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The effects of storage of fibrous feeds on ruminant livestock in developing countries

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RNRRS FINAL TECHNICAL REPORT:

THE EFFECTS OF STORAGE OF FIBROUS FEEDS ON RUMINANT LIVESTOCK IN DEVELOPING COUNTRIES

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PROJECT FUNDING DETAILS

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EXECUTIVE SUMMARY

1. The objectives of this project were:
(a) to assess whether mycotoxins in animal products represent a genuine hazard to humans in developing countries;
(b) to conduct a preliminary investigation of whether storage of fibrous ruminant feeds is associated with a loss in feeding value, with particular reference to the formation of mycotoxins; and
(c) to assess whether mycotoxins can affect ruminant production.

2. ***Contamination of milk with mycotoxins***. A study of evidence in the literature (Jones and Coker, internal report) concerning the transmission of mycotoxins in milk showed that the carry-over rate of aflatoxin B1 from feed to milk as aflatoxin M1 (AFM1) was in the order of 1-2 percent. Ruminants do not absorb much ochratoxin A when the concentration in the feed is low because ochratoxin A is hydrolysed in the rumen. However, this mycotoxin has been detected in the milk of cows given high doses of the toxin. The rate of transmission of zearalenone from feed to milk is around 0.7 percent. T-toxin, hydroxy-T-2 toxin, hydroxy-HT-2 toxin and deoxynivalenol are also transmitted into the milk. Of all known mycotoxins, the transmission to milk of aflatoxin and, to a lesser extent, zearalenone may represent the most serious human health hazard. In view of this conclusion, a systematic milk sampling study was carried out in Calcutta and Bhubaneswar (Eastern India) during the winter of 1992. The results of milk analysis revealed concentrations as high as 0.34 ug AFM1/litre in samples taken from the more intensive production systems. This compares with a maximum permissible concentration of AFM1 in milk in Europe of 0.05 ug/ml (assumed to be the safe level).

3. ***Mycoflora-related problems in stored maize and sorghum stover in Zimbabwe***. A study was carried out on the effects of storing maize and sorghum stover. A stover storage trial was conducted at Matopos Research Station in Zimbabwe to test the effects of different storage conditions on nutritive value. Stovers from maize and sorghum (two varieties) were sampled at harvest, and following storage for up to 120 days under the following conditions: standing in field; standing on an open air platform; and kept under shelter (roof) protection. The results showed that storage had an important effect on nutritive value, as assessed by the *in vitro* gas production method, with the samples left in the field having a significantly lower gas production, overall. There were also indications that gas production (which is strongly correlated with the degradability of feeds) is lowered during the first 60-90 days, but for sorghum, some of this loss might be recovered by 120 days of storage. Although several mycotoxigenic fungi (belonging to the genera *Fusarium*, *Phoma*, *Alternaria*, *Penicillium* and *Aspergillus*) were isolated from stored stovers, the study was of a limited nature so that their relationship to productivity problems in ruminants could not be determined.

4. ***Mycoflora and mycotoxins in one- year-stored rice straw and other fibrous feeds from Eastern India and Bangladesh***. The mycoflora of a range of mixed ruminant feed and forage samples collected in Eastern India and Bangladesh were determined. The most frequently isolated fungi were: *Aspergillus versicolor* (Vuill), Tiraboschi, *Penicillium citrinum* Thom, *Eurotium* species, *Wallemia sebi* (Fr) v Arx,

Aspergillus penicilloides Speg. and yeasts. *Fusarium moniliforme* Sheldon and *Cladosporium* species were among the most prevalent field fungi in straw but not in other feeds. Levels of certain individual fungi, particularly thermotolerant types, were higher in Bangladesh rice straw although overall counts were similar to those of the Indian samples. Analysis showed the presence of significant amounts of aflatoxin in a number of feed samples from Eastern India, but not in rice straw from India and Bangladesh. Zearalenone only occurred in a *Paspalum* straw sample and three other feed samples from India. Screening for other mycotoxins was not conducted.

5. ***Effects of mycotoxins on rumen degradability of feeds.*** A limited study using the *in vitro* fermentation method (incubation of feed plus added mycotoxin with sheep rumen inocula) showed that aflatoxin B1 reduced gas production significantly, indicating that the fermentability of feeds in the rumen is lowered. Preliminary indications are that other mycotoxins, notably tenuazonic acid, may also depress rumen function.

BACKGROUND

6. One of the major constraints to ruminant livestock production in developing countries is lack of sufficient feed, and there is, therefore, a need to maximise the efficiency with which available feed resources are utilised. Although this applies to feeds of all classes, fibrous crop residues of low nutritive value are of particular importance as sources of nourishment for ruminant livestock during the dry season, with stovers from maize, sorghum and millet being valuable in countries of sub-Saharan Africa, and rice straw in those of South Asia. Although in most situations crop residues are stored of necessity following harvest, in many situations it also makes economic sense to store feed for use during periods of shortage, or when the animal's requirement is greatest. There is, however, concern that under some conditions of storage, fungal growth and mycotoxin contamination may occur in feeds, which could then produce adverse effects on ruminant performance, and on human health from the consumption of livestock products, or from exposure to such feeds in production systems.

PROJECT OBJECTIVES

7. The specific objectives of this project were:

(a) to assess whether mycotoxins in animal products represent a genuine hazard to humans in developing countries;

(b) to conduct a preliminary investigation of whether storage of fibrous ruminant feeds is associated with a loss in feeding value, with particular reference to the formation of mycotoxins; and

(c) to assess whether mycotoxins can affect ruminant production.

RESEARCH ACTIVITIES

8. The work plan consisted of the following:

(a) assessment of the risk to humans from milk contaminated with mycotoxins by conducting a study of the literature, and by examining milk samples from commercial sources in Eastern India;

(b) assessment of the significance of mycoflora and mycotoxin contamination during storage of fibrous ruminant feeds by surveying feeds, and establishing feed storage trials; and

(c) prediction of the effects of mycotoxin-contaminated feed on ruminant livestock by testing selected mycotoxins in the *in vitro* gas production assay.

RESULTS:

(a) Contamination of milk with mycotoxins

(i) Literature review

9 A study of evidence in the literature concerning the transmission of mycotoxins in milk showed that the carry-over rate of aflatoxin B1 (AFB1) from feed to milk as aflatoxin M1 (AFM1) was in the order of 1-2 percent. Ruminants do not absorb much ochratoxin A when the concentration in the feed is low because ochratoxin A is hydrolysed in the rumen. However, this mycotoxin has been detected in the milk of cows given high doses of the toxin. The rate of transmission of zearalenone from feed to milk is around 0.7 percent. T-toxin, hydroxy-T-2 toxin, hydroxy-HT-2 toxin and deoxynivalenol can also be transmitted into the milk. The full review paper by Drs B.D. Jones and R.D. Coker is available as an internal NRI report.

10. On the basis of this literature review, it would appear that, of the mycotoxins that have been studied, the transmission to milk of aflatoxin and, to a lesser extent, zearalenone may represent the most serious human health hazard in developing countries.

(ii) Milk sampling in Eastern India

11. Between November 1991 and January 1992, milk samples were taken from four small-scale dairy farms in Calcutta and from two in Bhubaneswar, in addition to samples from the Operation Flood milk suppliers in the two cities. The results of HPLC analysis for AFM1 revealed concentrations as high as 0.34 µg AFM1/litre in samples taken from the more intensive production systems (Table 1). These figures should be considered against the maximum permissible concentration in Europe, which is 0.05 µg AFM1/ml.

Table 1. Aflatoxin M1 concentrations found in milk samples taken from commercial outlets in Calcutta and Bhubaneswar.

Source of milk	Proportion of samples AFM1-contaminated	Concentration range for AFM1 (µg/litre)
Calcutta:		
Farm A	2/5	0-0.04
Farm B	2/4	0-0.17
Farm C	0/6	
Farm D	0/3	
Mother Dairy	3/3	0.14-0.18
Bhubaneswar:		
Farm E	1/5	trace
Farm F	3/3	0.01-0.17
Omfed	2/2	0.26-0.34

12. Another important question arising from this study concerns the effects of pasteurisation and other financially-viable forms of processing milk on the fate of any AFM1 that was present. The literature on the effects of pasteurisation is unclear, but it is significant that in the above study milk, which was boiled before being transferred to sample bottles, still retained high levels of the toxin, although the concentrations before boiling were, or course, not known.

(b) Mycoflora-related problems in stored fibrous ruminant feeds

(i) Maize and sorghum stover storage trial in Zimbabwe

13. A stover storage trial was conducted at Matopos Research Station in Zimbabwe to test the effects of different storage conditions on nutritive value. Stover from maize (variety R201) and sorghum (varieties DC75 and SV2) was sampled at harvest (F), and following storage for 60, 90 and 120 days under the following conditions: standing in field (SF); standing on an open air platform (SOP); and keeping under shelter (roof protection) (SRP). The results (presented in Appendix 1) showed that storage had an important effect on nutritive value, as assessed by the gas production method, with the samples left in the field producing significantly less gas, overall. There were also indications that fermentability was lowered during the first 60-90 days (P=0.05), but for sorghum, some of this loss was reversed by 120 days of storage (Table 2). However, due to the limited nature of this experiment, the effects of 'storage period' and 'storage method' became confounded. It is recommended that an improved experimental design that is still practical be adopted in any follow-up study to identify all the issues of concern.

14. The samples generated in the trial were not analysed for any mycotoxins. However, it is noteworthy that the fungi isolated (belonging to the genera *Fusarium*, *Phoma*, *Alternaria*, *Penicillium* and *Aspergillus*) are all capable of producing mycotoxins. Both maize and sorghum stovers were stable in storage, with there being only minor differences in fungal contamination between stover stored in the field, or those stored in the open or on sheltered platforms. Whilst no major correlations could be detected between total fungal counts and gas produced during *in vitro* fermentation, appropriate methods for studying mycoflora-related nutritive value problems need to be developed (that incorporate an analysis of mycotoxins), before any firm conclusions can be drawn.

15. A paper describing the findings of this study was written for publication (Appendix 2).

Table 2. Least square means of main effects in the stover storage trial on gas produced (45 hr).

