THE DEVELOPMENT AND SIGNIFICANCE OF FUNGI IN POULTRY REARING SHEDS

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Indira A. Mylvaganam

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SUMMARY

A study was conducted at the Poultry Research Centre, Massey University, to determine the numbers, types and patterns of development of fungi that occur in poultry rearing sheds. Two sheds, a broiler shed and a layer shed were analysed for the fungal population by weekly sampling of litter, feed and air. Particular emphasis was placed on the presence of Penicillium and Aspergillus as these genera contain species of potential significance to flock health as infectious or toxigenic agents. Use of the dilution plating method with modified Potato Dextrose Agar and Aspergillus Differential Medium for culture resulted in successful isolation of these genera. Further selectivity was attained with the use of a modification of the strip-bait method of Luttrell (1967). Air sampling was by the exposed agar plate method.

The poultry houses were found to contain high levels of viable fungal propagules. Of the total fungi, Penicillium, Aspergillus and Scopulariopsis were the major genera. Other genera included Rhizopus, Mucor, Cladosporium and Geotrichum. Penicillium was found throughout the trials. Scopulariopsis was present at low levels in fresh litter and feed but counts increased greatly with time. Counts of species of Aspergillus, (A. flavus and A. fumigatus) also showed increases mainly towards the end of the trials. Other genera (Geotrichum, Cladosporium etc.) were found on certain days only.

As litter aged, its pH and moisture content increased and these increases were correlated to the increase in total fungal numbers during the period of housing. Patterns of fungal succession in the litter were similar to those in air. For some species the increases in numbers in litter were preceded by a similar peak in the air (eg. A. flavus) whereas for others the peak in litter was earlier than the peak in air (S. brevicaulis). The correlation between patterns in air and those in feed and litter was more obvious in the layer shed, where a more active flock created greater air movement than in the broiler shed where the birds were docile and caused very little circulation of air.

A. flavus isolates were screened for the production of aflatoxin on coconut agar. Approximately 30% of all A. flavus isolates tested were toxigenic. Also, it was possible to extract the toxin after growth in

semisynthetic liquid medium from isolates which had been strongly fluorescent on coconut agar, but not those which had been weakly fluorescent.

Feed and litter samples tested for the presence of aflatoxin and other mycotoxins (T-2, ochratoxin and zearalenone) were negative for these mycotoxins.

The fungal population of poultry houses has been shown to include species which may be of economic importance. The large amounts of A. flavus (potentially toxigenic) and A. fumigatus (infectious), in particular, should not be ignored as they may well affect poultry health and productivity.

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INTRODUCTION

Fungi are abundant in the poultry house environment, moulds and yeasts being regularly found in the air and dust (Bacon, Burdick and Robbins, 1974) and in litter and compound feed (Lovett, Messer and Read, 1971). As it has become necessary to have high bird densities in modern poultry operations due to the high cost of equipment and housing involved, problems associated with fungal contamination of the birds' environment can be accentuated as these microorganisms can be of significance to poultry production in various ways. They may reduce the nutritional value of feed, by using up essential vitamins and reducing digestibility; they may cause infectious diseases, eg. pulmonary aspergillosis; and they may be toxigenic, mycotoxins being produced as a result of fungal growth within the immediate environment, or on feed-stuffs during their production.

The poultry industry is currently undergoing a period of rapid expansion in New Zealand, and therefore it is important for poultry farmers to be aware of any factors that affect production. Investigations into the types and relative densities of fungi which occur in the poultry environment can provide useful information to stimulate recognition of the problems which may be caused as a result of fungal growth. In turn, this would enable precautions to be taken to avoid diseases and poor productivity. Presently there is a lack of such information in relation to the New Zealand poultry industry, and this was the initiative for the present study.

1. THE GENERAL ENVIRONMENT WITHIN POULTRY REARING SHEDS

1.1 Shed Design and Construction

The housing of poultry for either egg or meat production is important because it allows the poultry farmer to have many environmental factors under his control and ensures maximum use of feed energy for productive purposes. The design of the poultry house should therefore be such that is provides the favourable conditions necessary for maximum production, and at the same time reduce the chances of contamination of the environment with disease-producing agents such as fungi.

Although the actual dimensions of poultry houses will vary in relation to the size of the overall poultry operation and on its ultimate objectives, a poultry house must primarily provide a suitable environment for maintaining and growing the flock. This environment is partly determined by the design of the house. The configuration of the house, the insulation, heating, light and ventilation systems are designed keeping in mind such factors as optimizing production, housing costs and energy inputs. Ideally, the heating, ventilation and light systems should have a dynamic range, capable of responding to the requirements of the chickens and to the outer environmental conditions such as temperature, humidity, wind and solar radiation (Reece and Lott, 1982).

Poultry houses are insulated in order to conserve heat during the winter. Adequate insulation, in association with the high level of heat produced by the birds (especially in layer sheds where birds are active) enables an optimum temperature to be maintained. Suitable insulation with adequate ventilation enables laying houses to be filled to capacity. The body heat of the birds makes the inside of the house much warmer than the outside and insulation increases this difference between inside and outside temperatures. The warmer the air, the greater the amount of moisture it can hold; the warm, moist air will rise to the top of the house, where outlet ventilators can be placed to exhaust this moisture-laden air (Bell and Moore, 1949).

Ventilation provides Oxygen to the birds, keeps down the levels of dust and gases such as Carbon Dioxide and Nitrogen, as well as controlling shed temperatures and carrying out excess moisture. The removal of moisture prevents condensation which could moisten litter, feed and walking paths, so encouraging the growth of microorganisms. Such moisture is produced by the hens in their faeces and in respiration. It is desirable in a poultry house to have a relative humidity of 50-70%. This will give a litter moisture content of 20-35% (Deaton and Reece, 1980). Thus ventilation and insulation are interrelated, and used together to give optimum results. Good insulation and well-regulated ventilation result in good temperature control and proper air distribution, and condensation becomes unlikely.

The lighting system used in a poultry house depends upon the nature of the operation. Many poultry farmers make use of artificial light to

obtain partial control of flock egg production. Broilers are generally subjected to continuous, low-intensity light throughout the growing period, but it has also been found that intermittent light regimes can give favourable results. McDaniel, Kloon and Flood (1977) found that birds, both male and female, grown under intermittent light, were much heavier than those grown under continuous light. The effect of such regimes on fungal growth is uncertain, but it is known that light intensity can markedly affect growth and spore production by many fungi (Griffin, 1981).

The floor of the house should be moisture-proof, durable, free from cracks and easily cleaned. A board floor, if properly laid, can be easily cleaned and may be free from cracks. However, it may not be durable, and at certain times of the year the circulation of cold air below such a floor may cause the floor to become so cold that condensation from the warmer air inside the house may occur; this causes the litter to become wet. Concrete floors are advantageous in that they are durable, and may be thoroughly cleaned out between batches of birds, thus reducing the chances of infection. They can be kept warm by properly bedding with litter.

1.2 Air Circulation

The air is an important component of the poultry house environment and the health of the chickens is dependent to a large extent on the composition of the surrounding air. Air components in a poultry house include gases, such as O_2, CO_2 and NH_3, moisture and a high level of dust. Dust is mainly flaky and cellular, consisting of epidermal fragments of skin, feed particles, faecal matter and feather particles (Koon, Howes, Grub and Rollo, 1963). Moulds, mould spores, and other microorganisms are also carried by the dust. Dust causes deterioration of equipment in poultry houses, increases the incidence and severity of poultry diseases by disseminating pathogens, and transports odours. Deaton and Reece (1980) found that in a poultry house with resting birds, about half the dust particles were smaller than 10μm, whereas with active birds only a quarter of the particles were under 10μm. Larger particles can carry more microorganisms.

It is important that the level of dust be kept fairly low. Dust levels tend to drop as air moisture increases, but the air should not be

so moist as to allow condensation. Thus, a suitable equilibrium should be maintained by insulation and ventilation systems.

The accumulation of CO_2 and NH_3 in the shed can be harmful to poultry. High levels of CO_2 have been shown to affect egg production and shell thickness (Helbacka et al., 1963) and exposure to high NH_3 levels can reduce weight gains and feed conversion (Reece, Lott and Deaton, 1980) and also increase disease problems such as infectious bronchitis (Kling and Quarles, 1972). Continuous air change and mixing of clean air through the shed is thus important, and this may be achieved by an efficient ventilation system, which will control moisture levels and temperature as well as gas concentrations. However, such systems render the house susceptible to continuous inoculation with potential pathogens from the outside, although the possibility of transmission of airborne disease agents, including fungi, via the incoming air may be reduced by the use of efficient air filtration.

Ventilating fans are used by most commercial poultrymen today. Cross-ventilation is carried out by drawing air in through fans placed in one side wall and extracting it through fans in the opposite side wall or the roof. The fans are usually located at the top ends of the side walls, close to the ceiling (Nowland, 1978).

The temperature of the incoming air, the ventilation rate and the production of body heat by the birds determine the air temperature inside the poultry shed. The rate of air change should be such that it maintains a suitable temperature equilibrium immediately surrounding the birds (Osbaldiston and Sainsbury, 1963); insufficient ventilation can lead to heat stagnation and excessive ventilation can remove body heat too rapidly, resulting in hypothermia. In both cases, the birds would become more susceptible to infectious diseases.

1.3 Management

1.3.1 Equipment

Poultry equipment and appliances vary from the most elementary to the most advanced electronic devices. The efficiency of feeders and drinkers, used to supply feed and water to the birds at all stages of growth, is crucial for the prevention of gross fungal contam-

ination of the environment. Feeders are constructed in such a way as to avoid waste, to prevent contamination of feed by droppings and litter and to be easy to clean and durable. Tube feeders are used in some broiler operations. These are cylindrical metal feeders, with a pan underneath into which feed flows from the main body. They can hold from 6 to 14 kg of feed (Oluyemi and Roberts, 1979).The use of automatic feeding systems can improve overall efficiency by avoiding spillage as well as regularly providing the birds with fresh feed. One form of automatic feeding system distributes feed from a feed hopper through a chain electrically driven and operated by a time switch. This system mixes the feed thoroughly and prevents crowding of hens around any feed trough.

