HGCA

PROJECT REPORT No. 26

STANDARD METHODS FOR THE ENUMERATION OF FUNGI IN CEREALS AND CEREAL PRODUCTS

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Standard Methods For The Enumeration Of Fungi In Cereals And Cereal Products

By

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Final report on a six month project commencing 1st Feburary 1990.
HGCA Project No. 0025/2/89

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HGCA Project no. 0025/2/89

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ABSTRACT

Three separate investigations involving the methodology of counting yeasts and moulds in cereals and cereal products are covered in this report. These are summarised separately below.

Counts in flour: 24 laboratories in different parts of the world were each sent subsamples from two samples of flour and asked to carry out counts of moulds and yeasts using the method normally adopted. The need for standardisation was emphasised by the wide diversity of media and methods used. Despite this, mould counts in the samples were reasonably uniform. Yeast counts varied widely and it is clear that some workers have difficulty in separating these organisms and that some media are better than others for this purpose. Problems with bacterial contamination occurred with some of the selective media used. Samples of domestic flours tested at the same time generally contained low counts of fungi (i.e. <1000 per g).

Effects of presoaking ad mixing: In a collaborative exercise involving 17 laboratories from around the world, six samples of cereal and cereal product were examined for counts of fungi after soaking in saline/peptone diluent for 0, 30 and 60 min and after mixing for 2 min and again after 10 min at each soaking time. Despite using the same method and materials, both the within and between laboratory variability with some samples was unacceptably high. Mixing for 10 min or soaking for 30 min saw a clear improvement in recovery over mixing for 2 min without soaking in most laboratories. Of these treatments the latter is preferred on grounds of convenience. Soaking for 60 min gave no additional benefit. Agreement between laboratories on the composition of the mould flora in the samples was remakably good.

Monitoring mycological media: Evidence is presented to indicate that tests to monitor the performance of each batch of mycological medium is not just desirable but is necessary if accurate estimations of the numbers and types of fungi in cereals and cereal products are to be obtained. Not only is there a tendancy for variation in performance of batches of medium from the same suppliers but also results can vary with the same medium from different suppliers. The effects of this variability are particularly pronounced with selective mycological media. A method whereby plates of medium are stab inoculated with a range of test moulds and yeasts and the colony diameter measured with incubation time is suggested for monitoring purposes.

OBJECTIVE

To reorganise and participate in collaborative studies aimed at finding the best media and methods to use for enumerating moulds and yeasts in cereals and cereal products.

BACKGROUND

The three papers included in this report were presented at an International Workshop on 'Standarised Methods for the Mycological Examination of Foods' which was held in Baarn, Netherlands, on 20-24th August 1990. The aim of this meeting was to study the data generated prior to and subsequent to the first workshop which was held in Boston, U.S.A., in 1984 and produce suggestions regarding standardised procedures.

The work required to organise and participate in the collaborative tests to determine the effects of presoaking and mixing on the recovery of fungi from food (Study 2) and some of the work described in the paper on monitoring mycological media (Study 3) was funded by the Home Grown Cereals Authority. The collaborative study on counts of moulds and yeasts in flours (Study 1) was carried some years ago but is included in this report largely to show the diversity of media and methods which can be used to determine numbers of fungi in cereals and cereal products.

STUDY 1

REPORT ON A COLLABORATIVE EXERCISE TO COMPARE COUNTS OF MOULDS AND YEASTS IN SAMPLES OF FLOUR

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INTRODUCTION

At the workshop on methods for the mycological examination of food held in Boston in July 1984, a number of papers were presented which showed widely different levels of count for fungi in flour. There was doubt whether the results were a true reflection of the range of count which can be expected in this commodity or whether the variations were due, at least in part, to the different methods of enumeration employed.

In an attempt to clarify the situation, a collaborative exercise was organised involving the majority of participants at the workshop. Each laboratory was sent samples of the same two flours and asked to cary out counts of moulds and yeasts in duplicate subsamples using the method normally adopted. In addition, the participants were also asked to test a sample fo domestic white flour obtained from the larder or local shop. Twenty four laboratories took part in the exercise.

METHODOLOGY

Details of the media and methods used by the differ
ent laboratories are given in Table 1.

More laboratories used the spread plate method (13) than the pour plate method (9) two laboratories compared both methods. The 0.1ml. spread plate was the most popular but a few American laboratories distributed 1ml. of the 10^{-1} dilution of sample over the surface of two or three plates. The reason for this procedure is not clear.

Eight different diluents were used of which 0.1% peptone and saline/peptone were most common. The ratio of sample to diluent varied greatly, no less than eleven different ratios being used.

The vast majority of laboratories (18) used the Stomacher for preparing the primary dilution. Mixing times varied from 0.5 to 3.0 min, with 2 min being used most often. Two laboratories used a Waring blender, three shook by hand and one used a mechanical mixer for 30 min. Only one laboratory reported soaking the sample in diluent prior to mixing.

No less than 18 different media were employed of which DRBC and OGY were most popular. Six laboratories compared counts on two or more media.

In general, plates were counted after 5 days at 24-27°C. (18) but two laboratories counted after four days.

RESULTS AND DISCUSSION

Counts in reference samples

The average counts obtained by the different laboratories for moulds and yeasts in the duplicate subsamples of the two reference flour are given in Table 2. Results from comparison of different media and methods are included.

With sample A the mean mould count for all laboratories was 1130 per g with a standard deviation of 520 and coefficient of variation of 46%. Sample B had a higher mean count of 2130 per g with a standard deviation of 1070 and coefficient of variation of 50%. While a variability of 46-50% is not particularly satisfactory, it is probably no more than to be expected when so many different media and methods were employed.

The low mould counts obtained by laboratories 4 and 15 may possibly be explained by the fact that the plates were examined after only 4 days incubation. Comparisons made by laboratories 17, 21 and 24 and the results from laboratory 15 suggest that reduced mould counts will be obtained on acidified media using the pour plate method.

Yeast counts for the same flour sample varied considerably. Some laboratories found hundreds and even thousands per g to be present whereas others detected very few if any. It is clear that many workers have difficulty in distinguishing these organisms. The counts with DRBC generally tend to be rather higher than with other media. Three laboratories commented that bacteria were present on the plates. Confusion between yeasts and bacteria may explain some of the higher counts obtained with this medium. Contamination with bacteria 11 and 16 who used media without acidification or antibiotic supplement. Yeasts counts were generally higher using a spread plate than a pour plate method.