Period of storage (days)	Gas produced (mls)	Storage Method	Gas produced (mls)	Stover variety	Gas produced (mls)
Least square means for main effects:					
0	226.9[a]	S	226.9[c]	R201	215.8[ab]
60	214.2[b]	SF	207.1[a]	DC75	212.7[a]
90	209.5[b]	SOP	214.9[b]	SV2	221.3[b]
120	215.7[b]	SRP	214.8[ab]		
Significance of main effects:					
Storage Period (P=0.0012)		Storage Method (P=0.0004)		Stover Variety (P=0.0425)	

Values in the same row with different superscripts are significantly different (P<0.05)

(ii) Analysis of one-year stored rice straw in Eastern India and Bangladesh

16. In view of the literature reports that AFM1 and zearaleone are transmitted to milk, and since dairy cows in this region consume more of fibrous feeds than concentrates, it was of interest to examine whether these mycotoxins could form during storage of rice straw in Eastern India. Between November 1991 and January 1992, rice straw and other fibrous ruminant feed samples were taken from dairy farms in Calcutta and Bhubaneswar. In February 1993, similar sampling was conducted in Bangladesh. These and concentrate ruminant feed samples taken in the region were analysed for the mycoflora present and aflatoxin and zearalenone contamination.

17. The results are summarised in Appendices 3-5. The most frequently isolated fungi were: *Aspergillus versicolor* (Vuill), Tiraboschi, *Penicillium citrinum* Thom, *Eurotium* species, *Wallemia sebi* (Fr) v Arx, *Aspergillus penicilloides* Speg. and yeasts. *Fusarium moniliforme* Sheldon and *Cladosporium* species were among the most prevalent field fungi in straw but not in other feeds. Levels of certain individual fungi, particularly thermotolerant types, were higher in Bangladesh rice straw, although overall, the counts were similar to those of the Indian samples.

18. Analysis for aflatoxins and zearalenone showed aflatoxins in significant amounts in a number of feed samples from Eastern India, but not in rice straw from India or Bangladesh. Zearalenone occurred in *Paspalum* straw samples and three other feed samples from India. However, there was a very high level of aflatoxins in groundnut cake, with minor concentrations in rice chaff, biri testa and one of the mixed concentrates; zearalenone was also found in one of the mixed concentrates.

19. A paper describing the findings of this study was written for publication (Appendix 6)

(c) Effects of mycotoxins on rumen degradability of feeds

20. The detection of aflatoxin B1 and zearalenone in a number of dairy feeds (including fibrous feeds) obtained from Eastern India in 1991-92, coupled with suggestions that metabolites of both mycotoxins could be discharged in ruminant milk, raised concern on the effects of these mycotoxins on ruminant productivity, and in particular, on fibrous feed degradation in the rumen. A method that measures the quantity of gases produced during rumen microflora fermentation of fibrous feeds appeared to offer an ideal bioassay system to quantify the effects of mycotoxins on rumen function.

21. Maize stover was ground through a 1 mm screen and used as the basal substrate for artificial contamination with the pure mycotoxins. Appropriate volumes from solutions of 10.61 μg AFB1/ml and 11.81 μg zearalenone/ml, constituted in methanol, were mixed with 1 gm of the stover, and the solvent allowed to evaporate over five days. The contamination levels (0, 50 and 100 μg toxin/l fermentation medium) were selected to permit the evaluation of effects that might take place in the rumen of an animal of 400 kg body weight when consuming feed contaminated at a maximum of 1 mg/kg of the toxins. The cumulative gas production during 28 hours of incubation is shown in Table 3.

22. Factorial analysis of variance showed that aflatoxin B1 reduced gas production significantly (P=0.0066), but the effect of zearalenone was not significant (P=0.1403). There was no interaction between these toxins. Other experiments confirmed that aflatoxin reduced gas production, and also indicated that a number of other mycotoxins, in particular tenuazonic acid, could be toxic to rumen microorganisms. However, this study was terminated as it became apparent that methodology development was necessary to optimise the conditions with regard to solvent and substrates before comprehensive testing can be carried out using this test system.

Table 3. Gas produced during *in vitro* fermentation of maize stover artificially contaminated with aflatoxin and zearalenone.

Ratio of aflatoxin:zearalenone (μg/litre)	Gas produced after 28 hours (mls)
0:0 (methanol control)	170
50:0	160
100:0	153
0:50	169
0:100	166
50:100	140
100:50	142
50:50	157
100:100	133

CONCLUSIONS

23. Although this present study was preliminary in nature, it has shown that mycoflora-related feed problems are important to consider in intensive dairy production systems. Aspects that require consideration, in order of importance, are:

(a) Transmission of mycotoxins or their metabolites to the milk supplied to large population concentrations

24. This study has highlighted the incidence of mycotoxins in milk as a public health hazard for urban populations in Eastern India. The milk survey showed concentrations of AFM1 of up to 0.34 ug /litre when the maximum concentration of AFM1 permitted in milk in Europe is 0.05 ug/ml. This needs to be assessed in relation to the fact that at least 20 per cent of milk produced in India is consumed by infants and children, who may suffer chronic exposure to mycotoxins at an age when they are most susceptible. The livestock production systems should be changed, with appropriate feed management measures being adopted (eg improved feed storage, chemical treatment of feed, or dilution of contaminated feed) in order to minimise the scale of the milk contamination problem. Detailed studies are warranted on the transmission rate of mycotoxins to the milk under field conditions.

25. Another important area for research and development concerns the effects of milk processing on any AFM1 that might be present; a subject on which the evidence in the literature is unclear. Whilst the results obtained in the present study show that boiled milk still retains high levels of AFM1, the level present before boiling is not known. Further research is recommended on this aspect and, more specifically, on whether or not in developing countries processing of contaminated milk might not offer a more cost-effective and practical method for containing the AFM1 hazard to humans than the alternative of adopting feed management strategies. Further, since a number of secondary products are made from milk in India, a survey of these foods for AFM1 from retail outlets is also recommended.

(b1) Changes taking place in the nutritive value of stovers and straws during different storage conditions arising from the utilisation of carbohydrates by fungi

26. In this study it was found that storage of maize and sorghum stover under different methods and length of period could cause differences in the nutritive value of the feed. Whilst not a novel finding, the degree of changes in nutritive value was quantified. This area is worthy of further investigation. In many of the countries of sub-Saharan Africa, cattle nutrition during the dry season may be dependent on a limited supply of maize and sorghum stover in addition to grasses of even lower nutritive value. This calls for appropriate feed storage and feed allocation strategies to optimise the use of available feed resources in livestock production. Such a study is not warranted while there remains a surplus of rice straw in the rice-producing regions of
Asia. However, with the proliferation of high-yielding but short-straw varieties of rice into the farming systems of the region, the situation needs to be kept under review.

(b2) Mycotoxins produced by field and storage fungi on fibrous feeds and feed concentrate.

27. This study has not resolved the fundamental question of whether storage of rice straw, maize stover and sorghum stover can lead to formation of mycotoxins. This is mainly due to the high cost of conducting multi-mycotoxin screening of samples preventing its use in the study. Whilst, selective screening for aflatoxin and zearalenone in rice straw from Eastern India and Bangladesh proved negative, these mycotoxins were detected in a number of other ruminant feed samples. Furthermore, a number of mycotoxigenic fungi, notably *Aspergillus versicolor* (Vuill), Tiraboschi, *Penicillium citrinum* Thom, *Eurotium* species, *Wallemia sebi* (Fr) v Arx, *Aspergillus penicilloides* Speg. and *Fusarium moniliforme* Sheldon, were detected in crop residues. Particular attention is drawn to the prevalence of *Wallemia sebi* in the rice straw samples, an association that is of concern because this fungus produces the toxin walleminol, and also because cattle diseases are known in South Asia for which there is no agreed cause (for example, Degnala disease of buffaloes). In view of the considerable importance of rice straw in South Asia and the Far East, basic research is strongly recommended on the ability of this fungus to grow on rice straw and to produce mycotoxins. Accordingly, a concept note was submitted to the Livestock Production Programme (Appendix 7), although it was not successful.

(c) the effects of mycotoxins on ruminant production

28. The preliminary *in vitro* studies suggested that the presence of specific mycotoxins depressed rumen microbial activity, which could be expected to result in decreased digestibility of feeds and hence decreased productivity. It is recommended that the direct effect of mycotoxin on milk yield should be determined by undertaking a collaborative study through an EMC with the National Dairy Research Institute in India.

APPENDIX 1. RESULTS OF ANALYSIS OF MAIZE AND SORGHUM STOVER FROM THE STORAGE TRIAL IN ZIMBABWE

Sample	Variety	Storage period (days/ code)	Fungal counts after incubation at 25°C9 MEA medium	DG18 medium	MEA@45°C medium	Gas produced (mls)[1] 45 hrs	116 hrs	(%DM) hrs	ADF (%DM)	NDF protein	Crude (%DM) (%DM)	Ash
1	R201	0 F	$6.76x10^6$	$6.93x10^6$	$7.92x10^4$	76.8	232.9^{g}	296.1	32.7	57.6	9.63	9.25
2	DC75	0 F	$7.92x10^6$	$7.26x10^6$	$3.08x10^4$	76.0	219.3^{cdef}	289.7	32.5	59.7	5.66	10.64
3	SV2	0 F	$7.26x10^7$	$3.50x10^7$	$4.08x10^4$	84.2	228.5^{efg}	293.2	31.2	55.8	7.70	10.88
4	R201	60 SF	$1.35x10^7$	$1.24x10^7$	$5.49x10^3$	68.3	213.8^{bcd}	280.8	39.8	62.1	7.99	15.56
5	R201	60 SOP	$9.41x10^6$	$9.41x10^6$	$4.70x10^4$	72.0	213.7^{bcd}	274.2	37.9	61.5	9.30	13.07
6	DC75	60 SF	$1.45x10^7$	$9.74x10^6$	$2.00x10^4$	71.1	216.2^{bcde}	283.6	36.7	62.3	5.99	12.97
7	DC75	60 SRP	$1.91x10^7$	$1.88x10^7$	$2.31x10^4$	77.8	216.7^{bcde}	281.5	36.2	61.6	5.72	12.78
8	DC75	60 SOP	$8.08x10^6$	$1.49x10^6$	$1.45x10^4$	64.2	198.9^{a}	273.5	34.2	61.3	5.01	12.26
9	SV2	60 SOP	$8.91x10^6$	1.65x107	$1.03x10^4$	88.2	230.2^{fg}	306.0	31.4	55.7	6.41	8.98
10	SV2	60 SF	$2.15x10^7$	1.01x107	$3.91x10^4$	67.2	206.4^{ab}	282.9	36.6	62.8	6.66	11.71
11	R201	90 SF	$2.97x10^6$	$1.58x10^6$	$1.66x10^3$	57.6	198.2^{a}	278.6	39.4	63.1	8.60	12.82
12	R20	90 SOP	$6.93x10^6$	$1.16x10^7$	$6.59x10^4$	62.8	223.3^{adefg}	295.8	35.3	60.0	10.40	9.56
13	DC75	90 SF	$4.29x10^7$	$2.81x10^7$	$6.16x10^4$	56.7	200.4^{a}	279.5	37.3	65.5	5.29	11.45
14	DC75	90 SRP	$4.62x10^6$	$3.33x10^6$	$3.68x10^4$	70.9	206.8^{ab}	276.9	36.3	62.8	5.00	15.85
15	DC75	90 SOP	$3.63x10^6$	$1.81x10^6$	$5.56x10^4$	65.1	200.5^{ac}	281.0	38.1	65.2	4.35	12.36
16	SV2	90 SOP	$3.46x10^7$	$8.09x10^6$	$5.81x10^4$	77.3	222.6^{defg}	299.9	32.2	57.2	6.79	10.60
17	SV2	90 SF	$1.81x10^7$	$1.14x10^7$	$1.12x10^4$	61.4	207.4^{abc}	294.9	36.6	64.6	6.48	10.14
19	R201	120 SOP	$3.67x10^7$	$9.41x10^7$	$9.90x10^3$	68.1	205.7^{ab}	272.7	41.5	65.9	9.95	15.72
21	DC75	120 SRP	$8.25x10^6$	$8.47x10^6$	$1.32x10^4$	77.1	220.8^{de}	299.9	33.9	61.0	5.82	9.93
22	DC75	120 SOP	$2.97x10^7$	$2.31x10^7$	$3.30x10^3$	72.9	215.5^{bcdf}	294.9	37.9	64.3	5.97	10.91
23	SV2	120 SOP	$9.41x10^7$	$1.16x10^8$	$1.49x10^4$	81.0	223.9^{defg}	303.0	35.2	61.3	7.20	9.66
Pooled standard error of means						0.95						