The drinkers used in many poultry operations are trough-shaped, and made of galvanised iron or stainless steel. Troughs may be filled manually, or may be attached to a supply pipe. The top of each long side in a trough curves inwards, limiting the spillage of water. To keep the water clean, the troughs should be high enough so that litter cannot be disseminated into it, and located so that contamination with droppings cannot occur.

1.3.2 Litter

There are two different kinds of litter management. One is 'unchanged' litter, i.e. the simple spreading of wood shavings, sawdust etc. on to the floor of the pen and this is left undisturbed for the rest of the operation. The other is the 'built-up' or deep litter system. Built-up litter starts with a minimal level of litter to which more is added periodically until a depth of 25-30 cm is reached. This is considered an effective sanitary procedure without the liberation of offensive odours.

Ammonia is absorbed by the litter, which becomes alkaline as a result. Reports have shown that built-up litter can have dietary factors needed for the growth of chickens (Kennard and Chamberlin, 1948, 1951), but litter also represents a good substrate for fungal growth, with a concommittant danger to the health of the birds.

1.3.3 Feed

Poultry feed contains sources of carbohydrate, protein, fats, vitamins and minerals, but storage conditions must be carefully controlled to avoid spoilage by fungi. Carbohydrate, and thus energy, is derived mainly from cereals. Corn has a high energy content. Other cereals such as sorghum, barley and wheat have been shown to be satisfactory sources of metabolizable energy (Maust, Scott and Pond, 1972). The protein supplement is either of animal origin, or may be a substitute such as soybean meal. Vegetable sources of protein also include peanut meal. Fish meal is a good source of amino acids. Fats contain a high level of productive energy and mixtures of animal and vegetable fats are used in poultry rations. Vitamins are given either in their natural sources, or in synthetic form. High quality alfalfa meal supplies carotene to help meet Vitamin A requirements and also furnishes B complex vitamins, calcium and protein. It is added to layers' ration to produce a desired yellow colour in the egg yolk. Distillers' dried grains and solubles are also used in feeds (Waldroup, Owen, Ramsey and Whelchel, 1981). As an example, the ingredients of the standard feed used at the Poultry Research Centre at Massey University are listed in Table 1.

Pelleted feed is obtained by pressing the mash at high temperature and under high steam pressure. Pelleted feed is slightly more expensive than mash, but has advantages. There is less wastage and the birds receive a good balance of vitamins. With mash they tend to pick out the larger pieces of feed and leave out the finer particles which contain vitamins and minerals. Overall flock performance is similar with stock fed on mash or on pressed feeds (Nowland, 1978).

The poultry farmer may mix the feed himself or purchase a ready-made feed. The deciding factor is the cost of the finished feed and its formulation, but in both cases the feed, particularly if a mash, must represent a continuing source of fungal inoculum from the outside. It is the feed which is also likely to be the principal source of mycotoxins to the growing poultry.

The use of antifungal agents may sometimes be necessary to control mould growth and subsequent mycotoxin formation in feeds and litter. Among the most popular are propionic acid, copper sulphate and Gentian

violet. Stewart, Wyatt and Ashmore (1977) found that propionic acid was fungicidal at >3 µg/ml of medium, while crystal violet (cv) was fungicidal at >2 µg/ml. Cv also retarded aflatoxin production, and the sporulation of A. parasiticus. Different species were found to vary in their susceptibility to cv. Dilworth et al. (1979) investigated the effect of fungistatic compounds in broiler diets. The addition of sorbic acid was found to improve growth rates.

Dixon and Hamilton (1981a, b) found that the inhibitory properties of propionic acid could be altered depending on the feed ingredients (eg. the addition of fat enhanced activity). They also noted that the inhibitor must be evenly distributed throughout the feed. The smaller the feed particles and inhibitor particles, the better the inhibition.

Table 1. Ingredients and Nutrient Composition of a Typical Standard Rearing Diet. *

	Weight (g)
Premix	0.2
Coccidiostat (Amprol)	0.04
Barley	1.346
Maize	29.1
Wheat	25.0
Bran	8.0
Lucerne	2.5
Pollard	25.0
Peas	4.16
Blood	1.4
Meat and Bone meal	7.65
Brewers' Grain	7.0
Soybean	5.0
Apex Meal	0.03
Limestone	0.5
Fish Meal	5.57
	122.496

* Poultry Research Centre,
Massey University.

2. FUNGI IN POULTRY HOUSES

It is apparent from the foregoing discussion that even in the best-run poultry house, contamination of the environment with fungi or their metabolites is unavoidable. Thus a knowledge of potential changes in the fungal flora within the house is a useful adjunct to the maintenance of efficient production.

2.1 Fungal Isolation Techniques Relevant to Poultry Houses

Certain techniques which have been used in isolating soil fungi may be applied to the isolation of fungi from poultry feed and litter. Of these techniques, the most widely used is the dilution plate method. Several investigators have used the dilution plate method to analyse samples from the poultry environment (Halbrook et al., 1951; Schefferele, 1965, Lovett et al., 1971, and Dennis and Gee, 1973). Dennis and Gee (1973), for example, in their study of poultry house mycoflora, added 0.5 g litter or dust to 10 cm^3 of diluent, made dilutions and plated 0.1 cm^3 aliquots of each dilution onto various media. Counts were made after 5 days' incubation at 25^oC.

Stotzky (1972) and others have pointed out that the dilution plating method has some limitations. According to Stotzky, the majority of species that develop colonies on the plates are those that produce abundant spores. Also, dilution plating techniques give essentially no information on the real location of the fungi in the substrate. Although there are these limitations, soil microbiologists have used this method to get a reasonable picture of the general composition of the soil microflora and provided that standardised sampling and cultural techniques are used when a survey involves a time factor, useful comparative information can be obtained. Preliminary experiments can be used to determine the most satisfactory media, temperatures of incubation and dilutions for the particular substrate under examination and for any genera of particular interest (Booth, 1971).

A number of modifications to the dilution plating method have been proposed. For example, Barron (1971) used weak agar solution as the diluting agent, as it keeps dispersed particles in suspension longer. The samples were also blended with an Osterizor; the shearing forces of the blender allowed the recovery of large numbers of fungi of low

sporulating capacity or those in which the spores are strongly attached to the parent hyphae. Barron also states that it may be advantageous to add an inhibitor to the dilution blanks, to restrict further fungal growth in order to prevent colonies merging, and so facilitating identification.

Rose and Bradley (1980) have outlined a procedure for determining spore densities which was found to be more accurate than previously-used methods such as serial dilutions. Samples are collected from different locations and depths in the substrate; dilutions are made in buffered saline and mixed with an electric stirrer. Then a known amount is transferred to a Petroff-Hausser Counting chamber and spore numbers are counted under a microscope. With this method, mould spores and yeast cells may be differentiated and quantiated, thus making the use of this chamber statistically more accurate than previously-used methods.

To overcome the various problems imposed by dilution plating, a variety of other techniques have been used for fungal isolation. Mueller and Durrell (1957) described an immersion tube for isolating soil fungi. In this method, fungi grow through holes made in a centrifuge tube containing nutrient agar. The tube is immersed in the substrate and this allows the colonization of the agar under natural conditions of moisture and temperature. After a period of time the agar and the invading fungi are removed and transferred onto rose-bengal agar plates. This method is good for isolating actively-growing fungi, but its disadvantage is the small area sampled.

A similar method is the screened immersion plate developed by Thornton (1952). In this method, a screen containing two rows of holes is placed over a glass slide which has an agar film. Fungi grow through the holes onto the slide and may be isolated with a needle under a binocular microscope. Again, this method samples only a small area.

Andersen and Huber (1965) developed the plate-profile technique, which can sample a larger area. This method involves placing a plastic plate (20 x 30 cm) with 1 cm deep holes filled with agar, against a soil profile in the vicinity of roots. Fungi grow into the agar medium. With this technique, they were able to identify more than 10,000 fungi, representing 87 genera.

These methods are advantageous in that they allow the growth of fungi from selected loci in the soil directly into agar baits which can be varied in composition. However, the agar baits have different moisture and gaseous contents from soil; also they cannot be left in soil for long periods to study the succession of fungal genera and they are tedious to prepare and handle.

Hence Luttrell (1967) developed a strip-bait method, which retains the advantages of the previous techniques and corrects for some of their deficiencies. This method was also used by Bacon and Burdick (1977). Strip-baits are made of two strips of perforated electrician's tape sealed together with stacks of 5 nutrient-soaked filter paper discs between them. Strip-baits can be used to study the fungal flora in soil, poultry feed, litter and even air, by suspending them from a rod.

A modification of this method is described in detail in a later section as in the present study membrane filters have been used to replace the central disc of the stack.The membrane filter has been a valuable aid in the cultivation and identification of microorganisms. It has largely been used in bacteriological analyses of food products and water, but can also be applied to the cultivation of fungi. This application was described by Funder and Johannessen (1957). They demonstrated that fungi could be observed microscopically while growing through the membrane filter, and hence identified easily. Thus when membrane filters are incorporated into the strip-bait of Luttrell, we potentially have both a means of isolation and a system allowing direct observation and identification of fungi from a substrate.