Counts in domestic flours

The counts of moulds and yeasts in the samples of domestic flour tested by the different laboratories are listed in Table 3. Almost without exception the laboratories obtained lower counts in the domestic flour than in the reference flours. The average mould count for all samples examined was 520 per g. In general, counts were higher in samples examined by European than by American or Australian laboratories (average 850 and 410 moulds per g. respectively). Yeast counts were very variable but mostly they were low at under 100 per g.

Other observations
Counts on duplicate samples of the same flour and on duplicate plates at each dilution were similar at the majority of laboratories. Calculated counts from plates inoculated with consecutive tenfold dilutions of sample were similar in 8 of the 20 laboratories who provided this information. With the other laboratories the calculated counts from plates inoculated with the higher dilution of sample were 30-210% higher than the calculated counts from plates inoculated with the next lower dilution of sample. This dilution error tended to be more pronounced with laboratories using the pour plate method.

CONCLUSIONS

This exercise serves to stress the diversity of media and methods used for enumerating fungi in food. Bearing in mind the differences in methodology, the mould counts in the reference samples are reasonably uniform. However, yeast counts varied widely and this is a matter for further investigation.

There is evidence to indicate that the spread plate method is preferable to the pour plate method in that the former appears to give better recovery of yeasts and dilution errors are reduced. Using acidified media with the pour plate method and reading plates after a short incubation period also gave reduced counts. The results suggest that problems of bacterial contamination occur with media containing no acid or antibiotic and also, on occasion, with DRBC.

The low counts of moulds and yeasts quoted for flour by most participants at the workshop in Boston are fully justified on the basis of the results from the domestic flours tested by the different laboratories. Based on the results from this exercise, it is unlikely that differences in methodology can account for the large variations in level of count reported. The discrepancies can probably be explained by differences in the climatic conditions for growing and harvesting the wheat from which the flour was derived and also differences in the post harvest storage time and conditions prior to testing the sample. Flours from freshly harvested wheat contain relatively high numbers of field fungi and yeasts. Many of these do not survive for long under the dry conditions exisiting in most flours.

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Table 1 Media and Methods used by different laboratories

Lab.	Method	Diluent	Primary dilution	Mixing	Medium	Incubation
1 EUR	spread	saline pep	40/310	St. 2.0	OGY	5/27
2 EUR	spread	pep Ringers	10/90	St. 2.0	RBC,DRBC,DG18	5/25
3 EUR	spread	pep Ringers	1/10	St. 1.0	OMEA	5/25
4 EUR	pour	Ringers	10/90	St. 1.0	RBC	4/25
5 EUR	pour	saline pep	20/180	shake 30	DG18,MSSA,Malt	5-7/25
6 EUR	spread	saline pep	40/360	St. 2.0	DRBC	5/24
7 EUR	pour	saline pep	20/180	St. 0.5	OGY	5/24
8 EUR	pour	saline pep	20/180	St. 1.0	OGY	5/25
9 EUR	spread	buffered pep	40/360	soak St. 2.0	OGGA	5/24
10 EUR	spread	saline pep	8/72	St. 2.0	DRBC	5/25
11 EUR	spread	0.05% Tween	Various	St. 3.0	MEA	5/25
12 EUR	spread	pep	10/90	shake?	OGY,DRBC,GYC,GYA	7/RT
13 Aus	spread	pep	11/99	St. 2.0	DRBC	5/25
14 Aus	spread	pep	20/180	St. 0.5	DRBC	5/25
15 USA	pour	pep	20/180	St. 1.0	PDA 3.5	4/25
16 USA	pour	dist. water	11/99	shake 2.0	PDA	5/27
17 USA	pour	pep	50/450	shake?	PDACC;PD	5/25
18 USA	pour	buffer	25/225	blender 2.0	SMACC	6/25
19 USA	spread	pep	50/450	St. 2.0	ORBC	5/25
20 USA	pour	buffer	25/225	blender 2.0	PDAC	5/25
21 USA	pour & spread	buffer	22/198	St. 0.5	Myc. 4.0;OGY	5/25
22 USA	pour & spread	pep	30/270	St. 2.0	PDACC	5/25
23 USA	spread	buffer	11/99	St. 3.0	DRBC	5/245
24 USA	spread	buffer Tween	40/360	St. 2.0	OGY;RBC;DRBC PDA 3.5 PCACC	5/25

Key
pep = usually 0.1; peptose, buffer = usually Butterfields, St. = Stomacher, RT = room temperature
Key to lesser known media
MSSA = malt, salt, sucrose agar, Malt = 2% malt agar, OGGA = oxytetracycline gentamicin glucose yeast extract agar, GYC = glucose yeast extract chloramphenical agar, GYA = acidified glucose yeast extract agar, PDACC = PDA + chlortetracycline + chloramphenical, PDAC = PDA + chortetracycline, SMACC = Standard methods agar (BBL) & chlortet. & chloram, Myc = mycological agar (Difco) pH. 4.0

Table 2 Counts in reference flours (organisms per g)

Lab	Method	Medium	Sample A		Sample B	
			mould	yeast	mould	yeast
1	spread	OGY	860	240	2600	500
2	spread	RBC	6700	100	3000	400
	"	DRBC	1000	<50	1100	50
	"	DG18	1800	<50	3000	230
3	spread	OMEA	1500	25	2800	100
4	pour	RBC	210	320	320	180
5	pour	DG18	370	<20	1700	20
	"	MSSA	360	<20	2300	20
	"	Malt	430	<20	2500	20
6	spread	DRBC	1500	280	1700	800
7	pour	OGY	580	30	2200	60
8	pour	OGY	1700	20	4900	35
9	spread	OGGA	1300	250	1500	700
10	spread	DRBC	1200	230	2900	50
11	spread	MEA	6200	94,000	5000	240,000
12	spread	OGY	1200	100	1200	100
	"	DRBC	700	100	750	100
	"	GYC	100	100	4600	100
	"	GYA	1400	100	750	100
13	spread	DRBC	1400	280	2400	980
14	spread	DRBC	2300	480	3400	300
15	pour	PDA 3.5	470	190	590	500
16	pour	PDA	1900	1200	3300	2300
17	pour	PADCC	690	<10	1100	10
	"	PDA 3.5	450	<10	320	10
18	pour	SMACC	1800	<100	3200	650
19	spread	DRBC	1500	130	2200	500
20	pour	PDAC	1700	100	4400	100
21	spread	Myc 4.0	1300	120	2500	90
	pour	Myc 4.0	750	10	1400	10
	spread	OGY	1300	180	2400	150
	pour	OGY	950	10	1500	10
22	spread	PDACC	1100	200	1400	360
	pour	PDACC	960	30	1100	70
23	spread	DRBC	480	70	580	85
24	spread	OGY	1300	400	2800	430
	"	RBC	1400	880	2200	250
	"	DRBC	1200	1400	1700	2400
	"	PDA 3.5	1300	130	1000	230
	"	PDACC	1900	350	2500	430