1.Values without common superscript are significantly different (P=0.05). Regression of gas produced (r^2)on MEA - 0.04; on DG18 - 0.01; on MEA@45°C - 0.04

Codes: F - freshly harvested; SF - standing in field; SOP - standing on open air platform; SRP - kept under shelter roof) protection. R201 - maize; SV2 - sorghum; and DC75 sorghum.

APPENDIX 2. DRAFT PAPER DESCRIBING MYCOFLORA IN ZIMBABWEAN STOVERS

The effects of storage on the mycoflora of maize and sorghum stover in Zimbabwe

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Key Words: conservation, forage crops, biodeterioration, fungi

Abstract. A study was carried out on the effects of short-term field storage on the mycoflora of sorghum and maize stover in Zimbabwe. The fungi isolated from 24 maize and sorghum stover samples were all potential mycotoxin producing fungi, belonging to genera *Fusarium*, *Phoma*, *Alternaria*, *Penicillium* and *Aspergillus*. A number of storage fungi (*Aspergillus* and *Penicillium* species) were detected in both freshly harvested and stored samples. Maize and sorghum stover was stable in storage with very little difference in fungal contamination between stover stored in the field or on open or sheltered platforms.

Introduction

Inadequate nutrition is one of the major constraints to the achievement of higher livestock productivity in developing countries. and there is a need to maximise the utilisation of available feed resources. The storage of crop residues for use by ruminant livestock during periods of feed shortage, or when the animal's nutritional needs are greatest, for example during pregnancy and lactation, may be the best option in some circumstances. However, the storage of feed may allow the growth of fungi, and the possible production of mycotoxins [1]; the deleterious effects of which on ruminant livestock is well documented [2]. In addition, mycotoxins may have been produced by field fungi on the plant prior to storage. Field isolations from maize commonly include: *Fusarium moniliforme* Sheldon, *Fusarium moniliforme v. subglutinans* Wollenw. & Reinking, *Fusarium graminearum* Schwabe, *Aspergillus flavus* Link and *Penicillium citrinum* Thom [3-5]. Fungal isolations from sorghum commonly include: *F. moniliforme*, *Fusarium pallidoroseum* (Cooke) Sacc., *Alternaria alternata* (Fr.) Keissler, *Alternaria tenuissima* (Kunze) Wiltshire, *Curvularia lunata* (Wakker) Boedijn and *Phoma sorghina* (Sacc.) Boerema, Dorenbosch & v.

[6-8]. In this study the effects of different farm storage procedures in Zimbabwe how these contribute to potential mycotoxin contamination are assessed.

and Methods

Stover samples from sorghum varieties DC75 and SV2, and maize variety R201 taken from research plots at Matopos Research Station in Zimbabwe between May December 1992. A systematic sampling procedure for pre-harvest fungal was carried out taking 20 plants *in situ*. The cobs were removed, the plant then harvested, sun-dried and air-freighted back to the United Kingdom.

For the storage trial, plants were treated in the following ways after removal of the heads; (a) plants left *in situ* in the field; (b) plants harvested and placed on an open-air platform; (c) plants harvested and placed on a roofed platform. Storage was for 60, 90 or 120 days

Mycological analyses. Total counts were performed on ground samples using 10g of stover in 290 ml of 0.2% agar; a decimal dilution series was prepared using 0.2% agar. Aliquots of 0.1 ml were spread over the surface of Dichloran 18% Glycerol agar (DG18) (Unipath, UK) and Malt Extract Agar (MEA) [9] plates and incubated at 25°C for 5 days. Two further MEA plates were spread with 10^{-1} dilutions per sample, and incubated at 45°C, for an assessment of thermotolerant and thermophilic fungi. Plates were then counted and fungi identified direct from primary enumeration plates, or from subcultures made of the different fungal types. Fungi were identified using the keys for *Penicillium* [11], *Aspergillus* [12], *Fusarium* [Brayford (unpublished) based on [14]] and other fungi [13, 9]. Yeasts were identified using API 20C test strips (BioMerieux, UK), in conjunction with [15].

Results

Total fungal counts of less than 10^4 colony forming units per g (cfu/g) were considered as low; and greater than 10^6 cfu/g as high. The same criteria were applied to individual species. A high count of a potentially mycotoxigenic fungus does not necessarily indicate that mycotoxins have been produced; nevertheless, it is more likely that mycotoxigenic fungi, isolated at counts of greater than 10^5 cfu/g, could have produced mycotoxins within the substrate.

Fungal counts for freshly harvested material ranged from 6.76×10^6 to 7.26×10^7 cfu/g of stover (Table 1). R201 (maize) yielded the lowest count, and SV2 (sorghum) the highest. Overall there were no major significant ($p=0.05$) increases in counts with time during storage. Higher fungal counts were often obtained from samples left standing in the field in with the same variety which was stored either on open or sheltered platforms 1). There was no discernible difference between maize and sorghum stover in 1).

Levels of certain individual fungi were extremely high; *Fusarium* species comprised the component of the pre- and post-harvest mycoflora of both maize and sorghum stover. 2 and 3 give the mean counts for fungi isolated from the three cultivars. *F.* was isolated from all samples, and on average at very high levels: usually 10^7-10^8 cfu/g (Tables 2 and 3). There was no evidence that field fungi declined storage; fungal counts of *Alternaria*, *Fusarium* and *Cladosporium* were still high after days. Yeast counts were high throughout, at 10^6-10^8 cfu/g, principally composed of

Rhodotorula glutinis, *Cryptococcus laurentii*, *Cryptococcus albidus* and *Candida guillermondii*.

The 120 day standing samples from the field may have been damaged by livestock, therefore limited cross-comparisons could be made for the final samples. Levels of thermotolerant fungi were relatively high at around 10^4 cfu/g, for example *Emericella nidulans*, *Aspergillus flavipes*, *Aspergillus fumigatus* and *Aspergillus niveus*; these were the most commonly isolated storage fungi, apart from *Aspergillus flavus* and *Aspergillus ochraceus* (Tables 2 and 3). Table 4 shows the changes in the occurrence of fungi with time. The incidence of field fungi did not generally decline with time. *P. oxalicum* was not isolated from 90 days onward, whereas *Penicillium citrinum* was isolated from 90 days onward. Table 5 shows the percentage of samples in which levels of individual species were more than 10^5 cfu/g. Fungi isolated at greater than 10^5 cfu/g may have produced mycotoxins. For *Aspergillus niger*, although mean counts were high (Tables 2 and 3), it can be seen from Table 5 that this was from relatively few samples with high counts. *F. moniliforme* was always isolated at greater than 10^5 cfu/g from samples.

Discussion

Production of mycotoxins can occur either in the field prior to harvest, or after harvest whilst the crop is still wet. Many of the fungi isolated can produce mycotoxins on the growing crop in the field, for example: *F. moniliforme* - fumonisins, *P. sorghina* - tenuazonic acid, *A. alternata* - tenuazonic acid, alternoxins, alternariols, *F. moniliforme var. subglutinans* - moniliformin [16]. Toxin production by the fungi isolated depends on many factors including field environmental conditions. However, the high levels at which the fungi were isolated indicates a potential mycotoxin problem with these types of stover.

The field fungi isolated will not grow or produce toxins once the crop has dried to below approximately 19-21% moisture content (mc), corresponding to a water activity (a_w) of 0.87-0.92 [16]. However, any toxins produced pre-harvest will still be present in the dried crop.

The fungal content of stored maize and sorghum stover was relatively stable with few increases in overall fungal counts during storage. Levels of field fungi remained high even after storage for 120 days; storage fungi increased slightly. A small increase in fungi was associated with the standing crop in the field. Unfortunately, the loss of the final field samples at 120 days means that this cannot be substantiated. Pre-harvest contamination of the maize and sorghum plants by field fungi thus constituted the main spoilage problem, for the storage trial material, by the potential production of mycotoxins.

F. moniliforme can produce fumonisins, implicated in human oesophageal cancer [17]; leucoencephalomacia (ELEM); and pulmonary oedema in pigs [18]. *F. moniliforme*

var. *subglutinans* is associated with the production of moniliformin and *Fusarium sambucinum* with Type A trichothecenes [16]. Type A trichothecenes include; T-2 toxin, neosolaniol, and diacetoxyscirpenol. Type A trichothecenes have been implicated in haemorrhagic disease in ruminants [2]. Probably of more importance are the immunosuppressive effects of subclinical doses of trichothecenes upon ruminants. Calves are particularly susceptible, and exhibited decreases in thymus weight when fed 0.6 mg/kg/day of T-2 toxin [2]. Tenuazonic acid contamination of plant material by *P. sorghina* has been reported in the aetiology of onyalai, a blood disorder mainly found in Africa [19,20]. Tenuazonic acid has been found to be toxic in different bioassays, including the gas production system using rumen liquor, and the mung bean germination test (unpublished data) and the brine shrimp bioassay [21]. In poultry it is responsible for haemorrhagic disease [22].