The agar medium used for the isolation of fungi plays an important role in the types of fungi cultured. The incorporation of antibiotics such as chloramphenicol and streptomycin is effective in preventing the growth of bacteria. Overgrowth by spreading moulds such as Rhizopus and Mucor species has always been a problem in fungal enumeration. This problem may be overcome by the addition of certain substances. Curl (1968) incorporated 0.5 g phosphon (2,4-dichlorobenzyl tributyl phosphonium chloride per litre of medium). Another substance which has been used is Dichloran (2-6, dichloro-4-nitroaniline). King, Hocking and Pitt (1979) and Henson (1981) found that a combination of dichloran and the dye Rose-Bengal was effective in reducing the colony diameters

of these moulds. Those spreading moulds which were not inhibited by dichloran, were inhibited by Rose-Bengal.

A number of media partially selective for specific types of fungi have been developed, eg. Aspergillus Differential Medium (ADM) (Bothast and Fennell, 1974), which has been used extensively in the present study. This is a rose-bengal-containing medium useful for screening for Aspergillus flavus and A. parasiticus. The medium contains ferric citrate, which stimulates the production of a yellow-orange pigment by isolates of A. flavus and A. parasiticus.

ADM alone does not entirely inhibit bacteria and the spreading moulds, and more recently Hocking (1982) has described "Aspergillus flavus and parasiticus agar" (AFPA) which has the ingredients of ADM, with antibiotics and dichloran added.

In addition, interest in the ability of A. flavus to form aflatoxin has led to the development of media useful in screening for toxigenic strains. Hara, Fennell and Hesseltine (1974) reported the use of a modified Czapek's solution agar containing corn-steep liquor for detecting aflatoxin-producing strains of A. flavus. After the plates were incubated for 10 days at 28^{o}C, any aflatoxin present could be detected under UV light by the blue fluorescence of the agar surrounding the fungal colony.

Lin and Dianese (1976) developed an agar medium containing commercial coconut extract for detecting toxigenic A. flavus strains. On this medium, the aflatoxin-positive isolates showed a characteristic blue or blue-green fluorescence in the surrounding agar, under long-wave UV light. An incubation time of 3 days is sufficient to detect aflatoxin, and the fluorescence increases in intensity as the incubation time is lengthened. Coconut agar is easier to prepare than the corn-steep liquor agar of Hara et al. or various other media which have been proposed. The method enables quick detection of any toxigenic strains isolated and can be conducted before carrying out confirmatory extraction of toxin by organic solvents.

2.2 Presence and Patterns

Studies have been performed on the mycoflora of litter, feed and dust within poultry houses. Litter is composed of woodshavings, sawdust, straw etc. which can harbour microorganisms. Several bark residues which are used for broiler litters (pinewood bark, hardwood bark etc.) were examined by Thi So, Dick, Holleman and Labosky (1978) and shown to contain fungal spores. Fungi isolated from the broiler bark litters included A. flavus, A. fumigatus, A. niger, Fusarium moniliforme, Candida albicans and species of Geotrichum, Penicillium and Mucor.

Fungi are found in varying numbers in litter of all ages. Species of Penicillium, Aspergillus, Paecilomyces and Trichoderma are among genera which may be present (Bacon, Burdick and Robbins, 1974). However, wood shavings initially have a low moisture content; they tend to contain fungal species which are characteristic of low moisture environments, such as members of the Aspergillus glaucus group. The presence of these species is significant because they can initiate growth at low moisture levels and then gradually release water by their metabolic activities, thus facilitating the growth of other moulds such as A. candidus, A. flavus and species of Penicillium (Dennis and Gee, 1973).

As litter becomes older, certain changes take place in its composition and texture. The colour of the litter progressively darkens due to the droppings, and the moisture content increases. Fungi tend to be found in increasing numbers and types. The dust in the poultry house is believed to serve as a major source of the microorganisms found in the litter. Dust contains particulate matter such as feathers and feed particles, which are suitable substrates for fungal proliferation.

Dennis and Gee (1973) studied litter and dust samples from broiler houses at different localities in the U.K., before and after being used by a single crop of birds for 56 days. They found that in the dust, 90% of the moulds were species of Penicillium. Other species present were A. candidus, A. repens and Scopulariopsis brevicaulis. There was little difference in counts between initial and final dust samples. Fungal counts for dust were consistently higher than those for litter. In fresh litter, species of Penicillium (90%), Paecilomyces and Trichoderma were present. Where species of Aspergillus and S. brevicaulis

were found in fresh litter, they were present in much lower numbers than in the corresponding dust samples. In final litter, the counts of Penicillium and Paecilomyces decreased markedly, while Aspergillus and S. brevicaulis predominated.

Dennis and Gee found that total fungal counts in initial litters were lower than in the final litters. The maximum mould counts ranged from 10^5 g^{-1} in initial litter to 10^7 g^{-1} in final litter. Environmental conditions in the poultry houses appeared to favour the development of organisms present in the dust, rather than those present in the initial wood shavings. The fungal flora of the initial and final dust samples was similar to that of the final litter. Thus dust seemed to be the major source of microbial contamination for each lot of fresh litter.

Litter, feed and dust samples from broiler houses were studied by Bacon and Burdick (1977); some were 'problem' houses, i.e. with histories of mycotoxicoses or with problems of unknown aetiology, and others were normal houses, used as controls. Fungi were isolated by dilution plating, and by the strip-bait method of Luttrell (1967). They found that in the control houses, S. brevicaulis was present in high frequencies and high relative densities, with some others such as A. fumigatus being present as well. In the problem houses, Scopulariopsis was infrequent, and the more frequent fungi were species of Graphium, Petriella, Penicillium and Aspergillus.

In an earlier study, Bacon and Burdick (1974) examined poultry feed and litter samples from broiler houses in North-East Georgia. In fresh litter, the fungi found most frequently were Graphium penicilloides, Trichoderma viride, Trichothecium roseum and A. candidus. In used litter, those predominating were S. brevicaulis, Fusarium poa and species of Aspergillus and Penicillium. Again, dust was found to be the main source of inoculum, since the fungi in dust samples were the same as those in old litters. Feed and feed ingredients from feed mills were also examined. Among the fungi isolated were A. flavus and A. ochraceus and there was a correlation between fungi in the feed ingredients and the mixed feed.

Lovett et al. (1971) studied litter and feed samples from four farms in Southern Ohio, over 12 weeks. Samples were analyzed by dilution

platings on various media. They found a wider variety of moulds in the litter than in the feed. Seventeen fungal genera were identified from litter samples, with the dominant ones being Penicillium, Scopulariopsis and Candida. A succession was observed: when the litter pH was acidic Penicillium was dominant, but when the litter became alkaline Scopulariopsis became dominant. Twelve mould genera were identified from feed, and of these the dominant isolates were Penicillium, Aspergillus, Fusarium and Mucor.

The microbial flora of built-up litter was investigated by Halbrook, Winter and Sutton (1951), and by Schefferele (1965). In the study conducted by Halbrook et al., moulds and other microbes increased in the litter up to 8 weeks; but old, built-up litter contained fewer microbes than unchanged litter. Unchanged litter had over one hundred times more moulds than built-up litter. The built-up litter became more alkaline than unchanged litter, and it was suggested that this higher pH accounts for the decrease in moulds in built-up litter, since the effect was made more obvious by the addition of lime. This is in agreement with the results of Schefferele.

In the studies of Lovett et al. (1971), litter which was in use for one month or more was alkaline. Both litter pH and mould counts increased with litter usage up to one month, and then declined slightly and remained constant. In feeds, the pH's were in the acid range, with moisture contents about 12%. Fungal densities (7×10^2 - 3×10^5) appeared unrelated to pH or moisture content.

Species of Aspergillus, Penicillium, Petriella and Scopulariopsis made up 77% of the fungal isolates from a turkey confinement brooder house examined by Pinello, Richard and Tiffany (1977). These fungi were recovered from air and litter samples. Other fungal genera isolated included Cladosporium, Fusarium, Geotrichum, Paecilomyces, Mucor and Rhizopus. Conditions in the house had an effect on the fungi present. High temperature ($34^{o}C$) seemed to be detrimental to the growth of some fungi, as did the spraying of the house with antimicrobial agents. The highest number of fungal isolations was made when the windows and doors were closed and the temperature range was $22\text{-}26^{o}C$.

Moulds and other microbes increase in the poultry house air when the number of birds increases. Petersen, Sauter, Parkinson, Dixon and

Stroh (1978) reported that when birds were housed at 0.42 m^3/bird, there were greater microbial numbers in the air than when housed at 0.84 m^3/bird. A similar observation was made by Sauter, Petersen, Steele and Parkinson (1981).

Fungi have frequently been found in feed and feed ingredients which make up poultry rations. Moulds may invade seeds before they are harvested, or invade during storage, under certain levels of temperature and relative humidity. The microbiological quality of poultry feed and ingredients was studied by Tabib, Jones and Hamilton (1981). Samples of normal and suspect feeds were collected from turkey and broiler chicken operations, and the moisture content, pH, mould count and coliform count were measured. Mould counts were found to be in a range of values from 5 g^{-1} 10^6 g^{-1}. Pelleted feeds contained lower mould counts than unpelleted feeds.

Fungi can survive for long periods in feed ingredients. Samples of white corn which were assayed for aflatoxin and A. flavus in 1972 were re-examined in 1982 (Hesseltine and Rodgers, 1982). A. flavus was still present, though in lower counts. Reduced counts were also obtained for A. niger, Fusarium and Penicillium. Thus A. flavus can survive for years in naturally infected corn.

2.3 Significance

The presence of fungi in the poultry environment can give rise to a variety of problems. The production of mycotoxins by certain fungi in the feed and litter may result in serious economic losses. The nutritional value of feed may also be reduced by the fungi present. Infectious diseases can be caused when poultry inhale or ingest large numbers of fungal spores.