Table 3 Counts in domestic flours (organisms/g)

Laboratory	Sample	Mould	Yeast
1	A	110	100
	B	500	100
	C	300	<100
	D	800	400
	E	1400	<100
	F	1000	800
	G	600	100
	H	900	100
	I	800	100
	J	4000	200
	K	800	<100
	L	500	<100
	M	1900	200
2		730	250
4		320	30
5		530	<20
6		320	<100
7	A	300	110
	B	400	120
8		1000	10
9	A	1000	400
	B	700	600
10		680	50
12		300	<100
13	A	150	<100
	B	200	100
14		650	350
15	A	660	480
	B	410	280
16	A	210	30
	B	180	10
	C	220	20
17		1000	<10
18		150	<100
19		950	150
20	A	200	10
	B	150	10
21	A	240	20
	B	370	30
	C	120	10
	D	150	10
	E	300	140
	F	220	90
22	A	760	50
	B	730	80
23	A	700	20
	B	700	70

STUDY 2

REPORT ON A COLLABORATIVE EXERCISE TO DETERMINE THE EFFECT OF PRESOAKING AND MIXING TIME ON THE RECOVERY OF FUNGI FROM FOODS

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INTRODUCTION

The first SMMEF workshop held in Boston in 1984 results were presented which indicated that soaking cereals, cereal products and cereal based foods in diluent for 30 or 60 min. prior to mixing usually caused significant increases in the counts of fungi over that when the samples were not soaked prior to mixing (Seiler 1986). Some evidence was also presented to show that counts increased with mixing time using the Stomacher.

These results caused considerable discussion at the workshop and it was agreed that further information was required on this aspect of the counting procedure. Accordingly, a collaborative exercise was organised in Spring 1990 involving 17 laboratories (4 each from the UK and USA; 2 each from Australia, Germany and the Netherlands; and one each from Denmark, Hungary and Turkey). Six cereal samples were selected which varied in consistency and fungal population and 250g quantities sent to each laboratory together with enough of the same batch of DG18 basal medium and antibiotic supplement to complete the tests.

METHODOLOGY

Outline of test

With each sample 6 primary dilutions of 40g in 360ml of saline peptone diluent were prepared and duplicates soaked for 0, 30 and 60 min. After each soaking time the pairs ofsubsamples were mixed using the Stomacher 400 for 2 min and again after a further 8 min (10 min altogether). The plan of test is shown in Table 1. Mould and yeast counts were determined using a 0.1ml spread plate technique with DG18 medium. Specific instructions were given on the method of preparing dilutions, drying and spreading plates, and preparing the medium. The laboratories were asked to record separately the numbers of mould, yeast and total colonies on the duplicate plates at all countable dilutions after 7 days incubation at 25^{o}C but to note any increase in count from 5 to 7 days. In addition, some of the laboratories agreed to make a broad identification of the moulds present on the countable plates from the subsamples which had been soaked for 60 min and mixed for 10 min.

Samples

The samples selected for test and the mean total counts of moulds and yeasts obtained by all laboratories are given in Table 2. Unfortunately, only four of the samples (A, C, D and F) proved to have sufficiently high counts to make meaningful comparisons between treatments. It is particularly disappointing that the flour sample (E) gave such a low count since one of the aims of the exercise was to compare the effect of

presoaking and mixing in homogenous and non-homogenous materials.

RESULTS and DISCUSSION

Bacterial contamination

Only one of the 17 laboratories reported the presence of bacteria on the plates from one of the samples (F). Perhaps these workers mistook bacteria for yeasts or failed to add antibiotic to that batch of medium. Taken as a whole the results indicate that the combined effects of low water activity and chloramphenicol in the DG18 medium are adequate to prevent bacterial growth in 7 days at 25°C.

Incubation time

Most laboratories noted an increase in count from 5 to 7 days incubation. Usually the increase was small and rarely more than 10%. With samples A and C which contained large numbers of *Wallemia sebi* a number of laboratories commented that counts may have increased because of secondary contamination from the first colonies to appear. This and the small increase in count suggests that an incubation time of 5 days at 25°C is to be preferred.

Mould counts: within laboratory variability

The coefficient of variation in mould count between subsamples for all soaking and mixing treatments, but eliminating the effect of treatment, are shown for each laboratory for samples A.C.D and F in Table 3. With each sample, laboratories showing a high coefficient of variation of over 50% are underlined. Missing figures indicate that the laboratory concerned either did not test the sample or only tested a single subsample per treatment.

The within laboratory variability is higher with sample A than with the other samples, 6 laboratories showing coefficients of variation of over 50%. This result may suggest that the mould contamination is less evenly distributed in the wheat sample than in the other cereal samples. The variability in laboratories 3, 11 and 14 with most samples is such that their counts should be viewed with caution.

Mould counts: between laboratory variability

The mean $\log_{10}$ count of moulds obtained for all treatments with samples A, C, D and F are shown in Table 4 for laboratories where sufficient data was available.

The variation in count between laboratories is much less with samples C and F than with samples A and D. The reason for this is not clear but may be linked with type of fungi present and ease of counting colonies on the plates.

The large differences in the level of count obtained by laboratories for the same sample tested under the controlled conditions of this exercise is a matter of some concern in relation to microbiological standards. If, for example, the $\log_{10}$ mould count per g. specification for rejection of a sample is 4.50, sample D would be failed by half the laboratories but passed by the other half. Such a situation could give rise to some difficulty in the case of a dispute involving international trade.