Penicillium oxalicum is a pre-harvest contaminant of maize; of six samples contaminated with this fungus, four were of maize variety R201. *A. flavus* was isolated from more maize samples than sorghum. Although both these fungi are commonly associated with stored commodities, they can also invade plants prior to harvest [23,24]. In ruminants, ingestion of aflatoxin contaminated feed has been associated with hepatic damage, anorexia, lethargy, ascites and bloody diarrhoea. In addition, consumption of aflatoxin contaminated feed has been implicated in bovine immunosuppression and infertility [2].

There is some evidence that some mycotoxins may be degraded by the rumen flora. Intact rumen fluid, and isolated fractions of rumen protozoa and bacteria, had no effect on aflatoxin B^1 and deoxynivalenol, however, ochratoxin A, T-2 toxin, diacetoxyscirpenol were degraded to less active derivatives. [25]. Zearalenone was degraded to zearalenol A and B, which are more toxic to the ruminant than zearalenone.

Some of the fungi are known to cause mycoses or allergenic responses; these may be important factors in animal health. *Alternaria* spp can cause allergic effects in humans and animals [26,27], and has been isolated from bovine tissue samples [28]. *Aspergillus* and *Penicillium* spp have also been isolated from bovine tissue lesions. Trichothecene mycotoxins produced by Fusaria can cause dermatitis and destructive skin lesions [29]. The high incidence of potentially pathogenic yeasts is of some concern. *Cryptococcus laurentii* and *Cryptococcus albidus* have been isolated from humans and animals, including bovines, although these species are not as aggressive pathogens as *Cryptococcus neoformans* [15,28].

From the data presented there did not appear to be appreciable risks of post-harvest mycotoxin production during short-term outdoor storage of maize and sorghum stover under drought conditions, in Zimbabwe. The possibility of potential mycotoxin hazards to associated with the high levels of pre-harvest contamination of maize and

sorghum by *F. moniliforme* and *P. sorghina* and their associated mycotoxins, under normal rainfall conditions is worthy of investigation.

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Table 1. Viable fungal counts from samples of maize and sorghum stover (25°C)

Sample	Variety	Storage length (Days,code)[1]		Fungal counts (cfu/g) at 25°C		
				MEA 25°C	DG18 25°C	MEA 45°C
Maize	R201	0	F			
Maize	R201	60	SF			
Maize	R201	60	SOP			
Maize	R201	90	SF			
Maize	R201	90	SOP			
Maize	R201	120	SOP			
Sorghum	DC75	0	F	$7.92X10^6$	$7.26X10^6$	$3.08X10^4$
Sorghum	DC75	60	SF	$1.45x10^7$	$9.74x10^6$	$2.00x10^4$
Sorghum	DC75	60	SRP	$1.91x10^7$	$1.88x10^7$	$2.31x10^4$
Sorghum	DC75	60	SOP	$8.08x10^6$	$1.49x10^6$	$1.45x10^4$
Sorghum	DC75	90	SF	$4.29x10^7$	$2.81x10^7$	$6.16x10^4$
Sorghum	DC75	90	SRP	$4.62x10^6$	$3.33x10^6$	$3.68x10^4$
Sorghum	DC75	90	SOP	$3.63x10^6$	$1.81x10^6$	$5.56x10^4$
Sorghum	DC75	120	SRP	$8.25x10^6$	$8.47x10^6$	$1.32x10^4$
Sorghum	DC75	120	SOP	$2.97x10^7$	$2.31x10^7$	$3.30x10^3$
Sorghum	SV2	0	F	$7.26X10^7$	3.5010^7	$4.08X10^4$
Sorghum	SV2	60	SOP	$8.91x10^6$	$1.65x10^7$	$1.03x10^4$
Sorghum	SV2	60	SF	$2.15x10^7$	$1.01x10^7$	$3.91x10^4$
Sorghum	SV2	90	SOP	$3.46x10^7$	$8.09x10^6$	$5.81x10^4$
Sorghum	SV2	90	SF	$1.81x10^7$	$1.14x10^7$	$1.12x10^4$
Sorghum	SV2	120	SOP	$9.41x10^7$	$1.16x10^8$	$1.49x10^4$

1

F	Freshly harvested	SOP	Standing on open air platform
SRP	Kept under roof protection	SF	Standing in field

Table 2. Incidence of individual fungi in Zimbabwe maize stover samples

Fungal species	Maize R201	
	MVC[1]	%[2]
	c.f.u. per g	incidence
Alternaria sp	$9.96X10^5$	83
Cladosporium sp	$3.02X10^6$	67
Drechslera sp	$8.32X10^4$	17
Nigrospora oryzae	$2.77X10^4$	17
Chaetomium sp	$1.93X10^2$	17
Phoma sp	0	0
Phoma sorghina	$2.24X10^6$	67
Trichoderma sp	$8.32X10^4$	17
Fusarium moniliforme	$1.84X10^7$	1
F. mon. v. subglutinans	0	0
F. oxysporum	$2.77X10^5$	17
F. chlamydosporum	$2.77X10^5$	17
F. sambucinum	$6.05X10^6$	50
Aureobasidium sp	$6.47X10^6$	83
Yeasts	$3.12X10^6$	100
Absidia sp	$5.55X10^2$	17
Rhizopus sp	$2.77X10^5$	17
Aspergillus flavipes	$5.49X10^3$	67
A. niveus	$1.99X10^4$	83
A. niger	$2.11X10^5$	10
A. fumigatus	$1.93X10^2$	33
A. ochraceus	$3.96X10^5$	83
A. flavus	$4.70X10^4$	67
A. sydowii	0	0
A. wentii	$2.77X10^4$	17
Eurotium sp	0	0
Emericella nidulans	$3.26X10^3$	50
Penicillium oxalicum	$6.79X10^5$	67
P. citrinum	0	0
P. duclauxii	$2.77X10^6$	17

[1] MVC = mean viable count
[2] % incidence = percentage of samples in which species were present

Table 3. Incidence of individual fungi in Zimbabwe sorghum stover samples

Genera and species	Sorghum SV2		Sorghum DC75	
	MVC[1]	%[2] Incidence	MVC[1]	%[2] Incidence
Alternaria sp	$1.87X10^7$	67	$2.37x10^6$	22
Cladosporium sp	$2.19x10^7$	83	$3.99x10^6$	89
Drechslera sp	$5.83x10^5$	17	$1.68x10^7$	22
Nigrospora oryzae	0	0	$1.83x10^6$	22
Chaetomium sp	0	0	0	0
Phoma sp	$2.75x10^4$	17	0	0
Phoma sorghina	$3.3x10^4$	17	$8.29x10^5$	22
Trichoderma sp	$4.51x10^6$	83	$2.07x10^6$	44
Fusarium moniliforme	$1.96x10^8$	100	1.08x107	100
F. mon. v. subglutinans	$1.79x10^7$	33	$1.68x10^6$	22
F. oxysporum	$1.65x10^6$	17	$3.31x10^4$	22
F. chlamydosporum	0	0	0	0
F. sambucinum	$2.77x10^5$	17	$3.48x10^5$	22
Aureobasidium sp	$5.35x10^7$	67	$2.10x10^7$	44
Yeasts	$7.92x10^7$	50	$1.01x10^8$	67
Absidia sp	$1.67x10^3$	33	$6.59x10^3$	33
Rhizopus sp	0	0	$8.50x10^4$	22
Aspergillus flavipes	$3.89x10^5$	100	$5.60x10^4$	44
A. niveus	$5.05x10^4$	83	$1.41x10^5$	100
A. niger	$1.71x10^6$	67	7.33x105	100
A. fumigatus	$3.22x10^5$	83	$1.85x10^6$	44
A. ochraceus	$1.65x10^6$	17	$2.01x10^5$	44
A. flavus	$1.65x10^7$	17	$5.68x10^5$	56
A. sydowii	$1.65x10^7$	33	0	0
A. wentii	0	0	$5.01x10^5$	22
Eurotium sp	0	0	$5.82x10^5$	22
Emericella nidulans	$8.33x10^3$	17	$1.66x10^5$	22
Penicillium oxalicum	0	0	$2.17x10^6$	44
P. citrinum	0	0	$5.14x10^5$	33
P. duclauxii	$2.33x10^7$	50	$1.66x10^5$	

[1] MVC = mean viable count

[2] % incidence = percentage of samples in which species were present

Table 4. Changes in occurrence of fungi with time

Fungal species	Fresh n=3	Stored 60 days n=7	Stored 90 days n=7	Stored 120 days n=4
Alternaria sp	2	5	3	1
Cladosporium sp	3	5	6	3
Phoma sorghina	0	5	1	2
Trichoderma sp	0	2	6	2
Fusarium moniliforme	3	7	7	4
Aureobasidium sp	2	2	4	4
Yeasts	3	4	4	4
Absidia sp	3	3	0	0
Aspergillus flavipes	1	4	7	2
A. niveus	3	6	7	2
A. niger	3	7	5	4
A. fumigatus		6	2	2
A. ochraceus		3	4	3
A. flavus	2	3	3	2
Emericella nidulans	1	1	3	2
Penicillium oxalicum	2	4	0	0
P. citrinum	0	0	1	2

Figures are number of samples where the fungus occurs, out of total number of samples 'n'

Table 5. Percentage of samples infected with specific fungi at levels of more than 10^5 per g

Fungi	Percentage of maize and sorghum samples infected with fungi
Field fungi	
Fusarium moniliforme	100
Cladosporium sp.	67
Alternaria sp.	48
Aureobasidium pullulans	43
Phoma sorghina	38
Fusarium sambucinum	29
Fusarium moniliforme var. *subglutinans*	14
Fusarium oxysporum	14
Storage fungi	
Aspergillus niger	43
Aspergillus ochraceus	33
Aspergillus niveus	29
Aspergillus flavus	24
Penicillium oxalicum	24
Penicillium citrinum	19
Yeasts	43

Yeasts include: *Rhodotorula glutinis*; *Cryptococcus laurentii*; *Cryptococcus albidus*; *Candida guillermondii*

APPENDIX 3. PREDOMINANT FUNGI PRESENT IN RICE STRAW SAMPLES AT AN INCIDENCE OF MORE THAN 10^3 FUNGI PER G: COMPARISON BETWEEN BANGLADESH AND INDIAN SAMPLES