Fritz, Mislivec, Pla, Harrison, Weeks and Dantzman (1973) moulded poultry feed with fungi isolated from feeds which had been known to produce a haemorrhagic syndrome in pigs. Most of the moulds, eg. A. candidus, A. repens, P. cyclopium, F. moniliforme, A. ochraceus, did not produce a toxin but affected the chicks in other ways. In most cases, growth was slightly depressed. F. moniliforme was found to contain a thiaminase which destroys thiamine in the diet. Thus a deficiency was caused, which could be prevented by adding more thiamine to the diet.

This deficiency was also prevented when chicks were fed pre-sterilized feed. Beasley, Blalock, Nelson and Templeton (1980) isolated P. lanosum from feed from a house with wet litter and chickens showing poor growth. When corn inoculated with this fungus was fed to broiler chicks, they developed diarrhoea and their growth rate was reduced. Their gizzards were also affected, and P. lanosum was isolated from these gizzards.

Bartov, Paster and Lisker (1982) studied the nutritional effects of feeding stored, mouldy grains of normal and increased moisture content to broiler chicks. Mouldy grains were analysed to be free of mycotoxins. They found that in the moistened grains the fat content was decreased markedly. During the experimental period the mouldy grains, which contained less metabolisable energy, depressed performance. They concluded that the decreased energy level in diets containing mouldy grain is an important factor in their reduced nutritional value.

The digestibility of amino acids can also be affected by fungi in the feed. Nelson et al. (1982) have shown that when corn moulded with Penicillium sp. and A. ochraceus is fed to chicks, the amino acid digestibility, dry matter digestibility and energy utilization are reduced.

Most of the fungi in the poultry environment disseminate large numbers of spores. Penicillium and Aspergillus are both highly-sporing genera. Inhalation of large numbers of spores by poultry can result in problems such as pulmonary aspergillosis. Pulmonary aspergillosis is caused by the inhalation of large numbers of A. fumigatus spores, and has high mortality rates in young birds (Ghori and Edgar, 1979). Inhalation of a considerable number of spores may occur when the litter or feed is heavily contaminated. Pneumonia of young chicks is the most common form of the disease, which is usually acute. In older birds, there may be difficulty in breathing, reduced food intake and occasionally diarrhoea and convulsions (Hungerford, 1969). The lungs are most frequently affected. Lesions produced may be either localized in the air sacs or generalized with mycelial masses spreading throughout the air passages and bronchi. The lesions are greyish-yellow necrotic nodules varying in size, and in chronic cases may coalesce to form solid masses. Ghori and Edgar (1979) observed that chicks which were exposed to A. fumigatus spores had pulmonary lesions, and also showed suppressed growth. Thus pulmonary aspergillosis is a serious poultry disease,

because it can cause death among young chicks, and depressed performance in older birds.

Ogundero (1980) pointed out that poultry feed contained many fungi, such as A. fumigatus, Mucor and Penicillium that pose health risks to poultry. Similarly, Katoch,Bhowmik and Katoch (1975) found that poultry feeds and litter samples contained a variety of opportunistic fungi such as Mucor, Rhizopus, P. purpurogenum, P. rugulosum, P. oxalicum, A. niger and F. oxysporum. P. rugulosum can produce a toxin, rugulosin, which causes necrosis of hepatic cells, giving rise to hepatic lesions in experimental animals. P. oxalicum has been implicated in experimental air sac infections. Mucor and Penicillium species can be found in pulmonary mycoses, especially in mixed infections. The presence of these fungi in the surroundings of poultry can be the source of infection under adverse conditions.

Among the fungi which have been isolated from poultry sheds, many have been shown to have the potential for toxin production. The fungi isolated from the litter and feed of two poultry farms in Ohio, U.S.A. by Lovett (1972) were screened for toxin production. Chick embryos were inoculated with fungal culture filtrates and embryo death at 9 days was used as the toxicity indicator. Toxic fungal strains isolated included 2 A. terreus strains, 5 P. cyclopium strains, one strain each of A. fumigatus, Scopulariopsis sp., Fusarium sp. and 2 strains of P. patulum. These represented 13% of the total number of isolates screened. The genus which yielded the highest proportion of toxigenic isolates was Aspergillus; out of the 8 isolates screened, 4 were toxic, giving a value of 50% toxic for this genus. Penicillium yielded >10% toxic isolates. The toxigenic fungi were all from the feed, except for A. fumigatus and Scopulariopsis which were from the litter.

Toxigenic fungi were also found in the study conducted by Bacon and Burdick (1977) on fungi in broiler houses. Potentially toxic species isolated were: A. flavus, A. fumigatus, P. cyclopium, P. patulum and species of Cladosporium, Trichoderma and Fusarium. Houses known to have problems had A. flavus, whereas control houses did not. However, other toxigenic fungi were not found only in problem houses; some were also in the control houses. In their earlier study, Bacon and Burdick (1974) examined several fungi which they had isolated fom poultry feed and houses for their ability to produce mycotoxins on laboratory media.

They found that A. flavus produced aflatoxins, Gibberella zeae produced zearalenone and A. ochraceus produced penicillic acid and ochratoxin A. Zearalenone was the only toxic compound they found in corn; also in poultry feed the ability of A. ochraceus to produce ochratoxin and penicillic acid was a complex interaction between moisture and temperature.

Sharby, Templeton, Beasley and Stephenson (1972) have also shown that fungi isolated from poultry diets may be of danger to chickens. Chicks were fed with corn experimentally moulded with fungi which had been isolated from rations in use on poultry farms. The corn in the diets was replaced by varying levels of corn separately moulded with isolates of F. moniliforme, F. roseum, A. niger and A. flavus. The chicks that were fed these experimental diets were found to have depressed weight gain and feed efficiencies; excreta from these chicks were dark and sticky and the chicks showed a reduced dry matter digestibility. The impaired feed utilization could be due to an alteration in the ease of digestion or to some damage to the Gastro-intestinal tract. The experimental chicks also showed severe leg abnormalities. This was characterized by a bowing outward or slanting inward of the legs. Post-mortem observations showed mild enteritis and distended caecae, i.e. gastro-intestinal involvement.

In poultry sheds, toxigenic fungi can be found in the air as well as in feed and litter. Toxin-producing moulds which may be present in the litter can also be subsequently found in the air. Sauter et al. (1981) isolated moulds from the air of poultry houses; the two genera Penicillium and Aspergillus represented over one-half of the total mycoflora, and both contained mycotoxin-producing species. Wicklow and Shotwell (1983) investigated the presence of aflatoxin in the spores of toxigenic A. flavus and A. parasiticus strains. Aflatoxins were detected in the conidia as well as sclerotia of these strains. The distribution of the aflatoxins between conidia and sclerotia of individual isolates varied according to species and strain. Wicklow and Shotwell have shown that the levels of aflatoxin present in the conidia can be high enough to affect chickens which are in an environment containing a large number of these conidia. The presence of high levels of A. flavus conidia in the poultry house air could therefore be of considerable significance to flock health.

Toxigenic fungi can grow and elaborate their toxins in many substrates, such as crops, stored grain and compound feed, provided the environmental conditions are favourable. Many factors, eg. moisture levels, aeration, insect damage and storage conditions can influence the production of toxin. Moisture content of the substrate is particularly significant, because moulds can grow at moisture levels much lower than other microorganisms. Many of the mycotoxins are heat-stable, and are not destroyed by pelleting or by fungistatic compounds such as propionic acid.

Mycotoxicoses can assume one of three different forms (Pier, Cysewski and Richard, 1977). Acute primary mycotoxicosis occurs when high quantities of toxin are consumed and overt clinical disease is produced. Chronic primary mycotoxicosis occurs when low levels of mycotoxin are ingested over a long time. This chronic effect shows up in various ways, such as a slower growth rate, reduced feed consumption, increased susceptibility to bruising, decreased egg production and so on. Secondary mycotoxicoses are produced from the intake of very low levels of mycotoxins; the immune system and native mechanisms of resistance can become impaired.

It is very rarely that mycotoxins occur in high enough levels to give acute clinical disease. In poultry mycotoxicosis, one sees mainly decreased production and also an impairment of immunity which gives rise to disease susceptibility (Smith, 1982). There are 5 main mycotoxins of importance to poultry (Table 2). Of these, aflatoxin is the most widely known, and has received much attention since its discovery in the early 1960's. Chickens have a relatively low susceptibility to aflatoxins, with the main response being a reduced growth rate, although some acute cases have been described (Archibald, Smith and Smith, 1962).

The toxin can also affect the immune system, the main effect being the impairment of the function of lymphocytes and macrophages (i.e. cell-mediated immunity) mediated through a reduction of lymphokine activity (Pier, Richard and Thurston, 1978). Aflatoxin consumption has been shown to enhance the susceptibility of poultry to such diseases as salmonellosis, coccidiosis and candidosis. Reports by Pier et al. (1978) and Giambrone, Ewert, Wyatt and Eiden (1978) indicate that low levels (0.5 ppm B1) of aflatoxin can affect cell-mediated immunity and that

higher levels (2-10 ppm) can affect bursal activity, decreasing antibody production.

Aflatoxin also interacts with lipid, protein, carbohydrate, vitamin and mineral nutrition and has effects on blood, destroying the intrinsic blood coagulation system that is operative in the chicken (Doerr and Hamilton, 1977). All levels of aflatoxin reduce bone strength, by increasing bone fragility, and may make chickens more susceptible to bruising, even at very low levels, (eg. 0.6 ppm) (Tung, Smith and Hamilton, 1971).

Smith (1982) has mentioned (from Pier et al., 1980) the levels of aflatoxin at which various effects are noted in poultry. At 5-10 ppm toxin, the acute effects such as hepatic necrosis and death occur in the broiler. Bruising occurs at 0.6 ppm; reduced resistance at 0.6-10 ppm. In layers, decreased egg production occurs at 2-8 ppm.