Yeast counts: between and within laboratory variability

Significant numbers of yeasts were only encountered in samples D and F. The coefficient of variation in yeast count between subsamples and the

mean $\log_{10}$ count for all treatments found by the different laboratories for these samples are given in Table 5.

The within laboratory variability is greater than that encountered with moulds (Table 2) with more than half the laboratories showing coefficients of variation greater than 50%. Large differences also exist betweeen the mean yeast counts obtained by the laboratories. The results suggest that many laboratories have difficulty in identifying and counting these organisms.

Dilution Error

It is usually found that the mould count calculated from the average number of colonies per plate at the highest countable dilution is higher than the count calculated from plates at the next lower tenfold dilution, i.e. there is a dilution error. Table 6 shows the number of times that the count at the higher dilution was more than that at the next lower dilution for the various laboratories and samples examined in this collaborative exercise.

It is apparent that the extent of the dilution error varies considerably between samples and laboratories. With samples D and F the majority of laboratories obtained counts at the higher dilution, which were only 50% or less greater than those from the next lower dilution. With samples A and C, on the other hand, the majority of counts were more than twice as great at the higher dilution than at the lower dilution. i.e. a reading of 2.0 or more. With the latter samples the larger errors are probably due to inaccuracies in counting the large numbers of colonies present on plates at the lower dilution. Some laboratories encountered higher dilution errors than others. This was particularly evident with laboratory 13 whose counts were similar at the two dilutions. Taken over all samples, 8 of the 15 laboratories who counted at two dilutions showed a high mean dilution error of over 2.0.

Calculating counts in samples

In view of the errors described above it is not desirable to average counts at two dilutions when calculating the count per g of sample. Certainly, the use of the Farmiloe equation (Farmiloe et al 1954), where the greatest weight is placed on the countable plates with the largest number of colonies, cannot be justified. It can be argued that it is best to count plates at the highest countable dilution even if the plates contain 10 or less colonies. However, it must be considered dubious practise to rely on counts obtained from such low numbers per plate unless a number of replicate subsamples are compared. A better asessment may be achieved by counting plates containing 10-50 colonies, but it will be difficult to obtain such counts on all occasions using a tenfold dilution series. Perhaps there is a case for using a 5 fold dilution series where accurate mould counts are required.

Effects of presoaking ad mixing

The results with samples A.C.D and F have each been analysed statistically. Comparisons of the difference in mould count between a number of the soaking ad mixing treatments are given in histogramic form for each laboratory in figure 1.

When the mixing time was increased from 2 min to 10 min without soaking the majority of laboratories (79%) obtained an increase in count. With the exception of sample C, the within laboratory variability was such that most of the increases were not statistically significant. Nevertheless

the results present good evidence to indicate that the aditional mixing will improve recovery. When the counts after for 10 min without soaking are compared with those when the samples were soaked for 30 min and then mixed for 2 min, only half the laboratories noted an increase and only in four occasions was the difference significant. Most laboratories (67%) found an increase in mould count when the mixing time was increased from 2

to 10 min after soaking for 30 min. However, the differences in count were small and only statistically significant in five cases. By increasing the soaking time from 30 to 60 min followed by mixing for 2 min less than half the laboratories obtained an increase in count and only on three occasions was the increase significant. An important general finding was that the variability in count between laboratories was reduced when the samples were soaked or when mixed for 10min.

The results indicate that equivalent mould counts can be expected either by mixing the sample for 10min or by soaking for 30min and mixing for 2 min. There is some evidence to suggest that increasing the mixing time to 10 min after soaking will increase recovery, but the effect is small. No additional increase in count occurred by soaking for 60min rather than 30 min. On grounds of convenience, a soaking time of 30 min followed by mixing using the Stomacher for 2 min is recommended for mould counts in cereals and other foods where the fungal contamination is likely to be deep seated.

Identification

Ten laboratories took part in tests to identify the moulds present in the samples. Identifications were only carried out on the four countable plates resulting from the duplicate sub-samples which had been soaked in diluent for 60 min and then mixed with the Stomacher for 10 min. Because of the very low counts obtained, the results with samples B and E have been omitted from the analysis. The mean percentage incidence of the various mould genera and species obtained by the ten laboratories for the other four samples is shown in Table 7.

It is interesting to observe that the mould flora in the four samples varies considerably. With samples, A, C and D (wheat, oats and barley) the population is dominated by xerophiles. *Eurotium* species are the most common moulds present in sample C whereas *Wallemia sebi* predominates in sample D. Both of these fungi are found in sample A but a range of other

organisms are also present. The flora of sample F (mixed animal feed) is quite different with *Aspergillus candidus* and other Aspergilli making up the majority of the population and only small numbers of *Eurotium* and *Wallemia* were detected.

To a bakery microbiologist these findings are of interest since *Eurotium* and *Wallemia* are the principle causes of spoilage of low a_w products such as cakes. If the high counts of these organisms in the cereal are transferred through to the flour (which is likely) the mere handling of such a material in the bakery would increase atmospheric counts to an extent that the shelf life of products is likely to be reduced. Fortunately, experience with counts in flour (Seiler 1990) suggest that the miller is unlikely to use such high count cereals.

Agreement between laboratories on the composition of the mould flora in the different samples was remarkably good. If we take as an example the results from sample F (Table 8) it is apparent that only laboratories 2, 5

and 7 differ substantially from the others. These laboratories appear to have difficulty in separating Aspergilli from Pernicillia and in identifying *Aureobasidium and Aspergillus candidus*. Taken overall, the results are encouraging and indicate that most laboratories make a good job of separating the different moulds on DG18 medium.

CONCLUSIONS

1. Despite using the same sample material, the same batch of culture medium and antibiotic supplement and the same strictly specified method of test, there is a worrying variability between subsamples which is more pronounced with some samples and some laboratories than with others. Moreover, the large variation in both mould and yeast count obtained by different laboratories for the same sample is a matter of some concern.

2. There is good evidence to indicate that, without presoaking, increasing the mixing time from 2 to 10 min causes an increase in recovery of moulds and yeasts in the cereal samples examined. The extra mixing time tends to reduce the variation in count between laboratories.

3. The level of count and variability between laboratories after presoaking for 30 min and mixing for 2 min is similar to that obtained by mixing for 10 min without soaking and is preferred on grounds of convenience. There appears to be little additional benefit in soaking for 60 min.