Species/type	% of samples infected by individual fungi at a level of > 10^3 per g	
	Bangladesh	India
Field fungi:		
Grey, non sporing sp.	74	?
Cladosporium oxysporum	47	40
Stachybotrys sp.	47	0
Grey restricted, non sporing sp	42	0
Fusarium sambucinum	26	0
Acremonium sp.	21	0
Fusarium moniliforme	11	33
Aureobasidium sp.	11	0
Fusarium pallidoroseum	0	20
Storage fungi		
Wallemia sebi	100	40
*Penicillium spp**	84	53
Yeasts	74	13
Eurotium spp (A. glaucus gp.)	74	27
*Humicola lanuginosa***	68	0
*Emericella nidulans***	68	0
*Aspergillus versicolor gp.***	63	53
*Aspergillus fumigatus***	53	7
*Rhizomucor pusillus***	53	0
Geotrichum candidum	37	0
Vinaceous, non sporing sp.	32	0
*Aspergillus terreus***	26	0
Aspergillus niger	21	7
*Aspergillus candidus***	16	7
Aspergillus flavus	11	7
Aspergillus restrictus gp.	11	20
*Thermoascus type***	11	0

* *P. citrinum, P. purpurogenum, P. raistrickii, P. glabrum, Eupenicillium cinnamopurpureum*

** fungi association with heating

APPENDIX 4. SUMMARY OF FUNGAL COUNTS OF STRAW AND OTHER FEED SAMPLES FROM INDIA AND BANGLADESH

Samples	Country	Isolation Medium[a]	Number of samples (n)	Mean fungal count (c.f.u. per g x10^6)	± Standard Error (x10^6)
Straw	Bangladesh	MEA	19	3.40	0.63
		DG18	19	3.70	0.54
		MEA 45	19	1.10	0.45
Straw	India	MEA	17	19.60	7.30
		DG18	17	17.00	5.50
		MEA 45	17	0.06	0.05
Other feeds	India	MEA	13	3.90	2.90
		DG18	13	2.50	2.00
		MEA 45	13	0.02	0.002

[a] MEA: Malt Extract Agar; DG18: Dichloran Glycerol Agar; MEA 45: Malt Extract Agar incubated at 45°C.

APPENDIX 5. SUMMARY OF AFLATOXIN AND ZEARALENONE CONCENTRATIONS IN STRAW AND FEEDS FROM EASTERN INDIA AND BANGLADESH

Sample	Zearalenone (µg/kg)	Aflatoxins (µg/kg)			
		B_1	B_2	G_1	G_2
Rice straw[a]	-	4			
Rice straw	-	3			
Rice straw	-	3	-	-	
Paspalum palidosum straw	422	-	-	-	
Wheat bran	-	3	-	3	
Wheat bran and chana testa	-	3	-	3	
Wheat bran and chana testa	-	-	-	3	
Wheat bran and chana testa	316	4	-	-	
Groundnut cake	-	3182	487		
Rice chaff	-	16	3		
Biri testa	-	22	4	-	
Mung testa	-	4	-	3	
Mixed concentrate	-	13			
Mixed concentrate	-	-			
Mixed concentrate	843	7			
Mixed concentrate	-	7			
Mixed concentrate		-		3	

Notes: a - 13 rice straw samples from India and 19 from Bangladesh were negative for aflatoxin and zearalenone; - not detected (minimum limit 2 µg/kg for aflatoxins and 50 µg/kg for zearalenone.

APPENDIX 6. DRAFT PAPER DESCRIBING MYCOFLORA AND MYCOTOXINS IN EASTERN INDIA AND BANGLADESH

The incidence of mycoflora, aflatoxin and zearalenone in dairy feed and forage samples from Eastern India and Bangladesh

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Key Words: mycotoxins, fungi, animal feeds, spoilage, Southern Asia

Abstract. The mycoflora of a range of mixed ruminant feed and forage samples collected in Eastern India and Bangladesh were determined. Most fungi isolated from the samples were those associated with low moisture contents. Several potentially mycotoxigenic fungi and thermotolerant species were present particularly in the rice straw. The most frequently isolated fungi were: *Aspergillus versicolor* (Vuill) Tiraboschi, *Penicillium citrinum* Thom, *Eurotium* species, *Wallemia sebi* (Fr.) v. Arx, *Aspergillus penicilloides* Speg. and yeasts. *Fusarium moniliforme* Sheldon and *Cladosporium* species were among the most prevalent field fungi in straw but not other feeds. Levels of certain individual fungi, particularly thermotolerant types, were higher in Bangladesh rice straw although overall counts were similar to those of the Indian samples. Mycotoxin analysis for aflatoxins and zearalenone were carried out on all samples. Aflatoxins were detected in significant amounts in some feed samples from Eastern India but not in rice straw from India and Bangladesh. Zearalenone occurred in a *Paspalum* straw sample and three other feed samples from India.

Introduction

Crop residues are an important source of feed for ruminant livestock in developing countries; rice straw forming a high proportion of this type of feed particularly in India and Bangladesh. Mould contamination of feeds and feed ingredients is common and mycotoxin contamination can occur from a variety of sources particularly in mixed feeds [1,2]. In conserved forage crops, there are numerous reports of mycotoxins and problems of unspecified toxicity in ruminants [3]. However, there is a lack of quantitative data on the natural occurrence of the mycoflora and mycotoxins concerned. As part of an investigation into the susceptibility of rice straw and feeds to fungal contamination and potential hazard to human and animal health, a survey of feeds and conserved forage crops from farms and shops in India and Bangladesh was undertaken. The incidence of the mycotoxins aflatoxin and zearalenone was investigated.

Materials and Methods

Sampling. Nineteen samples of rice straw were collected from farms in five different regions of Bangladesh; of these, two originated from the Jute Research Institute, one from a rice farm and the remainder from dairy farms. Sampling was carried out during

January and February 1993. The typical climate for this region is characterised by: average humidities of 65 to 82% with higher values from July to January; a rainy season from April to September; minimum temperatures ranging from 11 to 26°C with a cool period from November to April and maximum temperatures from 25 to 36°C, June being the hottest month. All straw samples were taken from heaps of three to five metres in height and diameter, the traditional method of storing straw in Bangladesh.

Thirty feed and fodder samples were collected between December 1991 and January 1992 from farms, villages and traders in three different regions of Eastern India: the Lake Town area of Calcutta, Bhubaneswar and Baikunthapur near Bhadrakh, Orissa. Of these, sixteen consisted of rice straw, one of *Paspalum palidosum* straw, five of mixed concentrates, three of wheat bran with chana (*Cicer aeritinum* L.) testa, and one each of: groundnut cake, rice chaff, biri (*Vigna mungo* L.) testa and mung (*Vigna radiata* L.) testa. Each sample comprised numerous aliquots, taken from different parts of the total feed that was available at each location. Apart from two freshly harvested straw samples, all others had been stored for 10 to 12 months from the previous harvest and therefore exposed to the full range of climatic conditions of the region during 1991. Most of the samples collected in urban dairy farms originated in different villages and thus give a reasonable representation of the variation in straw quality in West Bengal and Orissa for that year. During 1991 the area experienced a relatively hot spring and summer (maximum day temperature 36°C) followed by a wet and humid autumn and a colder than normal winter. The maximum winter day-time temperature ranged from 20 to 25°C; the night-time minimum from 9 to 16°C.

Mycological analysis. To analyse the mycoflora, total fungal counts were performed on ground samples, using either 10g of straw or 30g of other types of feed in 290ml or 270ml of 0.2% agar. A decimal dilution series was prepared from this initial dilution using 0.2% agar. Aliquots (0.1ml) of each dilution were spread over the surface of Malt Extract Agar containing chloramphenicol (MEA) [4] and Dichloran Glycerol Agar (DG18) (Unipath, UK) plates. MEA is a general medium used for isolation of a wide range of fungi; DG18 is used for the isolation of xerophilic fungi, capable of growth at low moisture contents. Plates were incubated at 28°C for 5 to 7 days. Additional plates of MEA were incubated at 45°C for isolation of thermotolerant and thermophilic fungi. After incubation, fungi were counted and the proportion of different types assessed and identified. Identifications were made of *Penicillium* species [5], *Aspergillus* [6], other fungi [4,7] and *Fusarium* [8].

Mycotoxin analysis: sample preparation. Straw samples, mixed feeds and feed ingredients were ground in an Apex knife mill fitted with a 3mm screen.

Aflatoxin analysis: extraction and sample clean-up. A 25g sample was extracted by shaking (40 min) with 80% acetone in water (150ml) and filtered using Whatman No 4 paper. Aliquots (5ml) were applied on pre-conditioned phenyl bonded phase columns (Bond-Elut, Varian) in 60ml aqueous methanolic acetic acid (92.7:6.7:1, v/v) and 3ml lead acetate solution (20% w/v). The column was washed with water (10ml) and the aflatoxins eluted with chloroform (7ml) through an anhydrous sodium sulphate column and dried in a flow of nitrogen at 45°C.

Determination of aflatoxin levels by High Performance Thin Layer Chromatography (HPTLC). Dried eluates were redissolved in 200µl benzene:acetonitrile (98:2, v/v) and spotted on silica 60 plates. Plates were first developed in anhydrous diethyl ether and air dried. Interfering impurities were removed by discarding the top 15mm of silica gel from the plate. The plates were then rotated through 180° and developed twice in chloroform:xylene: acetone (6:3:1, v/v), air dried and briefly heated at 105°C (2min). Aflatoxins B_1, B_2, G_1 and G_2 were determined by comparison with mixed standards, using an integrating scanning densitometer (CAMAG Scanner II) at an activation wavelength of 366 nm and a K400 filter.

Zearalenone analysis. Zearalenone was determined by HPTLC using a modification of the method of Bennett *et al.* [9].

Zearalenone extraction and sample clean-up. Samples of rice straw (25g) or feed concentrate (50g) were extracted by shaking (40 min) with water (20ml), diatomaceous earth (25g) and chloroform (250ml). Filtrate (50ml) was shaken in a separating funnel with 2% sodium hydroxide (50ml), saturated sodium chloride solution (10ml) and chloroform (50ml). The chloroform layer was discarded, a further 50 ml of chloroform added, shaken and again discarded. The aqueous layer was recovered, acidified with 10.6% citric acid solution (50ml) and extracted twice with dichloromethane (50ml). Combined extracts were dried on a bed of anhydrous sodium sulphate (40g) and evaporated to near dryness on a rotary evaporator and to dryness in a vial under a flow of nitrogen.

Determination of zearalenone levels by HPTLC. Extract residues were dissolved in 200µl benzene:acetonitrile (9:1, v/v), spotted on silica 60 plates and developed in

toluene:ethyl acetate:formic acid (6:3:1, v/v). Plates were dried and sprayed with fast violet B salt which produces purple spots in the presence of zearalenone. Zearalenone was determined by comparison with standards, using an integrating scanning densitometer (CAMAG Scanner II).