The above are some of the many effects of aflatoxin on poultry. Research over the last decade has indicated that a wide range of other fungal toxins can affect poultry health (Table 2).

Table 2. Mycotoxins of Importance to Poultry.

Mycotoxin	Fungal Species	Major Effects
Aflatoxin B1, B2, G1, G2	A. flavus A. parasiticus	Most toxic; severe alteration in protein and lipid synthesis -> poor growth, impaired feed efficiency, altered immunity; also carcinogenic properties.
Ochratoxin A, B	A. ochraceus P. viridicatum	Nephritis and nephrosis with increase in liver glycogen.
T-2 Toxin (Trichothecene)	Fusarium sp.	Oral inflammation in broilers and layers with an accompanying decline in feed uptake.
Zearalenone (F-2)	F. graminearum	Oestrogenic.
Citrinin	P. viridicatum A. ochraceus	Increased water uptake, resulting in severe diarrhoea.
Rubratoxin A1, B	P. rubrum P. purpurogenum	Hepatotoxic, especially rubratoxin B. Also haemorrhages in bladder.

2.4 Mycotoxin Extraction and Analysis

A number of mycotoxins can now be detected by direct extraction and analysis of toxins by biochemical methods. This can provide a final answer as to whether toxin is present in a sample of litter or feed. However, the amount of toxin present is likely to be very small. One may find only small pockets of toxin due to the sporadic growth of the mycotoxin-producing fungi. Thus it is difficult to obtain a representative sample and protocols for sampling and subsampling have been presented by Davis, Dickens, Freie, Hamilton, Shotwell and Wyllie (1980).

A commonly-used general procedure for the extraction and analysis of mycotoxins has been outlined by Jones (1977)as:

(a) Sample preparation for analysis: the sample (usually 1 to 5 kg) is ground and mixed until it is homogeneous. The actual sample to be analysed, about 50 g, is taken from the main sample by quartering procedures.

(b) Defatting: the ground sample may be defatted with hexane, a method applicable to most feeds, but not to material containing substantial amounts of water.

(c) Toxin extraction and purification: Several analytical methods are available for the extraction and purification of aflatoxin and other mycotoxins. These methods utilize organic solvents such as chloroform, and lipid solvents such as diethyl ether. Richard (pers. comm.) recommends that samples be extracted with chloroform and passed through a silica-gel column. Hexane can be used to remove any interfering lipids, and anhydrous ether to remove pigments. The mycotoxins can then be extracted by eluting the column with different solvent mixture.

(d) Separation and identification of mycotoxins: The eluates obtained from the column can be evaporated, re-dissolved in benzene/acetonitrile, and further analysed by thin-layer chromatography using known standards as markers. A wide range of solvent mixtures may be used for developing the TLC plates, the

most frequently used being chloroform/methanol and chloroform/acetone/ethanol (Moreau, 1979). TLC can be used for the identification and quantitation of individual mycotoxins.

There are also a number of reasonably rapid screening tests available, eg. minicolumns for aflatoxin detection. Minicolumns have been used extensively for the detection of aflatoxin from feed ingredients. The columns are prepared by plugging a 4 mm diameter glass tube at one end with glass-fibre filter paper, filling the tube (4.5 cm long) with silica gel and plugging the other end in the same way (Holaday, 1968). The minicolumns are developed in a chloroform:methanol extract of the sample. If aflatoxin is present in the sample, a blue fluorescent band will be observed when the column is exposed to UV light. This method is rapid and can also be used for screening of extracts before quantitation on TLC plates.

AIMS OF PROJECT

(a) To study the fungal flora of poultry houses, particularly the patterns of succession with time of the principal genera in the litter, feed and air of poultry houses.

(b) To examine the possibility of selectively isolating Aspergillus and Penicillium spp. from poultry litter and feed effectively.

(c) To investigate the prevalence of toxigenic Aspergillus flavus strains within the poultry house environment.

(d) To determine if any mycotoxins can be detected in the poultry feed or litter.

MATERIALS AND METHODS

1. SOURCE OF SAMPLES AND SAMPLING PROCEDURE

1.1 Poultry House Samples

Samples of litter and feed were collected from one layer and one broiler house at the Poultry Research Centre of Massey University. The layer shed measures 42 m x 12 m and is divided into several pens (Fig. 1) in which different experiments can be conducted. The largest pens in this shed measure 7.8 m x 4.8 m. The birds in these pens are crowded together, with over 700 birds in the larger pens. The broiler shed is somewhat smaller and has just four pens on either side, each pen being approximately 7 x 5 m. In the broiler operation, there are 150-200 birds in each pen.

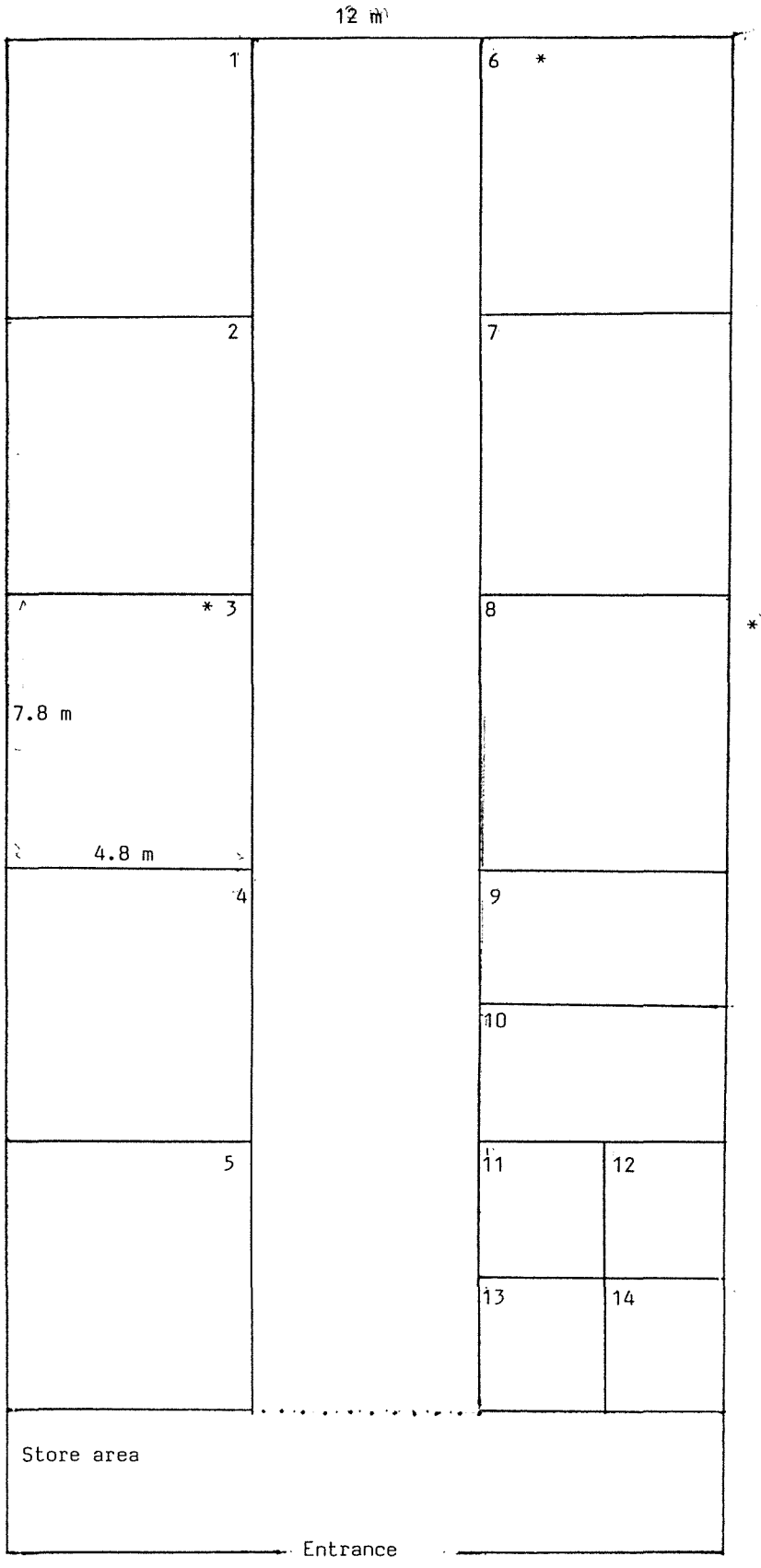
The sheds are thermally insulated, fan-ventilated and light-controlled. In the layer shed, the birds are tested for the effects of lighting on growth rates and feed conversions. During rearing, they receive continuous artificial light for the first week, then 10 weeks of photoperiod each day up to 14 weeks of age, at which time the light period is stepped up each week for 3 weeks. Thus at 17 weeks of age the birds receive 13 hours of light each day. At 18 weeks they are transferred from the shed to battery cages for laying.

Ventilating fans are fixed in the walls of both sheds. The air is drawn in by fans in one side wall and expelled through exhaust fans in the opposite wall so that the air flow is unidirectional.

In both sheds, the individual pens are fitted with a gate, in the iron surrounds. In the broiler shed, tube feeders are used. These consist of a cylindrical container fitted to a pan-base. Feed flows from the container downwards to the pan. In the layer shed, an automatic feeding system is used, consisting of two lines of pan-feeders, one to each side of the house. The pans are automatically topped up with feed at regular intervals, from a main feed container. Water is supplied in continuously-fed drinking troughs in both sheds.

The feed ingredients for broilers are obtained from Farm Products Ltd., Porirua, and mixed in the feed-processing unit at the Poultry

Figure 1. Poultry Research Centre Layer Shed.



Research Centre. For the layers, the feed ingredients are obtained from Manawatu Farm Products Ltd., and mixed at the P.R.C.