4. Serious dilution errors are encountered which are larger with some samples than others and vary with laboratory. The best method of calculating the count needs further consideration.

5. With few exceptions there was remarkably good agreement between laboratories on the composition of the mould flora in the different samples.

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Table 1: Plan of test

	Sample											
Soaking time (min)	0				30				60			
Subsample (40g in 360ml diluent)	A		B		A		B		A		B	
Mixing time (min)	2	10	2	10	2	10	2	10	2	10	2	10

= 12 counts per sample

= 72 counts per 6 samples

Table 2: Samples used in the collaborative exercise

Code	Description of sample	Mean count per g
A	Winter wheat (Slejpner)	3.1×10^5
B	Winter wheat (Avalon)	2.1×10^2
C	Spring Oats	4.6×10^5
D	Spring barley (Corniche)	5.2×10^4
E	Bread making flour	2.9×10^2
F	Mixed animal feed	3.8×10^4

Table 3: Moulds: Within laboratory variability (Coefficients of variation)

Laboratory Code	Sample			
	A	C	D	F
1	23.62	21.71	35.33	30.50
2	68.73	19.15	39.93	14.26
3	84.33	67.46	-	-
4	-	-	-	-
5	19.62	8.89	13.74	25.97
6	16.68	26.75	5.09	52.03
7	54.81	18.11	13.89	24.22
8	20.56	13.66	17.35	27.35
9	19.15	9.95	30.29	33.11
10	36.30	8.42	19.07	7.72
11	-	15.29	61.66	52.06
12	86.51	24.54	-	-
13	51.74	-	16.55	17.60
14	104.13	35.08	60.84	68.07
15	-	-	-	41.45
16	8.52	20.48	30.87	15.40
17	-	-	-	12.77

Table 4: Moulds: Between Laboratory Variability (Average $\log_{10}$ counts/g)

Laboratory Code	Sample			
	A	C	D	F
1	5.18	5.67	4.17	4.41
2	4.74	5.65	3.72	4.36
3	5.72	5.54	-	-
4	-	-	-	-
5	5.53	5.78	4.95	4.65
6	5.28	5.58	4.76	4.59
7	5.53	5.77	5.00	4.61
8	5.87	5.60	4.98	4.36
9	5.05	5.69	4.13	4.53
10	4.98	5.61	4.65	4.42
11	-	5.59	4.49	4.43
12	5.91	5.68	-	-
13	5.48	-	4.20	4.37
14	4.86	5.50	3.67	4.26
15	-	-	-	4.44
16	5.38	5.71	4.77	4.57
17	-	-	-	4.45

Table 5: Yeasts: Within and between laboratory variability

Laboratory Code	Within laboratory variability (coefficient of variation %)		Between laboratory variability (Ave. $\log_{10}$ count/g)	
	Sample D	Sample F	Sample D	Sample F
1	111.2	63.4	3.81	3.33
2	91.2	52.4	3.70	3.80
3	-	-	-	-
4	-	-	-	-
5	105.5	72.0	3.89	3.98
6	87.8	39.7	3.87	3.67
7	30.5	77.9	3.82	3.64
8	-	143.2	-	3.87
9	27.9	-	3.56	-
10	63.8	-	3.32	-
11	17.2	97.6	3.76	4.07
12	124.4	36.8	3.58	3.59
13	-	91.9	-	3.34
14	50.7	36.5	4.51	3.59
15	-	31.0	-	3.96
16	-	31.5	-	3.83
17	-	26.1	-	4.03

Table 6: Errors with mould counts at two countable dilutions

Laboratory Code	Dilution error* Sample				
	A	C	D	F	Mean
1	3.5	2.0	1.4	1.3	2.1
2	1.4		2.6	0.8	1.6
3	5.6			2.0	3.8
4	1.3	1.7	1.3	1.1	1.4
5	1.8		1.0	1.6	1.5
6					
7	2.8	2.4	1.1	1.4	1.9
8	3.9	2.4	1.7	1.6	2.4
9	2.4		0.9	0.8	1.4
10	1.9		1.1	1.1	1.4
11		3.1	1.8	1.2	2.0
12					
13	8.0		8.5	10.1	8.9
14	1.7	2.4	5.3	0.9	2.6
15	2.0	3.8	1.1	1.5	2.1
16	2.9	2.4		1.2	2.2
17				1.1	1.1

* Ratio of the average mould count at the highest countable dilution over the average mould count at the next lower tinfold dilution. A large figure represents poor reproducibility between counts at the two dilutions.