Results

Results of mean fungal counts are shown in Table 1 for rice straw samples from both India and Bangladesh and feeds or feed ingredients from India. For all samples, there was no significant difference ($p=0.05$) between counts on MEA and DG18 at an incubation temperature of 25°C. Certain xerophilic fungi will only grow on low water activity media like DG18 which may highlight count differences between media. Mean fungal counts for rice straw samples from Bangladesh were around 10^6 cfu per g compared with 10^7 cfu per g for Indian straw samples (Table 2) although these were not significantly different ($p=0.05$). In samples from both countries, lowest counts were of the order of 10^5 cfu per g. Other Indian feeds and feed ingredients (wheat bran, wheat bran with chana testa, mung testa, groundnut cake, biri testa, rice chaff and mixed concentrates) yielded a wider range of counts, from 10^3 to 10^7 cfu per g. Counts representing thermotolerant fungi (MEA incubated at 45°C) were significantly lower ($p=0.05$) compared with those obtained on plates incubated at 25°C. Thermotolerant fungi were more prevalent in Bangladesh rice straw samples than those from India (Table 2).

Field fungi were present in straw samples from both India and Bangladesh. Common to both countries were: several *Fusarium* species (*Fusarium sambucinum* Fuckel, *Fusarium moniliforme* and *Fusarium pallidoroseum* (Cooke) Sacc., *Cladosporium oxysporum* Berk & M A Curtis and *Alternaria alternata* (Fr.) Keissler (Figs 1 to 3). *Stachybotrys*, *Acremonium* and *Aureobasidium pullulans* (de Bary) Arnaud were present in the Bangladesh straw samples but not in the Indian straw. Field fungi were not present in the mixed feed and feed ingredient samples from India.

Storage fungi such as species of *Aspergillus*, *Penicillium* and Mucorales were present in all samples from both India and Bangladesh (Figs 1,2 and 3). Predominant storage fungi included: *Wallemia sebi*, species of *Eurotium*, *Aspergillus versicolor*, *Eupenicillium cinnamopurpureum* Scott & Stolk and yeasts. Fungi present at lower levels were: *Emericella nidulans* (Eidam) Vuill. , *Aspergillus fumigatus* Fres., *Aspergillus candidus* Link, *Aspergillus flavus* Link, *Aspergillus niger* v. Tieghem and several species of *Penicillium*. Species common to both Bangladesh and Indian

samples included: *E. cinnamopurpureum*, and *Penicillium citrinum* Thom. *Penicillium islandicum* Sopp and *Penicillium implicatum* Biourge were found in Indian straw and feed samples; *Penicillium raistrickii* G. Sm., *Penicillium purpurogenum* Stoll, *Penicillium glabrum* (Wehner) Westl. *and Penicillium oxalicum* Currie & Thom in the Bangladesh straw samples. Thermophilic and thermotolerant fungi were present in a high proportion of samples of Bangladesh rice straw. These included: *Thermomyces lanuginosus* Tsilinsky, a species of *Thermoascus*, *Emericella nidulans*, *Aspergillus terreus* Thom and *Rhizomucor pusillus* (Lindt) Schipper.

Results for aflatoxin and zearalenone analysis are presented in Table 2. A very high level (3700 µg/kg) of total aflatoxins was found in Indian groundnut cake. Significant aflatoxin levels (>10 µg/kg) were also found in Indian samples of rice chaff, mixed concentrate and biri testa (Table 2). Aflatoxin and zearalenone were not detected in either Indian or Bangladesh rice straw samples (Table 2). Zearalenone was found at levels of more than 300 µg/kg in three Indian samples of: wheat bran and chana testa, mixed concentrate and *Paspalum* straw.

Discussion

Many of the fungi isolated from the rice straw and feed samples are capable of producing mycotoxins; others may be associated with mycoses or allergenic responses and several with spoilage. All species found in this study have been isolated by other workers from mixed feeds and feed components [10-13]. The significance of the actual levels of counts is difficult to ascertain. Fungal counts are influenced by the degree of fungal sporulation and fragmentation of hyphae and may not relate to fungal growth. Unacceptable levels of fungi depend on the "normal" profile of each commodity and the use to which it is to be put [14]. Levels may be designated by particular commercial companies but often there is no standardisation. The presence of one particular species may be more significant than another in relation to spoilage or toxigenic potential. In general, counts of 10^5 per g are regarded as high; for cereal grains, a limit of 10^5 cfu per g has been recommended by the International Commission on Microbiological Specifications for Foods; for other dried foods, the limit for fungi is 10^4 per g [15]. In animal feeds, levels will depend on the treatment and nature of the feed components. In this study, high counts (more than 10^5 per g) were characteristic of all samples of rice straw but other Indian feeds had more variable fungal counts.

In comparison with the Indian feed and straw samples, a wider range of field and storage fungi were found in the straw samples from Bangladesh. Levels of some individual fungi were much higher in rice straw from Bangladesh although overall fungal counts were similar to those of the Indian straw samples. Since sampling was carried out in different years, the significance of this is not clear.

Field fungi such as *Alternaria*, *Cladosporium* and *Fusarium* were present in samples of rice and *Paspalum* straw from India and Bangladesh but not in other feeds and feed components from India. The presence of *Stachybotrys* in 47% of the Bangladesh straw samples may be of some concern . *Stachybotrys* is commonly found in hay and has been associated with chronic and acute toxic effects in horses and other animals including man [20]. The mycotoxin zearalenone was detected in Indian samples of *Paspalum* straw, a mixed feed and wheat bran with chana testa; however, species of *Fusarium* were only detected in the *Paspalum* sample.

Zearalenone has been reported in Indian mixed feeds by other workers [16]. Degnala disease of buffalo in the Punjab region of India and Pakistan has been attributed to *Fusarium equiseti* or *F. pallidoroseum* on rice straw [17], although specific toxins were not identified. In the UK, a batch of mouldy hay was linked to cattle infertility [18] and there are other reports of unspecified toxicity to ruminants in conserved forage crops [1]. Most of the fusaria isolated from the samples were species not normally associated with high zearalenone production. *Fusarium graminearum* Group 1 has been reported to produce high levels of zearalenone in wheat and barley straw [19] but this was not isolated from rice staw in the present study.

One of the main differences between straw samples from India and Bangladesh was the prevalence of thermotolerant and thermophilic fungi in the Bangladesh straw. The presence of fungi such as *Thermomyces* and *Thermoascus* which were found at high levels in the Bangladesh straw samples are indicative of heating just after harvest. This is likely to occur when the rice is stacked for some time in the field as stalk paddy prior to threshing. Under such conditions, these fungi can cause rapid heating of the stalk and grain [21].

Thermomyces lanuginosus and *Thermoascus* are true thermophilic fungi with maximum temperatures for growth of more than 50°C and minima of more than 20°C Toxicity implications of various thermophilic fungi are not clear. Studies on the toxigenic potential of several of these species (*Thermomyces*, *Thermoascus*,

Rhizomucor and *A. fumigatus*) indicated some toxic effects in bioassays [22]. *Thermomyces* is able to produce dicoumarol from o-coumaric acid, metabolites which may be associated with haemmorhaging in cattle [23]. Thermotolerant species isolated in this study included: *Emericella nidulans, Rhizomucor pusillus, Aspergillus versicolor, Aspergilllus fumigatus* and *Aspergillus terreus*. These have maximum growth temperatures of near 50°C and minima of less than 20°C.

Storage fungi were ubiquitous in both the feed and straw samples. Of these a high proportion are xerophilic, associated with growth at low moisture contents (commodiites in equilibrium with relative humidities of less than 85%). These included *Wallemia sebi, Eurotium species, Aspergillus versicolor, Eupenicillium purpurogenum, Penicillium citrinum* and species of the *Aspergillus restrictus* group. It is not clear whether these fungi would have grown during storage but their presence at high levels (more than 10^5 cfu per g) suggests that this is likely. The significance of yeasts which were frequently isolated from a wide range of samples is not known.

Aspergillus versicolor was commonly isolated from both India and Bangladesh samples. *A. versicolor*, which is often associated with both freshly harvested and stored cereals is capable of producing the mycotoxin sterigmatocystin [1]. Of the remaining commonly isolated aspergilli, *Aspergillus terreus, Aspergillus niger, Emericella nidulans* and *Aspergillus flavus* are all capable of producing toxins including citrinin, sterigmatocystin and aflatoxin. Various mycotoxicoses have been attributed to *Aspergillus fumigatus* although there is little evidence for the natural occurrence of its toxins. *Emericella nidulans*, another potential sterigmatocystin producer detected at high levels in Bangladesh straw has also been reported from hay, silage or straw [22].

The mycotoxin aflatoxin was detected at very high levels (3700 µg/kg total aflatoxins) in the sample of Indian groundnut cake with significant aflatoxin levels in three other Indian feeds. Statutory limits for aflatoxin B_1 and total aflatoxins in feeds for dairy cattle average 10 µg/kg and 20 to 50 µg/kg on a world-wide basis; levels for non-dairy animals are higher. India has a total level of 30 µg/kg for aflatoxin B_1 in all foods and a limit of 120 µg/kg in peanutmeal for export as a feed ingredient [25]. Aflatoxin was detected in Indian feed samples particularly groundnut components [16]. Mycological results revealed the presence of *A. flavus* in the groundnut cake and at low levels in a further ten samples. No significant aflatoxin levels were found in either the Bangladesh or Indian rice straw. *A. flavus* has not been commonly reported from

hays or straw although the potential for aflatoxin production has been demonstrated in inoculated hay [1].

Wallemia sebi was detected at high levels in all Bangladesh rice straw samples and in Indian straw samples but at lower levels. Mycotoxigenicity of this fungus is not well documented but it has been reported to produce a toxin (walleminol) of similar toxicity to citrinin and penicillic acid [26]. *Aspergillus fumigatus*, isolated from a high proportion of rice straw samples from India and Bangladesh, is frequently associated with heating of stored hay, particularly if it is stored damp. The highest level of *A. fumigatus* in this study occurred in a sample of Indian rice straw which had been wetted through storage on the farm building roof. Various mycotoxicoses have been attributed to *A fumigatus* although there is little evidence for the natural occurrence of its toxins [1, 27]. Most of the species of *Penicillium* isolated from rice straw samples were characteristic of those found in rice grains [9, 20]; several such as *P. citrinum*, *P. glabrum*, and *P. purpurogenum* are capable of producing mycotoxins [20] but these have not so far been reported as naturally occurring in straw or hay.