Wood shavings and dust which make up the litter are obtained from various timber companies.

1.2 Soil Samples

The soil specimens used in the testing of the strip-bait method (Section 3.3.1) were obtained from the orchard area of Massey University Campus and from a city council park at Milson, Palmerston North. The orchard soil was selected because of its fertility, and the Milson soil for its relatively high moisture content, as these conditions encourage a dynamic fungal population favourable to the testing of the strip-bait.

1.3 Duration of Sampling

The study of fungal growth and succession carried out in the broiler shed was called the broiler trial and that in the layer shed was called the layer trial. The broiler trial lasted 40 days; collection of samples commenced on 5.5.83 and was conducted weekly until the end of the trial, on 15.6.83. The layer trial lasted 17 weeks, the collection of samples beginning on 1.7.83 and continuing weekly until 28.10.83, when the chickens were transferred to laying cages. Air sampling was carried out on four occasions in the broiler trial and weekly in the layer trial.

1.4 Sample Collection

1.4.1 Litter

Litter was collected from two pens in each trial, pens numbered 3 and 10 in the broiler trial and pens labelled A and B in the layer trial (pens 3 and 6 in Figure 1). A spade was used to mix the litter in an area close to the centre of each pen and approximately 200 g transferred to a plastic bag. The bags were sealed with cellotape and stored at $4^{o}C$ until examination.

In the broiler trial, two strip-baits (Section 3.3) were also embedded in the litter each week, and removed and replaced by another two baits one week later.

1.4.2 Feed

Feed samples were collected fortnightly in the broiler trial from pen 10 and weekly in the layer trial from pen A. Approximately 500 g were collected from the tube or pan feeder after mixing, transferred to a plastic bag which was sealed with cellotape and stored at $4^{o}C$. In the broiler trial, two strip-baits were sealed to the base of the tube feeder in each pen and removed and replaced fortnightly with another two baits.

1.4.3 Air Sampling

Air sampling was carried out by exposing agar plates to poultry house air/dust. Two plates of Potato Dextrose Agar (PDA) (Section 2.1) and two plates of Aspergillus Differential Medium (ADM) were left exposed on a ladder for 30 min, about half-way between the floor and the roof. After the period of exposure, the plates were covered with their lids and sealed with cellotape. One plate of each medium was incubated at $25^{o}C$ and the other at $37^{o}C$, for 7 days.

1.4.4 Soil Samples

A spade was used to dig soil samples for the testing of the strip-bait method. The chosen area was well mixed and approximately 1000 g of soil were transferred to plastic containers. The containers were closed and stored at $4^{o}C$ until used.

2. MEDIA AND REAGENTS

2.1 Media

The following media were used in the investigation:

(a) Potato Dextrose Agar (PDA):

Potato infusion	200.0	g
Dextrose	20.0	g
Agar	15.0	g
Phosphon	0.05	g
Distilled water	1.0	L

Streptomycin (to a final concentration of 0.05 g/L dissolved in a little 95% alcohol) was added to the cooled medium after autoclaving for 10 min at 15 psi. Phosphon (2,4-dichlorobenzyl tributyl phosphonium chloride) was added to prevent overgrowth by spreading Zygomycetes such as Mucor and Rhizopus (Curl, 1968).

(b) Aspergillus Differential Medium (ADM):

Tryptone	15.0	g
Yeast Extract (Difco)	10.0	g
Ferric citrate	5.0	g
Agar	15.0	g
Rose Bengal	0.025	g
Phosphon	0.05	g
Distilled water	1.0	L

Streptomycin (0.05 g/L) was added after autoclaving for 10 min at 15 psi (King et al., 1979).

(c) Czapek-Dox Agar (for identification of Aspergillus species):

Sucrose	30.0	g
Sodium nitrate	3.0	g
Dipotassium phosphate	1.0	g
Magnesium sulphate	0.5	g
Potassium chloride	0.5	g
Ferrous sulphate	0.01	g
Agar	15.0	g
Distilled water	1.0	L

Autoclaved for 10 min at 15 psi.

(d) Coconut Agar (for preliminary screening for aflatoxin production by A. flavus isolates):

"Coconut Cream"*	200.0	cm^3
Distilled water	600.0	cm^3
Agar	15.0	g
pH	6.9	

* Samoa Tropical Products Ltd., (coconut cream, water and polysorbate 60). Autoclaved for 15 min at 15 psi and poured thickly into plastic petri dishes (Lin and Dianese, 1976).

(e) Semisynthetic Liquid Medium (SMKY):

Sucrose - analytical grade	200.0	g
Magnesium sulphate	0.5	g
Potassium nitrate	3.0	g
Yeast extract (Difco)	7.0	g
Distilled water	1.0	L
pH	5.5	

40 cm^3 quantities of the medium were placed in 500 cm^3 conical flasks which were then plugged with cotton wool and the plugged mouth sealed with foil before autoclaving for 10 min at 15 psi (Diener and Davis, 1966).

(f) Nutrient Solutions (for soaking of filter papers making up the strip-baits):

A: Dextrose Peptone Yeast Extract (DPY):

Dextrose	5.0 g
Peptone	1.0 g
Yeast extract	2.0 g
Ammonium nitrate	1.0 g
Dipotassium phosphate	1.0 g
Magnesium sulphate	0.5 g
Ferric chloride	trace
Sodium propionate	1.0 g
Distilled water	1.0 L

Chlortetracycline (0.03 g/L) and streptomycin (0.03 g/L) added prior to autoclaving for 10 min at 15 psi.

B. DPY (with antibiotics added, as above) and 20 g/L tannic acid added prior to autoclaving for 10 min at 15 psi.

C. DPY + antibiotics as in A, made with 40.0 g (4%) dextrose and autoclaved for 10 min at 15 psi.

D. DPY + antibiotics with 4% dextrose and 20 g/L tannic acid. Autoclaved for 10 min at 15 psi (Luttrell, 1967).

2.2 Reagents

(a) Mounting fluid - Lactophenol Cotton Blue:

Phenol crystals	20.0 g
Lactic acid	20.0 g
Glycerol	40.0 g
Distilled water	20.0 cm^3

Dissolved by gentle heat under a hot tap, and 0.05 g cotton blue (Porrier's blue) subsequently added.

(b) Mycotoxin Standards:

Aflatoxin standard (B and G):
A standard mixture of aflatoxins obtained from Sigma Chemical Co., U.S.A. was used. The benzene:acetonitrile (98:2 v/v) mixture contained 5.0 $\mu g/cm^3$ B1, 1.5 $\mu g/cm^3$ B2, 5.0 $\mu g/cm^3$ G1 and 1.5 $\mu g/cm^3$ G2.

T-2 (Trichothecene) Standard:
50 μg of T-2 toxin obtained from Sigma Chemical Co., U.S.A. and dissolved in 1 cm^3 acetonitrile.

Zearalenone Standard:
100 μg zearalenone obtained from Sigma Chemical Co., U.S.A. and dissolved in 1 cm^3 acetonitrile.

Ochratoxin Standard:
100 μg ochratoxin obtained from Sigma Chemical Co., U.S.A. and dissolved in 1 cm^3 acetonitrile.

(c) TLC development solvent:

TLC plates were developed in chloroform:methanol:formic acid (97:2:1), in an unequilibriated glass chromatography tank.

3. SAMPLE EXAMINATION

3.1 Moisture Content and pH Calculations

3.1.1 Moisture Content (m.c.)

20 g of the collected sample were weighed and placed in a large glass petri dish. The sample was then dried in an oven at $80^{o}C$ for 3 hours, at which time it was removed and weighed. The sample was then replaced in the oven and after one hour, removed and re-weighed. This was repeated until two consecutive readings were identical. The m.c. was expressed as the % of moisture in the original sample.

3.1.2 pH

The pH values of the litter and feed samples were measured by shaking 5 g of sample in 20 cm^3 distilled water, and measuring the pH of the suspension with an Orion Research digital ionalyzer/501 pH meter.

3.2 Dilution Plating

In order to obtain total and differential (genus) counts of fungi present in the samples of feed and litter, the dilution plating technique was carried out weekly. 5 g of each sample were placed in a universal bottle containing 20 cm^3 sterile distilled water (s.d.w.). This was shaken for 20 min on an automatic shaker. 0.5 cm^3 of the suspension were then transferred to 4.5 cm^3 of s.d.w. in a bijou bottle to give a 10^{-1} dilution. Preliminary experiments indicated that the greatest variety of genera and the most consistent results in replicative sampling could be obtained by culturing 10^{-1} dilutions on both PDA at $25^{o}C$ and ADM at $37^{o}C$. The numbers and types of colonies appearing on each of the plates were recorded.

3.3 Strip-bait Method

The strip-bait method of Luttrell (1967) was used together with the dilution plating method to isolate fungi from poultry feed and litter.

Strip-baits were made of filter paper squares sealed between two strips of perforated electricians' tape. Whatman No. 5 filter papers were soaked in one of the nutrient solutions (Methods 2.1.f) and air-dried. Small squares were cut from the dried paper and stored in an envelope. Two strips of plastic electricians' tape (approx. 7 cm long) were sealed together and three holes punched 2 cm apart. The two strips were then separated. In preliminary experiments stacks of 5 filter-paper squares were placed over the holes in one strip. Then the other strip was laid over the first strip so that the holes in the tape centred over the squares, and the two strips were sealed together. An adaptation of Luttrell's method involving the incorporation of a membrane filter (cut to size from Millipore 0.45 um membrane filters) as the centre square, was used in the main part of the investigation.

