorghum samples. Fermentation of mouldy sorghum samples with *C. tora* seeds resulted in higher amounts of *Lactobacillus* counts at 36 h. The increase in Total Plate Counts and *Lactobacillus* counts was observed to parallel the decrease in mould counts (Table 1).

Effect of fermentation on ergosterol and mycotoxins

Ergosterol levels decreased by 75% in mouldy sorghum during fermentation at 24 and 36 h (Table 2). The results of aflatoxin B1 analysis showed that fermentation of mouldy sorghum for 24 h did not affect aflatoxin level. However, at 36 h of fermentation the toxin levels were reduced to non- detectable levels in these samples (Table 2). Fumonisin B1 was not detected either at 24 or 36 h of fermentation of mouldy sorghum.

Effect of fermentation on nutritive value

It was observed that fermentation *per se* resulted only in marginal increase in nutritive value in mouldy sorghum. Although the increase was not statistically significant a trend could be observed in enhancement of nutritive value particularly with respect to protein, ash, fat, fibre and minerals owing to their high levels in *C. tora* seeds (Tables 3 and 4). In mouldy sorghum, protein levels increased by 33%, ash by 50% and fat by 87% upon addition of *C. tora* seeds. Crude fibre levels increased by almost 3 times while carbohydrates and energy values did not show much

Table 3. Results of proximate analysis of fermented mouldy sorghum and *C. tora**.

Sample	Fermentation time (h)	Proximate principles (g/100g)						
		Moisture	Crude protein	Total ash	Crude fat	Crude fibre	Carbohydrate	Energy (Kcal)
Mouldy sorghum	0	15.6	9.1	1.3	1.6	1.7	72	340
	24	5.8	10.4	1.4	2.2	2.1	80	382
	36	3.7	10.6	1.4	2.3	1.3	83	395
C. tora	0	2.5	17.8	6.1	6.0	13.4	60	366
	24	11.7	16.8	6.0	5.9	12.4	53	334
	36	14.9	18.1	6.3	6.4	14.3	46	316
Mouldy sorghum + *C. tora*	0	7.5	12.1	2.5	3.0	4.6	73	364
	24	8.0	12.7	2.6	3.1	5.0	72	366
	36	10.7	12.9	3.1	3.9	8.2	69	363

*Results are average of 2 replicate analysis.

Table 4. Results of analysis of mineral in fermented mouldy sorghum and *C. tora* seeds*.

Sample	Fermentation time (h)	Mineral content (mg/100 g)			
		Iron	Calcium	Magnesium	Zinc
Mouldy sorghum	0	3.6	92	90	1.6
	24	4.2	113	88	1.6
	36	4.4	84	91	1.9
C. tora	0	114	781	220	3.6
	24	121	662	191	3.6
	36	137	716	180	3.2
Mouldy sorghum + *C. tora*	0	52.6	187	85	1.8
	24	57.6	180	82	2.0
	36	56.4	238	87	2.1

*Results are average of 2 replicate analysis.

increase in mouldy sorghum when *C. tora* seeds was added. The increase in iron was most notable which was 15 times more in mouldy sorghum after addition of *C. tora* seeds. Fermentation with *C. tora* seeds for 36 h resulted in an increase of 27% in calcium levels in mouldy sorghum while magnesium and zinc levels increased by 2 and 17%, respectively.

DISCUSSION

The present study was undertaken to assess the feasibility of developing a probiotic feed ingredient from mouldy sorghum using spontaneous fermentation as a process to reduce mould and mycotoxin contamination. An attempt was made to assess the potential of addition of *C. tora* seeds to enhance the nutritive value of mouldy sorghum and also serve as a support base for fermentation. A significant finding in the present study has been a decrease in mould count, which paralleled the increase in total plate count and *Lactobacillus* count. Earlier studies on sorghum fermentation indicated the presence of various lactic acid bacteria species such as *Lactobacillus* and *Pediococcus* (Gassem, 1999; Mohammed et al., 1991; Chavan and Kadam, 1989), which constitute the predominant flora in the spontaneous fermentation of water/cereal meal mixtures. The increase in total plate count as well as LAB counts observed in the present study is comparable to various studies carried out on fermented cereals (Khalil, 2005; Sanni et al., 2002). An increase in total plate count and lactic acid bacteria counts with increase in fermentation time was observed in the preparation of *Khamir* a traditional bread made of sorghum in Saudi Arabia (Gaseem, 1999). Fermentation of maize dough with cowpea has resulted in an increase lactic acid bacteria upto 9.9 cfu/gm at the end of 72 h of fermentation (Sanni

et al., 2002). *C. tora* seeds used in the present study had highest Lactobacillus counts. Thus when added to sorghum sample it resulted in higher *Lactobacillus* counts than those achieved with fermentation of sorghum alone.