Many of the fungi isolated from the feeds and forage samples are capable of causing respiratory infections, mycotic abortions and digestive disorders including *Aspergillus flavus*, *Aspergillus fumigatus* and *Rhizomucor pusillus* [28]. Both *Emericella nidulans* and *Thermomyces lanuginosus*, detected at high levels in the Bangladesh rice straw samples have been reported to cause respiratory diseases in horses and other animals including ruminants; *E. nidulans* has also been associated with invasive aspergillosis in man [29]. Species of *Cladosporium*, *Alternaria*, *Rhizopus*, *Aspergillus* and *Fusarium* can also cause various allergenic reactions [1]. Many of these problems are associated with intake of mould spores by breathing contaminated air during handling or feeding. Additionally, mycotoxicoses can also be induced via this route due to high mycotoxin concentration in spores [30].

Conclusions

In this study, a wide range of fungi were isolated from rice straw collected in India and Bangladesh and feeds from India. Many of these fungi have the potential to produce mycotoxins. Analysis for aflatoxins and zearalenone revealed contamination of indian samples of *Paspalum* straw and some feeds with these mycotoxins. Aflatoxin and zearalenone were not present in Bangladesh straw which was characterised by a high incidence of thermophilic and thermotolerant fungi with undefined potential toxicity

problems. Mycological results indicated that analysis for citrinin and sterigmatocystin might have been appropriate to further define any potential toxicity risks.

The mycoflora results indicated the unbiquitous presence of storage fungi which may be involved with spoilage of feeds and straw. Thermophilic fungi linked to commodity heating were prevalent in Bangladesh rice straw. Field fungi were present in rice straw samples but not in feeds and feed ingredients. Several of the fungi, in addition to potential mycotoxin production may also be of significance in relation to causing allergenic reactions and mycoses.

Acknowledgement

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Figure 1: Incidence of different types of fungi at levels of more than 10^3 cfu per g expressed as percentage of samples in which these fungi occurred in Indian rice straw

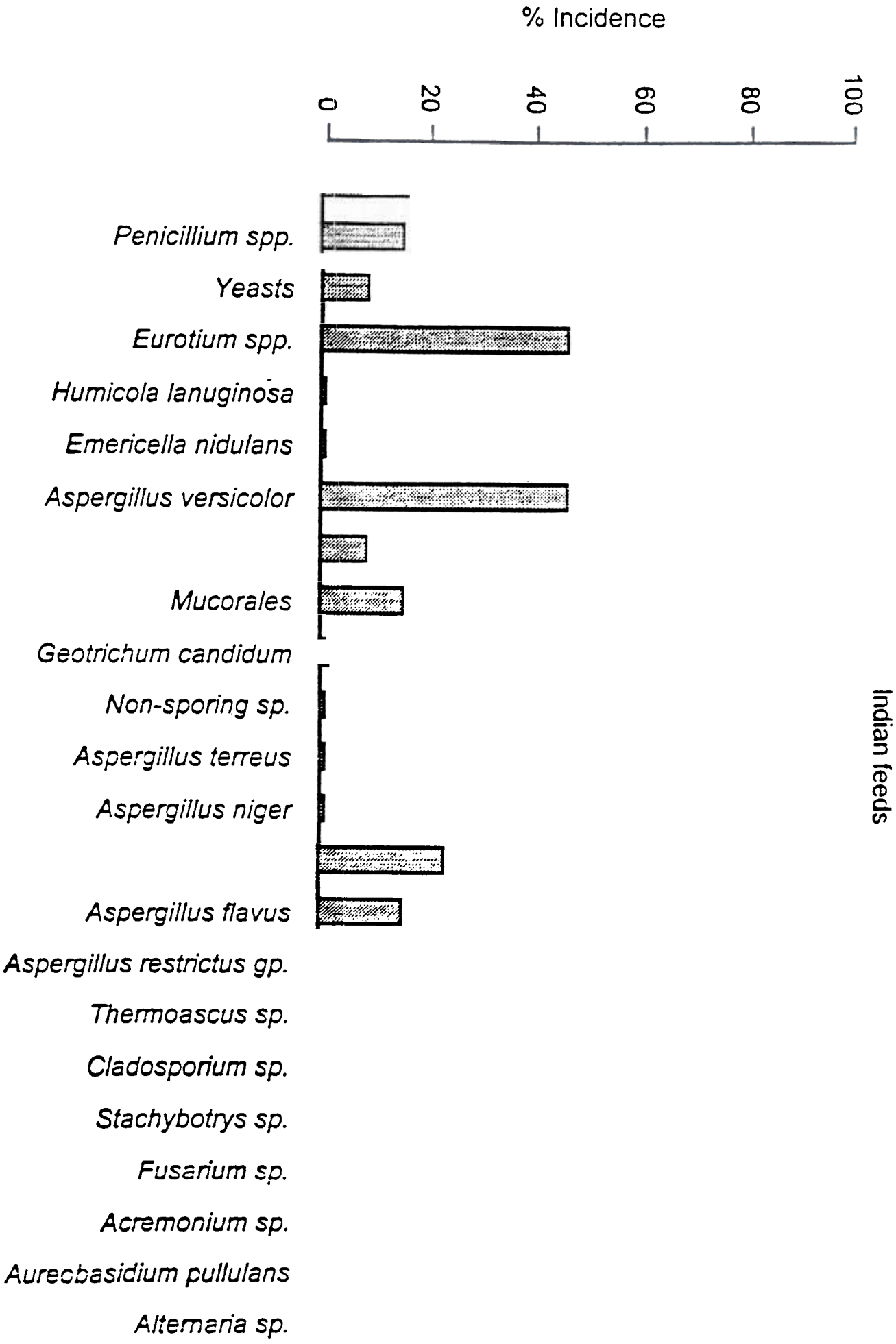
Figure 2: Incidence of different types of fungi at levels of more than 10^3 cfu per g expressed as percentage of samples in which these fungi occurred in Indian feeds and feed components

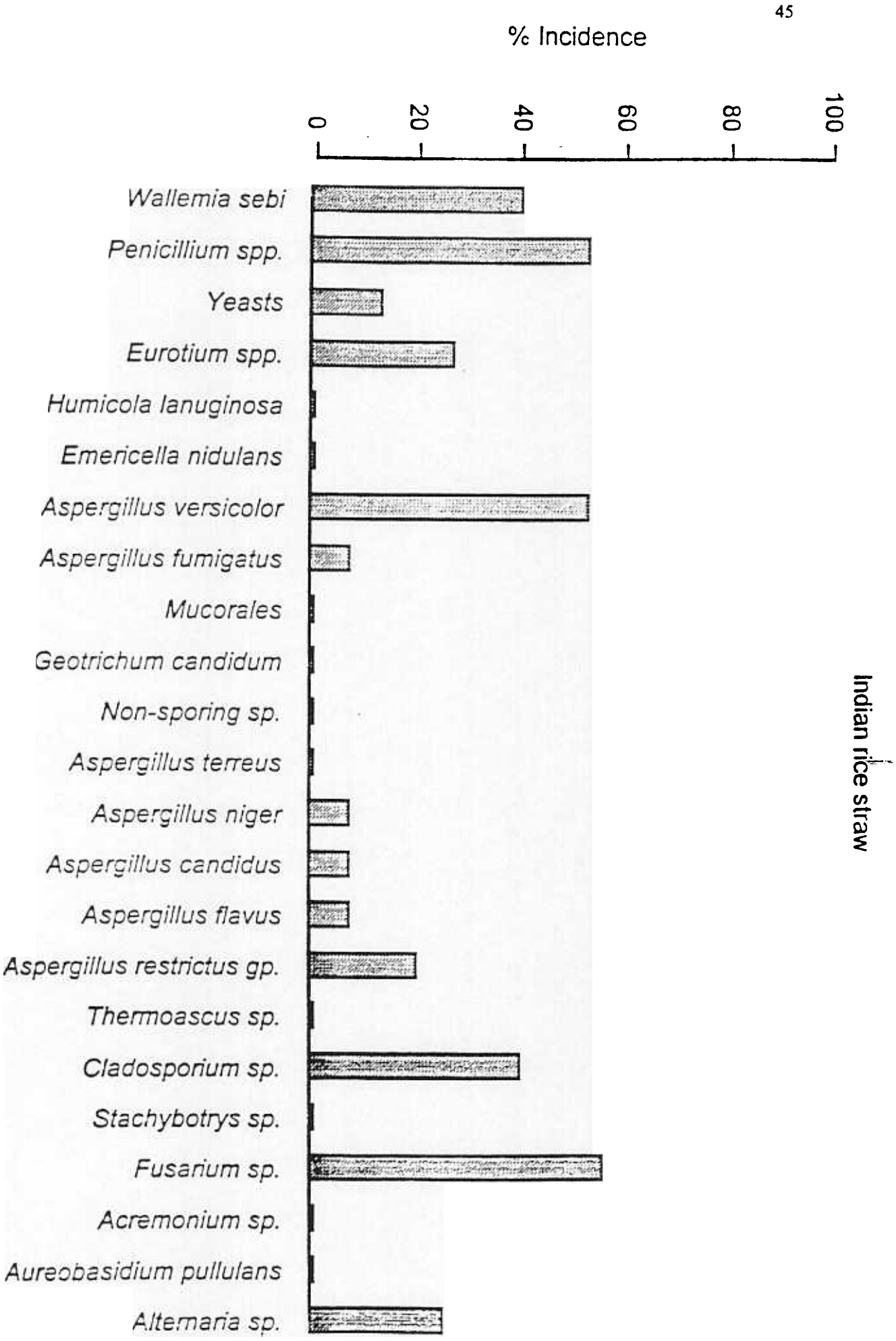
Figure 3: Incidence of different types of fungi at levels of more than 10^3 cfu per g expressed as percentage of samples in which these fungi occurred in Bangladesh rice straw