The strip-baits were inserted into the substrate (soil in the preliminary experiments and litter/feed in the poultry trials) and left for incubation. After the period of exposure, the baits were removed and the tapes pulled apart. The centre square in the stack of five was removed and transferred to agar plates, which were then incubated. Fungi which grew from the centre square were counted and recorded. The use of membrane filters enabled the fungal growth to be viewed microscopically on one cut half of the membrane while the other half could be cultured.

3.3.1 Preliminary Testing of the Strip-bait

The efficiency of the strip-bait and the conditions most favourable for its use were investigated using soil specimens.

a. Testing of membrane filter as the centre square: Two soil specimens were placed in separate petri dishes and labelled Milson soil and Massey soil. Two strip-baits were prepared, each with four holes, two containing stacks with a filter paper as the middle square and two with a membrane filter as the middle square. One strip was placed in the Milson soil and the other in Massey soil. After 5 days the baits were removed. One middle filter paper and one membrane filter from each soil was cultured on PDA and the other filter paper and membrane filter on ADM and incubated 7 days at $25^{o}C$.

b. Investigation of most favourable culture medium, nutrient solution and incubation temperature:

This was carried out by embedding strip-baits made with different nutrient solutions (A, B, C and D, Methods 2.1) in soil specimens, and after 8 days culturing the centre membrane filters on different combinations of media (PDA and ADM) and incubation temperatures ($25^{o}C$ and $37^{o}C$).

c. Investigation of substrate moisture content: The effect of substrate moisture content on the efficiency of the strip-bait technique was investigated by adjusting the moisture content of soil specimens to 5%, 20% and 50%, and embedding strip-baits made with nutrient solutions B and D. The membrane filters were removed and cultured at $25^{o}C$ on PDA and at $37^{o}C$ on ADM.

d. General efficiency: The ability of strip-baits to isolate fungi in proportion to their occurrence in the soil was studied. Soil samples were inoculated with varying levels of Aspergillus sp. and a fixed level of Penicillium sp. Strip-baits with squares soaked in solutions B and D were placed in the samples. The membrane filters were later removed and cultured on ADM at $37^{o}C$. This procedure was repeated with a fixed level of Aspergillus sp. and varying levels of Penicillium sp.

3.3.2 The Strip-bait as a Means of Fungal Isolation in the Poultry Trials

50 g of each weekly sample of feed and litter were placed in each of two plastic petri dishes. The m.c. of the sample in one petri dish was adjusted to a level 5% above the original m.c., and the other left unadjusted.

Two strip-baits (with squares soaked in solutions B and D) containing 3 stacks were embedded in each sample of litter/feed. The membrane filters from each stack were removed at 5, 8 and 14 days. On removal, each membrane filter was cut in two: one half was examined microscopically after staining with lactophenol cotton blue, and the other half was cultured on ADM + phosphon and incubated at $37^{o}C$ for 5-7 days. Colonies which appeared on the plates were recorded.

In the broiler trial, strips embedded in the feed/litter of the broiler house were removed every week and replaced with two more strips. Removed strips were analysed in the laboratory by culturing the membrane filters on ADM at $37^{o}C$ for 5-7 days.

4. METHODS OF IDENTIFICATION

The identification of fungal isolates was carried out on the basis of the macroscopic and microscopic appearances on PDA and ADM, with the aid of Ajello, Georg, Kaplan and Kauffman (1963), Barron (1968), Onions, Allsopp and Eggins (1981) and Raper and Fennel (1965). Isolates of Aspergillus were inoculated on Czapek-dox agar and incubated 2-3 weeks at 25°C, for identification.

For the microscopic examination, slides were prepared as needle mounts using lactophenol cotton blue as the stain, according to the standard methods described by Dring (1971). Cellotape mounts using lactophenol cotton blue as mounting fluid were also used. The slide culture technique (Booth, 1971) was used for some isolates to obtain an undisturbed picture of the hyphae and attached conidiophores and conidia.

5. MYCOTOXIN PRODUCTION

5.1 Screening of A. flavus Isolates for Aflatoxin Production:

5.1.1 Coconut Agar

A. flavus colonies encountered in the layer trial were subcultured onto PDA plates to obtain pure cultures. After 4 days' incubation at 37°C, these were inoculated onto plates of coconut agar and incubated at 25°C. At 3, 7 and 12 days of incubation, the reverse of the cultures on the plates were viewed under long-wave U.V. light (wave-length 365 nm) in a Chromatovue * cabinet, to detect the presence of any fluorescence. In the early part of the trial, when relatively few A. flavus colonies were isolated, all isolates were tested on coconut agar. Later in the trial when there were numerous A. flavus isolates, the numbers tested were limited to a range of 20-50 colonies, some being dilution plate isolates and others being strip-bait isolates.

* CHROMATO-VUE CABINET, Model CC-60, UVP, Inc.

5.1.2 Semisynthetic Liquid Medium (SMKY)

Several coconut agar isolates of A. flavus were tested for the production of aflatoxin on the semisynthetic liquid medium of Diener and Davis (1966) (Methods 2.1). Randomly-selected positive and negative isolates were grown on PDA slants for 12 days. Suspensions of A. flavus spores were made by adding 5 cm^3 s.d.w. and shaking the slants gently for 20-30 min. 0.5 cm^3 of each spore suspension were then transferred to a SMKY flask. The flasks were wrapped with dark-blue paper and incubated at 25°C for 10 days.

At the end of the incubation period, any aflatoxin produced in the SMKY medium was extracted with chloroform. 100 cm^3 chloroform and 90-100 g glass beads were added to each SMKY flask, and left for one hour with periodic shaking. Then the medium was filtered into a 25 cm^3 conical flask, through several layers of muslin cloth. The filtrate was re-filtered twice through Whatman No. 4 filter paper into a 250 cm^3 separatory funnel. The funnel was shaken and then left in a stationary position for the contents to separate into two layers. The lower layer of $CHCl_3$ was removed into a separate container, and concentrated by evaporation.

Chloroform extracts prepared in the above manner were re-suspended in 5 cm^3 $CHCl_3$ and stored in a bijou bottle plugged with cotton wool and covered with aluminium foil until analysis by thin-layer chromatography (TLC).

5.2 Multimycotoxin Analysis of Feed and Litter Extracts

Several samples of litter and feed from the layer trial were subjected to a multimycotoxin analysis (Richard, pers. comm.) for the detection of aflatoxin, ochratoxin, T-2 and zearalenone. The samples were selected from the early, middle and late sections of the trial, and the method involved silica gel 'clean up' column chromatography and TLC analysis.

5.2.1 Preparation of the Silica Gel Column

The glass column used was Quckfit CR 32/40. This was fitted to a 500 cm^3 separatory funnel (Quickfit D2/22) plus stopper as a

base. 5 g anhydrous sodium sulphate were placed at the bottom of the column. The column was half-filled with chloroform. 10 g of silica gel (Bio-Sil A, 100-200 mesh) activated by drying in an 80^{o}C oven for 1 hour, cooling in a dessicator and re-hydrating with 1% water, were added slowly to the column with gentle agitation with a long glass rod. When the silica gel had settled, excess $CHCl_3$ was removed wth a pipette until there were 8 cm $CHCl_3$ above the silica gel. Then 15 g of sodium sulphate were added carefully to the column and allowed to settle. Excess $CHCl_3$ above the sodium sulphate was drawn off, leaving approximately 1 cm $CHCl_3$ to prevent the column from drying.

5.2.2 Preparation of chloroform extracts

The feed or litter sample was ground finely, and 50 g added to a 500 cm^3 conical flask. Then 25 cm^3 s.d.w., 25 g diatomaceous earth ('Celite' filter-aid) and 250 cm^3 $CHCl_3$ were added in that order. The flask was placed on an automatic shaker for one hour. The contents of the flask were then filtered through Whatman No. 1 filter paper, and the first 50 cm^3 of chloroform filtrate were collected. The filtrate was transferred to a separatory funnel containing 150 cm^3 n-Hexane. The funnel was shaken and the contents left to settle. The hexane layer was then drained off, and the chloroform extract retained for the analysis.

5.2.3 Multimycotoxin method

The chloroform extract was placed on the silica gel column and allowed to elute. 150 cm^3 benzene was added to wash the column and both these eluates were discarded. Then the sample left in the column was analysed by eluting for the different mycotoxins.

To elute for zearalenone, 250 cm^3 of acetone:benzene (5:95) were added. To elute for T-2 toxin and aflatoxin, the column was first eluted with 150 cm^3 anhydrous ether, and then with 150 cm^3 methanol:chloroform (3:97).

To elute for ochratoxin, 250 cm^3 glacial acetic acid:benzene (1:9) was added.

The eluates from each sample were collected in separate bijou bottles, concentrated by evaporation, and re-dissolved in a mixture of

acetonitrile:benzene (2:98). The bottles were tightly plugged with cotton wool, covered with aluminium foil and stored in a dark container at $4^{o}C$ until analysis by thin-layer chromatography.

5.3 Thin-layer Chromatography (TLC)

SMKY (section 5.1.2) and column eluate (section 5.2.3) samples were analysed for the presence of mycotoxins by TLC.

5.3.1 TLC Plates

Aluminium sheets 20 cm x 20 cm, 0.2 mm thickness, precoated with silica gel 60 (non-fluorescent, No. 5553) obtained from E. Merek, Darmstadt, were used in all TLC analyses.

5.3.2 Spotting of samples and standards on TLC plates

TLC plates were used as whole plates (20 cm x 20 cm), half-plates or quarter-plates, depending on how many samples were being developed at any one time. A pencil line was drawn on the TLC plate 2 cm from the lower edge. 10 µl aliquots of the relevant mycotoxin standards and 10 µm aliquots of the test samples were spotted onto the spotting line at marked intervals, using a 10 µl disposable micro-pipette (Clay Adams Scientific Co.). After spotting was completed, the plates were air-dried for 15 min, and subsequently developed.