An important observation in the present study was a decrease in ergosterol, aflatoxin and fumonisin contents on fermentation at 24 and 36 h, which could be attributed to the presence of high amounts of *Lactobacillus* after fermentation. This is in accordance with many investigations that have reported the control of mould growth and mycotoxin production in liquid cultures and grains by lactic acid bacteria (Laitila et al., 2002; Peltonen et al., 2001; El-Nezami et al., 1998; Gourama and Bullerman, 1995; Karunaratne et al., 1990). Various traditional products from sorghum prepared by fermentation have shown reduction in mycotoxins through fermentation involving lactic acid bacteria (Adegoke et al., 1994; Zinedine et al., 2002).

Spontaneous fermentation has been shown to improve the nutritive quality of various cereals and legumes (Khalil, 2005; Sanni et al., 2002; Haikara et al., 1997; Chavan and Kadam, 1989). The enhancement in nutritive value particularly proteins has been shown to be more significant with the addition of legumes. In the present study, the overall effect of fermentation on nutritive value was marginal. Much of the enhancement in nutritive value came as a result of addition of *C. tora* seeds to mouldy sorghum particularly for proteins, minerals like iron, calcium, magnesium and zinc. Fermentation of cereals like sorghum, with traditional legumes has been earlier shown to improve the protein levels and digestibility (Nout and Sarkar, 1999; Chavan et al., 1988). Earlier studies on fermentation of sorghum with traditional legumes like green gram for 5 days has shown a 20% increase in protein content (Chavan and Kadam, 1989). In the present study, although fermentation of mouldy sorghum resulted in only marginal increases in protein level, addition of *C. tora* seeds improved the initial protein level by 43% at 36 h of fermentation.

Fermentation resulted in 37% increase in calcium levels in mouldy sorghum with addition of *C. tora* seeds. This may be attributed to degradation of phytates, tannins, trypsin inhibitors and oxalates that are known to bind minerals and decrease their bioavailability. Earlier studies on lactic fermentation of whole wheat flour showed degradation of phytic acid and greater solubility of calcium and magnesium thus improving their availability (Lopez et al., 2000).

In the present study the effect of fermentation on the anti nutritional factors present in *C. tora* was not assessed. However various studies have reported considerable reduction in anti-nutritional factors in traditional legumes by fermentation involving lactic acid bacteria (Aregheore, 1998). In addition to having high protein content various antioxidant, antimutagenic and antibacterial properties have been reported in *C. tora* seeds (Yen and Chuang, 2000; Choi et al., 1998). Thus while studies are yet to be carried out to assess the effect of fermentation on anti nutritional factors in *C. tora* seeds, the observations in the present study indicated their potential use as a high protein source in animal feeds. Using spontaneous fermentation the utility of mouldy sorghum and *C. tora* seeds for animal feed could be considerably enhanced through improving nutritive value and microbial safety.

Conclusions

The results of the present study showed that fermented products that have nutritional benefit and microbiological safety could be produced from the materials not hitherto utilized, namely mouldy sorghum and *C. tora*. The study also gains much relevance in areas where farmers do not get sufficient demand for their agricultural produce. This is often observed in areas where mould damage in sorghum is a frequent problem like the areas from where mouldy sorghum samples were collected in the present study. Under conditions of extensive damage/loss in terms of yield, the mould damaged sorghum is left in the field unharvested, since harvesting incurs expenditure in terms of labour. This results in underutilization of the grain as well as monetary loss to the farmer. Thus fermentation could be exploited as a viable process for utilization of underutilized or unconventional plant materials such as mouldy sorghum and *C. tora* for the development of a safe and nutritionally sound probiotic feed ingredient.

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