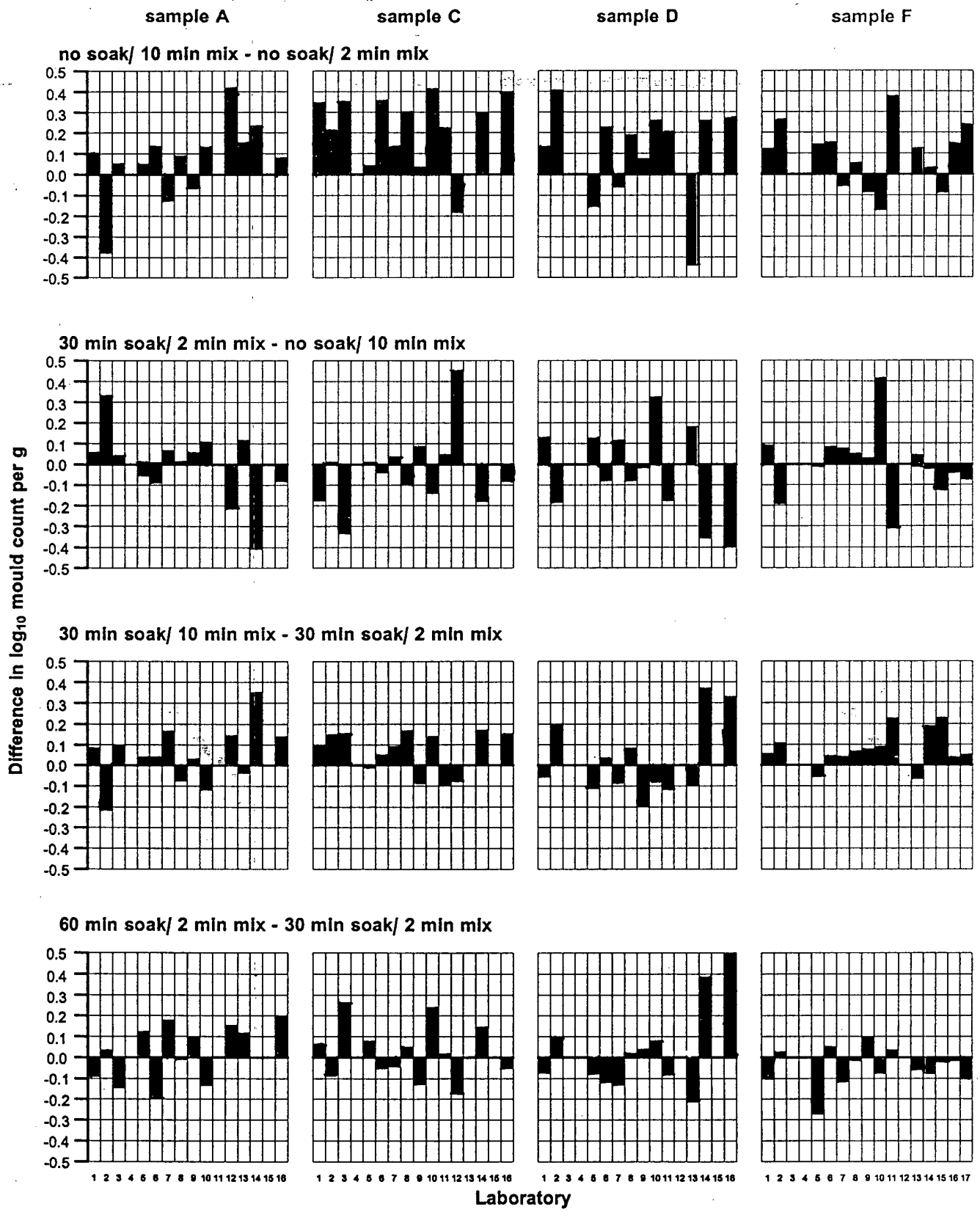
Table 7: Mean incidence (all laboratories) of moulds in four samples samples of cereal or cereal products

Mould Species	% incidence in sample			
	A (winter wheat)	C (spring oats)	D (spring barley)	F (mixed animal feed)
Alternaria	0.2	0.2	0	0
Aureobasidium	0.3	0.7	4.1	7.3
A. candidus	2.3	0.2	0.4	52.1
A. versicolor	1.4	1.7	1.3	3.4
A. restrictus	2.8	0	2.5	0.1
Aspergillus sp.	8.0	10.4	2.0	6.9
Cladosporium	0.8	1.2	0.6	1.7
Eurotium	39.8	71.0	10.9	5.1
Mucoraceae	0	2.4	0.3	1.8
Penicillium	8.3	7.9	6.6	11.9
Verticillium	1.4	0.2	0.7	3.1
Wallemia	20.7	0.2	57.4	1.7
Others	14.0	3.8	13.3	4.9

Table 8: Identification of moulds in sample F (mixed animal feed) by ten collaborating laboratories

Mould Species	% incidence found by laboratory number									
	1	2	3	5	7	8	9	11	12	16
Aureobasidium	3	18	10	33			3	5		1
A. candidus	77	22	64			56	91	69	57	85
A. versicolor		7		22				1	2	2
A. restrictus									2	
Aspergillus sp.		4		37	27				1	
Cladosporium		4		1	1	9			2	
Eurotium	7	4	9	3	1	4	3	8	8	4
Mucoraceae		11			2	1	2		1	1
Penicillium	13	30	7	3	33	11		11	6	5
Verticillum			10		14	7				
Wallemia				1	7			6	2	1
Others					15	12	1		20	1

Fig. 1 Comparison of various presoaking and mixing treatments



STUDY 3

MONITORING MYCOLOGICAL MEDIA

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INTRODUCTION

At the present time it seems certain that few laboratories routinely carry out tests to monitor the performance of new batches of dehydrated media before use. In a busy, cost conscious microbiology laboratory it can be argued that there is neither the staff nor time available to carry out tests to ensure each batch of the large number of different media used comes up to standard. However, from time to time most microbiologists meet problems with poor recovery, lack of sensitivity or contamination with unwanted organisms which suggest that the medium is at fault. Usually it is found that a mistake has been made in the weighing of ingredients or in the method of sterilisation or storage prior to use, but occasionally there is good evidence to indicate that the batch of dehydrated medium is at fault. Whatever the cause, such incidents can result in considerable loss in time and hence money moreover they can cause embarrassment and loss of face. The effort required for monitoring may seem well spent when such incidents occur.

Another reason why there is an urgent need to develop suitable methods for assessing the performance of microbiological media is the growing importance of laboratory accreditation in many countries. To become accredited routine testing of media is a necessity and the method used must be accurately defined.

Most problems of underperformance occur with selective media, where a slight variation in the concentration of the highly active selective agents can have a marked deleterious effect on their properties. In the case of mycological media it is important that levels of antibiotic supplements and antispreading agents, such as rose bengal and dichloran, are correct. Too little antibiotic will allow bacterial growth whereas too much may inhibit mould or yeast growth. With rose bengal, exposure to light will markedly increase its inhibitory properties and recovery of fungi can be seriously impaired if either the ready prepared dehydrated medium or poured plates are not kept strictly in the dark. Dichloran is a very powerful material which is normally used at a concentration of 2mg/l if rose bengal is also present or at 5mg/l when it is the only inhibitory agent in the medium. Only a small deviation from these concentrations can result in too little or too much inhibition.

MONITORING METHODS

With a selective bacteriological medium performance can be monitored by comparing the recovery of wanted and unwanted organisms against a suitable general purpose medium. Counts on overnight broth cultures are estimated using either a modified Miles-Misra method (Corry 1983) or by ecometric streaking (Mossel 1982). These procedures can also be adapted for assessing the recovery of certain yeasts and for ensuring that bacteria are adequately inhibited on a selective mycological medium. However, they are unsuitable for moulds and some yeasts which do not produce uniform suspensions and spread too rapidly to allow enumeration. Moreover, the selective principle with a mycological medium is different from that of a bacteriological medium in that one of its main functions is to restrict the growth of spreading moulds and allow their easy identification and this cannot be properly judged on the basis of recovery alone.