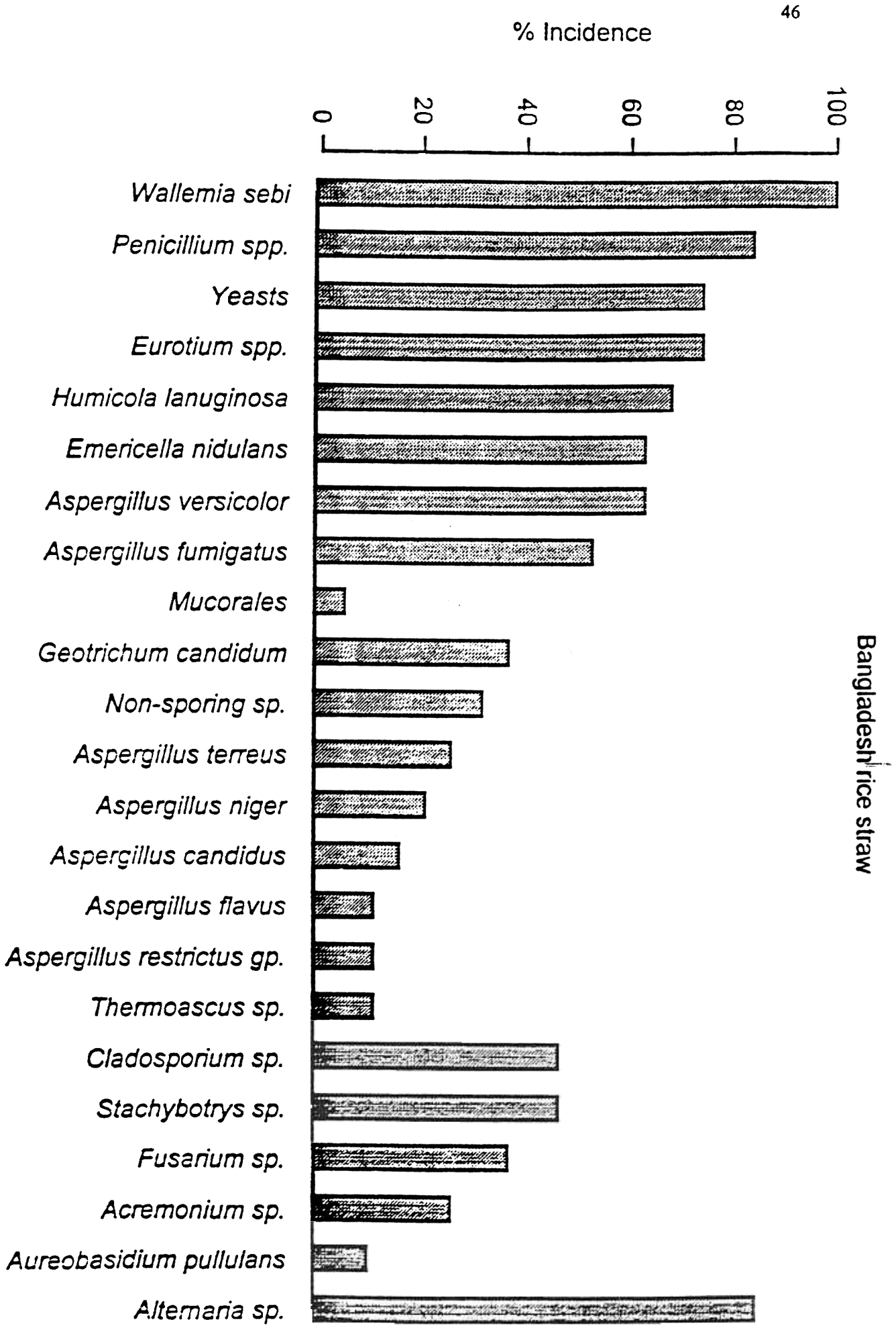
Table 1 **Mean fungal counts of straw and other feed samples from India and Bangladesh**

Samples	Country	Isolation Medium[a]	Number of samples (n)	Mean fungal count (c.f.u. per g $x10^6$)	± Standard Error ($x10^6$)
Straw	Bangladesh	MEA	19	3.40	0.63
		DG18	19	3.70	0.54
		MEA 45	19	1.10	0.45
Straw	India	MEA	17	19.60	7.30
		DG18	17	17.00	5.50
		MEA 45	17	0.06	0.05
Other feeds	India	MEA	13	3.90	2.90
		DG18	13	2.50	2.00
		MEA 45	13	0.02	0.002

[a] MEA: Malt Extract Agar
DG18: Dichloran Glycerol Agar
MEA 45 Malt Extract Agar incubated at 45°C


% Incidence
0
20
40
60
80
100
Penicillium spp.
Yeasts
Eurotium spp.
Humicola lanuginosa
Emericella nidulans
Aspergillus versicolor
Mucorales
Geotrichum candidum
Non-sporing sp.
Aspergillus terreus
Aspergillus niger
Aspergillus flavus
Aspergillus restrictus gp.
Thermoascus sp.
Cladosporium sp.
Stachybotrys sp.
Fusarium sp.
Acremonium sp.
Aureobasidium pullulans
Alternaria sp.
Indian feeds


% Incidence
0
20
40
60
80
100
Wallemia sebi
Penicillium spp.
Yeasts
Eurotium spp.
Humicola lanuginosa
Emericella nidulans
Aspergillus versicolor
Aspergillus fumigatus
Mucorales
Geotrichum candidum
Non-sporing sp.
Aspergillus terreus
Aspergillus niger
Aspergillus candidus
Aspergillus flavus
Aspergillus restrictus gp.
Thermoascus sp.
Cladosporium sp.
Stachybotrys sp.
Fusarium sp.
Acremonium sp.
Aureobasidium pullulans
Alternaria sp.
Indian rice straw


% Incidence
0
20
40
60
80
100
Wallemia sebi
Penicillium spp.
Yeasts
Eurotium spp.
Humicola lanuginosa
Emericella nidulans
Aspergillus versicolor
Aspergillus fumigatus
Mucorales
Geotrichum candidum
Non-sporing sp.
Aspergillus terreus
Aspergillus niger
Aspergillus candidus
Aspergillus flavus
Aspergillus restrictus gp.
Thermoascus sp.
Cladosporium sp.
Stachybotrys sp.
Fusarium sp.
Acremonium sp.
Aureobasidium pullulans
Alternaria sp.
Bangladesh rice straw

APPENDIX 7. CONCEPT NOTE SUBMITTED TO THE LIVESTOCK PRODUCTION PROGRAMME: TOXICITY OF SELECTED FUNGAL ISOLATES FROM RUMINANT FIBROUS FEEDS

Research Proposal for RNRRS Funding

PROJECT TITLE:	The incidence and toxicity of selected fungal isolates from ruminant fibrous feeds
RNRRS PROGRAMME	Livestock Production
RNRRS PROGRAMME PURPOSE:	To improve the performance of livestock in semi-arid crop/livestock and livestock production systems
PRINCIPLE INVESTIGATOR:	P W Wareing
ADDRESS: Maritime,	Natural Resources Institute, Chatham Chatham, Kent.
COLLABORATOR(S)	NDRI, Karnal, Haryana, India Jute Research Institute, Rangpur, Bangladesh
TOTAL COST OF PROJECT:	£79,360
DURATION OF PROJECT:	3 Years
DATE OF SUBMISSION	April 1995
LOCATION OF PROJECT:	NRI
UK Location(s) (if different from address above)	
Overseas Location(s):	India Bangladesh

BACKGROUND:

Fibrous crop residues are important feedstocks for ruminants, particularly during the dry season. Of particular note are maize, sorghum and millet stovers in sub-Saharan Africa, and rice straw in south Asia. Fungal contamination of feeds can lead to feed deterioration and the production of mycotoxins. Both factors can have adverse effects on livestock production; in the former case, lowered nutritive value, in the latter, feed refusal factors and toxic effects on ruminant metabolism.

One observation from the RNRRS project on the effects of storage of fibrous foods on ruminant livestock in developing countries (R5181) was the predominance of *Wallemia sebi* and *Aspergillus versicolor* in rice straw samples from eastern India and Bangladesh. *A versicolor* can produce the metabolites averufin, versicolorin sterigmatocystin. Sterigmatocystin was found from Bangladesh rice straw at moderate levels. Sterigmatocystin is a hepatotoxin and carcinogenic to laboratory animals: the toxicity of sterigmatocystin to ruminants is unknown., W. sebi can produce the secondary metabolite walleminol. The toxicity of walleminol to ruminants is unknown, as is the effect of the presence of *W. sebi* from rice straw on ruminant health. *W sebi* was isolated more frequently and at higher levels from Bangladesh than from India. Bangladesh straw samples were stored for longer periods of time than Indian samples: this may have been the main factor for the different incidence from the two countries.

PROJECT PURPOSE:
The aims of the project are a) to determine the incidence of *A. versicolor* and *W. sebi* in rice straw and other susceptible feeds, and the effects of storage on the incidence of the organisms; b) to determine the relative toxigenic potential of isolates; c) to determine the effects of sterigmatocystin and walleminol on a simulated rumen system. The information obtained would be used to determine the likely risks of sterigmatocystin and walleminol contamination of straws by current storage practices.

RESEARCH ACTIVITIES:
(i) Establish contacts.
(ii) Collection of samples of straw from typical farms.
(iii) Analysis of samples for the incidence of *A. versicolor* and *W. sebi* in straws.
(iv) Examination of the effects of storage on the incidence and level of *A. versicolor* and *W. sebi* from straws, through storage trials or the collection of samples of detailed history.
(v) Determination of the toxigenic potential of *A versicolor* and *W. sebi* isolates from straws.
(vi) Determination of the incidence of sterigmatocystin and walleminol from straw samples.
(vii) Evaluation of the effect of walleminol and sterigmatocystin on rumen liquor bacteria under a model system.

OUTPUTS:
(i) Information on the safety of fibrous feeds with respect to mould and mycotoxin contamination.
(ii) Recommendations to farmers on appropriate storage methods for straws to avoid excesive contamination of straws with fungi and toxins.

CONTRIBUTION OF OUTPUTS:
A better understanding of the contamination of straws with fungi and mycotoxins would contribute to the provision of recommendations for mproved on-farm storage practices. More information on the role and effects of *A. versicolor* and *W. sebi* in the straw storage environment.

BENEFICIARIES:
The main beneficiaries would be small-scale farmers who depend on the efficient ultilisation of limited resources.

RISKS AND ASSUMPTIONS:
(i) Samples containing *A. versicolor* and *W sebi* can be obtained from India and Bangladesh.
(ii) In-country storage trials can be set up.
(iii) Toxigenic isolates of *A. versicolor* and *W. sebi* can be obtained, and toxins isolated efficiently.

FINANCIAL SUMMARY:

ITEMS	Year 1	Year 2	Year 3	Total
Staff	28,960	14,480	14,480	57,920
Travel				
Airfare	4,500			4,500
Subsistence	7,200			7,200
Consumables				
Cultures, standards, chemicals	1,000	1,000		2,000
Capital Equipment	nil	nil	nil	nil
Training/Publications			500	500
Overheads				
Contingency				7,240
TOTALS				79,360