One approach to monitoring a mycological medium is to carry out counts on a substrate containing a variety of suitable moulds, yeasts and bacteria. The substrate could be a carefully selected sample of food or it could be a freeze dried mixed culture of test organisms. In either case there is a major problem in that the population will not remain stable during storage. Not only will the total numbers decline, but, more important, some individuals will decline more rapidly than others. Thus, it is unlikely that a material will be found which can be distributed round the world and used on a regular basis by laboratories to judge the performance of new batches of mycological media.

An alternative approach is to use a stab inoculation procedure and measure the rate of increase in colony diameter with incubation time. The work which has been undertaken to standardise such a technique and assess its usefulness for comparing the performance of different batches of mycological media is discussed in this paper.

THE STAB INOCULATION PROCEDURE

The method simply involves inserting a straight wire loaded with spores of the test organism into the agar medium. Experience has shown that dispersal of spores around the inoculation site can occur if the inoculation occurs from above the medium and it is necessary to turn the plate upside down and inoculate the agar carefully from below.

In preliminary tests it was found that the method of inoculation had a considerable bearing on the number of stabs that developed growth and the rate of colony increase during incubation. Stabbing the medium with a wire heavily loaded with dry spores (or mycelium) taken from a 7-14 day old culture of the test moulds not only ensured that every stab developed a colony but the average colony diameter in a given incubation time was always greater than when

a lighter spore inoculum was used. More important, there was less variation in diameter between replicate colonies.

The amount of medium poured per plate, the method of drying the surface of the agar prior to inoculation and the incubation temperature have all been shown to influence the rate of colony increase. The method eventually chosen involves pouring plates with 20ml of the medium under test, drying the surface with lids removed in an incubator either at 40°C for 2h or 50°C for 1h, stab inoculation from below with a wire heavily loaded with spores (or mycelium) of the test organism, measurement of the colony diameter at daily intervals during incubation at 25°C and calculation of the growth rate from the plot of the average colony diameter against time.

The number of inoculation sites per plate depends on the rate of development of the test organism and on the medium to be monitored. With rapid spreading species it is usually only possible to inoculate in one central position per plate. Fast and slow growing moulds can be inoculated on 3 and 5 positions, respectively, and yeast and bacteria can be inoculated in up to 8 positions per plate. To economise on the number of plates used, it is possible to inoculate on 5 positions with a representative mix of fast and slow growing moulds or in 8 positions with different yeasts and bacteria. However, in the case of moulds there is always the danger that the plates will become contaminated with spores of fast growing species in the course of inoculation and it may be better to use only 3 inoculations per plate. Whatever method is used, it is important to ensure that at least 3 colonies of each test organism on separate plates are used to obtain an average growth rate or colony diameter in a given incubation time.

This stabbing procedure was used to compare the growth rates of a variety of test organisms on the same batches of ready prepared mycological media made strictly according to the manufacturers instructions at weekly intervals over a period of 5 weeks. Plates which had been stored in a refrigerator for 5 weeks were also tested. With the media and test organisms employed, the growth rates rarely varied by more than 10% from the mean. Thus, providing the procedure is carefully followed, either growth rate or average colony diameter in a given time is a reliable indication of the performance of a medium.

TEST ORGANISMS

Wherever possible organisms exhibiting stable characteristics which do not change on repeated subculturing should be used. For monitoring a mycological medium it is necessary to include fast spreading moulds, strongly and weakly growing moulds, yeasts and bacteria. A list of the test organisms used in these investigations and which might be considered suitable for the purpose is given in Table 1. Obviously, it would not be necessary to use all these organisms but at least one or two should be selected from each group. It is clearly desirable that the test

organisms chosen should be related to the type of food to be examined and the nature of the medium. For example, with low or intermediate moisture foods, where it may be desirable to use a medium of reduced a_w such as DG18 (Hocking and Pitt 1980) a range of xerotolerant moulds and osmophilic yeasts which commonly spoil these foods, such as A. flavus, E. glaucus and Z. rouxii, might be included. For moist foods a range of less xerotolerant organisms may be more suitable.

EVALUATING THE STAB INOCULATION PROCEDURE

To assess the value of the stab inoculation technique for monitoring purposes two series of tests were carried out.

Comparing batches of different media from the same manufacturers
In the first test samples representing different production batches of five dehydrated mycological media were received from a prominant medium manufacturer. Each batch of each medium was prepared as per the manufacturer's instructions and poured in 20ml amounts into petri dish plates. After drying, triplicate plates were stab inoculated in three positions with a wire heavily loaded with spores or cells of the nine moulds, four yeasts and four bacteria listed in Table 1. The average increase in colony diameter at 25°C of the three rapidly spreading moulds and also the six other strongly and weakly growing moulds on the different batches of the five media are shown in histogram form in Figure 1.

The effect of rose bengal and dichloran in reducing the growth rate of fast spreading moulds without markedly affecting the growth of the other moulds is evident from this figure. With some media, batch to batch variations in growth rate were observed which were most pronounced with oxytetracycline glucose yeast extract agar (OGYE; Mossel et al 1962) and dichloran rose bengal chloramphenicol agar (DRBC; King et al 1979). With OGYE, the growth rates were significantly higher with batches 1 and 2 than with batches 3 and 4. With fast spreading moulds such differences will have a bearing on how soon plates become overgrown. With DRBC, the significantly lower growth rate on batch 3 may mean that incubation time is increased and recovery reduced when carrying out counts in foods. It should be mentioned that in routine laboratory tests other batches of DRBC have been found to perform rather poorly. Small but significant differences were also observed between batches of dichloran glycerol medium (DG18).

With some of the yeast species, growth rate was seriously reduced on DG18 and DRBC and it is possible that these might not be recovered in tests with mixed populations in food. As expected, all the test bacteria grew happily from a stab inoculation on malt extract agar (ME) which did not contain an antibiotic. While the oxytetracycline in OGYE successfully presented the growth of all the four test bacteria, the chloramphenicol in the other three selective media failed to prevent the growth of Pseudomonas aeruginosa. Whether this is important in terms of a mycological medium for food is uncertain but the finding does suggest than an

alternative antibiotic or combination of antibiotics may be required.

No tests were undertaken to determine count in the same food using these different batches of media. Hence, it is not possible to indicate the extent to which the differences in growth rate noted will affect the recovery of fungi from food. With OGYE it is not difficult to judge that, if fast spreading moulds are present, plates are likely to be overgrown before it is possible to obtain a count no matter which batch is used.

Comparing batches of the same medium from different manufacturers
In a second series of tests samples of rose bengal chloramphenicol medium (RBC: Jarvis 1973)) were received from three different media manufacturers. Plates prepared from each medium were used for stab inoculation tests with a range of moulds, yeasts and bacteria (not necessarily the same as those listed in Table 1) and also for spread plate counts with two samples of cereal. No growth of the test bacteria occurred on any of the batches in 7 days at 25°C. The growth rate of the test yeasts was essentially similar on all media. The mean mould counts, and the mean growth rate and colony diameter after 4 days at 25°C obtained with the test moulds using the stab inoculation technique, are given in Table 2 for the various batches of RBC medium.

The new batch of medium from manufacturer A and the medium from manufacturer B gave significantly lower mould counts than the other batches of the same medium. Significant differences were also observed in the mean growth rate and average colony diameter after 4 days at 25°C with the various test organisms using the stab inoculation procedure. However, there is no relationship between batches which give rise to reduced counts and those which give fast or slow growth rates of the test organisms. Perhaps this is not entirely surprising since it is only when rapid spreading species are present that counts are likely to be reduced and few such organisms were present in the two cereal samples used for counts in this test. Observations when counting the plates suggest that the higher number of moulds found when using the manufacturer A old stock medium may have been due to the reduced colony size which enabled more colonies to be counted.

In addition to the significant differences in count and growth rate there were marked differences in the appearance and colour of the test moulds in the various batches of RBC medium. This was particularly evident with the medium from manufacturer B where it was very difficult to make an identification of commonly occuring moulds on the basis of appearance.

The work which we have undertaken with the stab inoculation procedure suggest that it may prove suitable for monitoring purposes. A certain expertise is necessary when stabbing the medium to ensure that mould spores are restricted to the inoculation site but otherwise the method is straightforward. Moreover, it need not be particularly time consuming if it proves

possible to assess performance on the basis of any average colony diameter reading in a given incubation time rather than on growth rate. One of the main reasons why batches of medium are not monitored more frequently is the time required to prepare and maintain cultures of the test organisms. In our tests we inoculated from 7-14 day old cultures but it is likely that equally good results will be obtained from older cultures. Labour costs will be much reduced if it is possible to subculture the test organisms on a monthly basis.

CONCLUSIONS

From the tests described and general observations in the laboratory there would appear to be a real need for a simple and reliable method for assessing the performance of selective media. This need is becoming more urgent with the growing requirement for laboratory accreditation. The stab incoculation procedure is an approach which warrants attention but much work is required to assess its potential. Perhaps there is a case for spreading the load by carrying out a further collaborative exercise with the same test organisms and making comparisons of growth rate and counts on foods with the same batch of medium and other batches held in the laboratories or supplied by manufacturers.

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Table 1: Suggested test organisms for monitoring mycological media

Rapid spreading moulds	Mucor racemosus Rhizopus stolonifer Fusarium culmorum
Fast growing moulds	Aspergillus flavus Penicillium cyclopium Alternaria alternata
Slow growing moulds	Eurotium repens Cladosporium herbarum Verticillium alboatrum
Yeasts	Hansenula anomala Saccharomyces cereviseae Pichia burtonii zygosaccharomyces rouxii
Bacteria	Bacillus subtilis Pseudomonas aeruginosa Staphylococcus aureus Escherichia coli

Table 2: Comparison of mould counts and growth rates and colony diameter of test organisms on four batches of RBC medium

	A old stock	A new batch	B new batch	C new batch
Mean count per g x 10^5	2.1	1.5	1.6	2.0
Mean growth rate (mm/day/25°C)	3.26	3.74	3.97	4.33
Mean colony diameter (4 days at 25°C)	11.93	14.35	14.78	16.07

RECOMMENDATIONS FOR FURTHER STUDIES

Presoaking ad mixing (Study 2)
The results from this study indicate that either increasing the mixing time or soaking for 30 min prior to mixing will improve the recovery of fungi from cereals. It is not certain whether the increased count is due to better release of organisms from the deeper tissues of the grain or to recovery of stress damaged cells. The fact that useful data was not obtained from the flour sample included in this exercise is unfortunate since this may have resolved this uncertainty.

It is recommended that a further study be carried out, possibly on a collaborative basis, to determine whether presoaking or increasing the mixing time is beneficial for increasing the numbers of moulds and yeasts isolated from milling products of differing levels of homogeneosity such as bran, germ, offals, wholemeal and straight run flours and starches.

It is also recommended that this work be expanded to include tests to determine the effect of the water activity (a_w) of both the diluent and medium on recovery of fungi from these materials. Preliminary tests indicate that for cereals a reduction in a_w may be beneficial, particularly with the longer soaking and mixing times.

Monitoring media (Study 3)
At the Baarn workshop it was agreed that further extensive inter laboratory studies were urgently required to evaluate methods for monitoring the performance of mycological media. The following approaches were recommended.

1. With the same batches of media, compare the reproducibility of colony diameters with different test organisms during incubation using various stab inoculation techniques. The information from this preliminary study wil be used to determine which method and test organism to use.

2. Using a standardised method, compare diameters and appearance of colonies in a given incubation time using as many batches of the same medium from different suppliers as possible. Carry out counts on a standard test material with each batch of medium. It is hoped that the results from this study will make it possible to relate count with colony diameters and thus indicate the minimum and maximum acceptable diameters for different test organisms on the various mycological media.

Other work

1. A wide range of different selective media are being suggested for the isolation and identification of toxigenic fungi in food using a direct plating method. There is a need for studies, possibly of an inter laboratory nature, to compare these media and select the most suitable for the purpose.

2. At the Baarn meeting a number of papers were presented on the use of immunology for rapid detection of fungi in foods. The results of surveys on different foods using two commercially available mould latex immunoagglutination kits were shown to give promising results. Further work is required to evaluate these kits for screening cereals and cereal products for presumptive mycotoxin contamination.