5.3.3 Development of TLC plates

Plates were developed in 100 cm^3 chloroform:methanol: formic acid in a glass chromatography tank, until the solvent level reached the top of the plates, about 2 cm below the top edge. The plates were then removed and dried in a fume cupboard for 15 min.

5.3.4 Examination of TLC plates for the presence of mycotoxins

Dried TLC plates were examined under U.V. light for the presence of fluorescence. Samples being examined for aflatoxin were examined under long-wave U.V. light while those being examined for other mycotoxins were viewed under short-wave U.V. light.

RESULTS

1. MOISTURE CONTENT AND pH OF POULTRY HOUSE SAMPLES

1.1 Moisture Content

1.1.1 Litter

In both trials, the moisture contents of the litter samples increased with age. In pen 10 of the broiler trial, litter m.c. ranged from 6.1% (day 1) to 12.6% (day 40) and in pen 3 the litter showed a m.c. increase from 6.9% to 11.8% (Fig. 2). In the layer trial, m.c.'s of litter from pen A increased from 7.6% at day 1 to 18.0% at the end of the sampling period. Litter from pen B ranged from 6.3% to 19.1% m.c.

1.1.2 Feed

In the broiler trial, feed samples showed a limited m.c. range between 5.9% and 6.3% (Fig. 3). In the layer trial, the m.c. of feed samples showed a minimum of 5.0% and a maximum of 7.0%.

1.2 pH

In the broiler trial, the pH values of litter samples showed a steady increase from 5.4 to 8.0 in the litter of pen 10, and 5.4 to 7.9 in litter of pen 3 (Fig. 4). For the feed samples, pH varied from 5.5 to 6.2 (Fig. 5). Similarly in the layer trial, litter A showed a pH increase from 5.6 to 10.9, litter B from 5.71 to 11.12 and feed samples 5.8 to 6.39 (Figs. 4 and 5).

Figure 2. Variation of moisture content of poultry house litter with age.

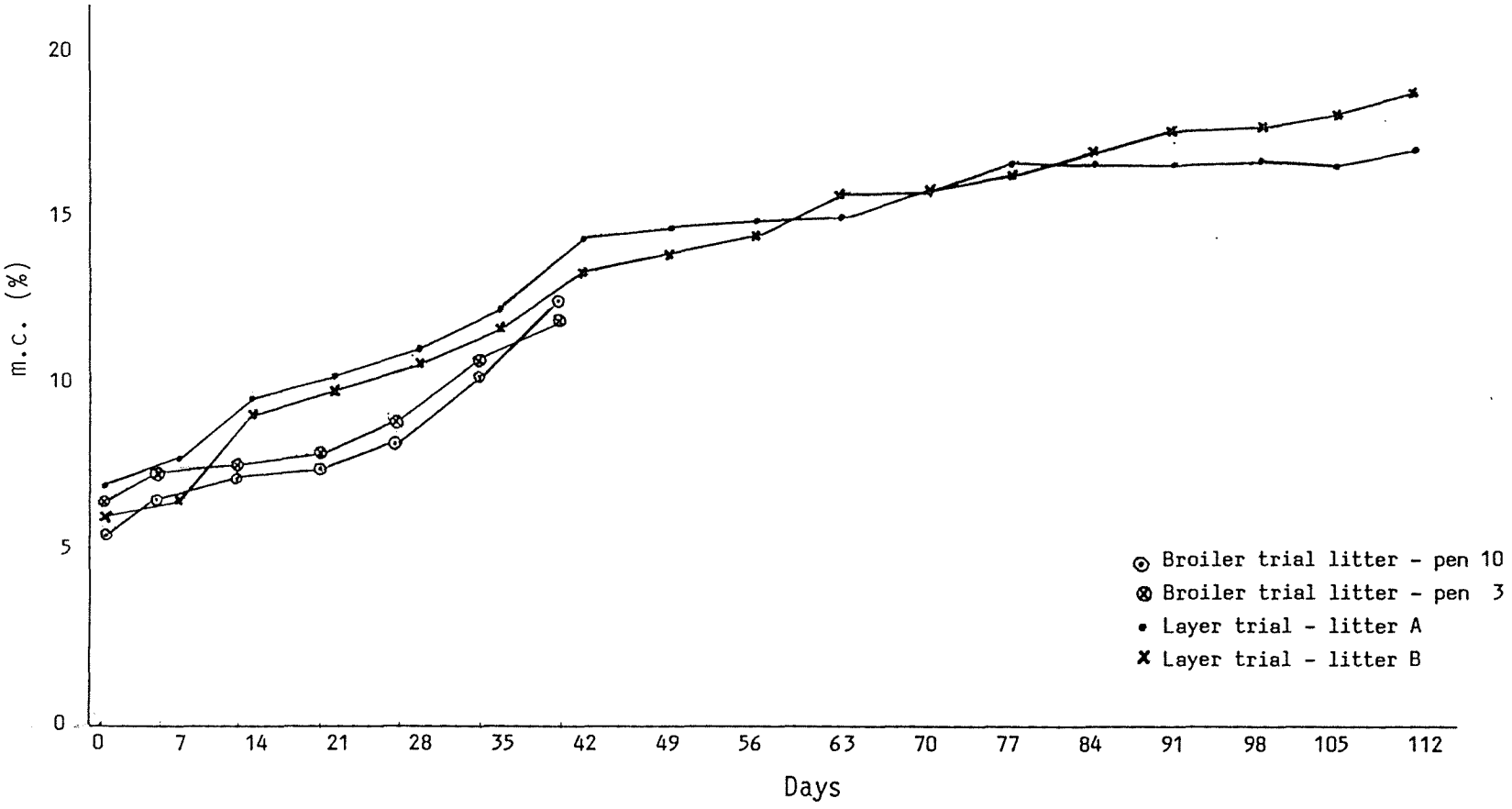


Figure 3. Variation of moisture content of poultry house feed with time of sampling.

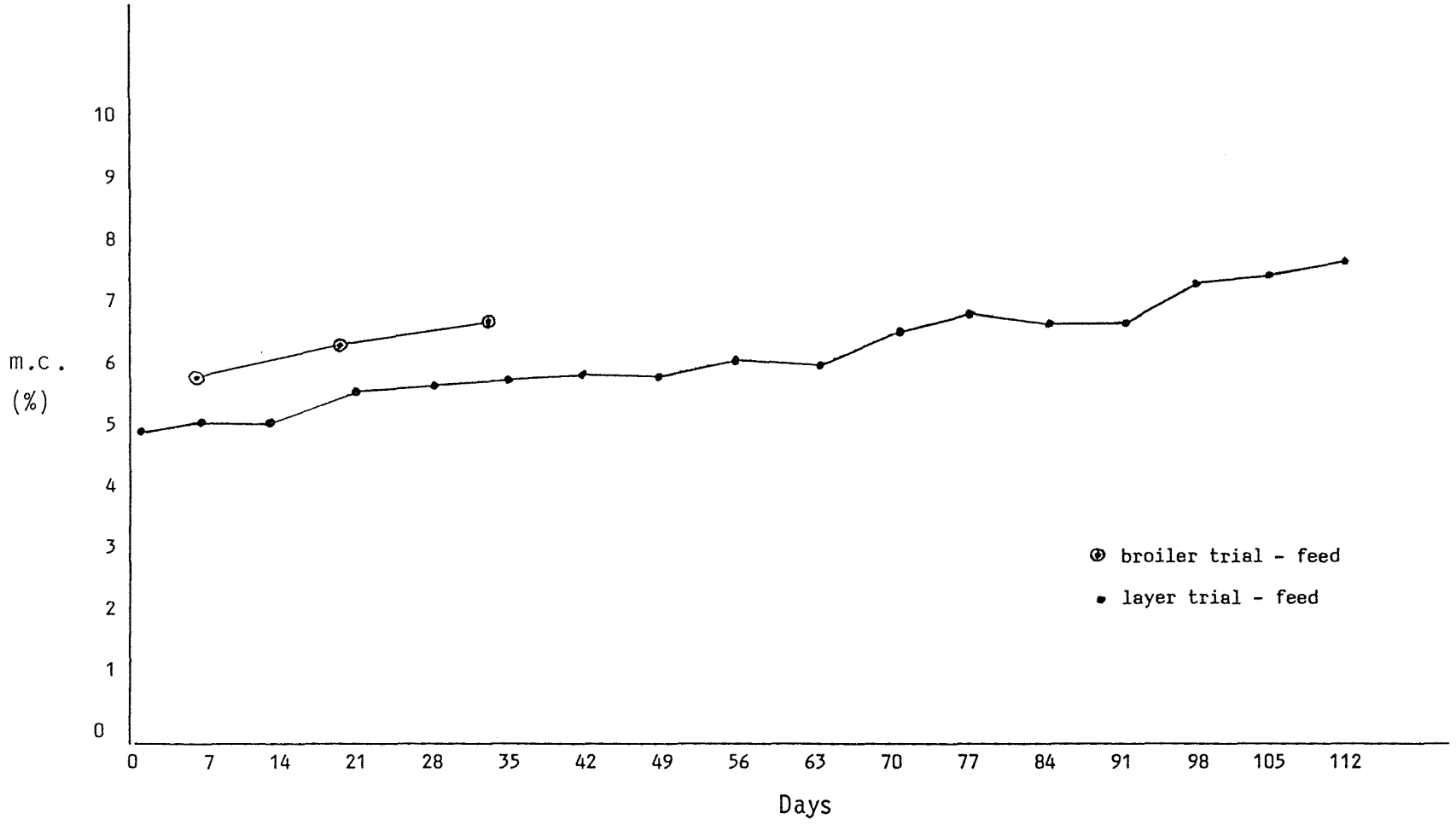


Figure 4. Variations in pH of poultry house litter with age.

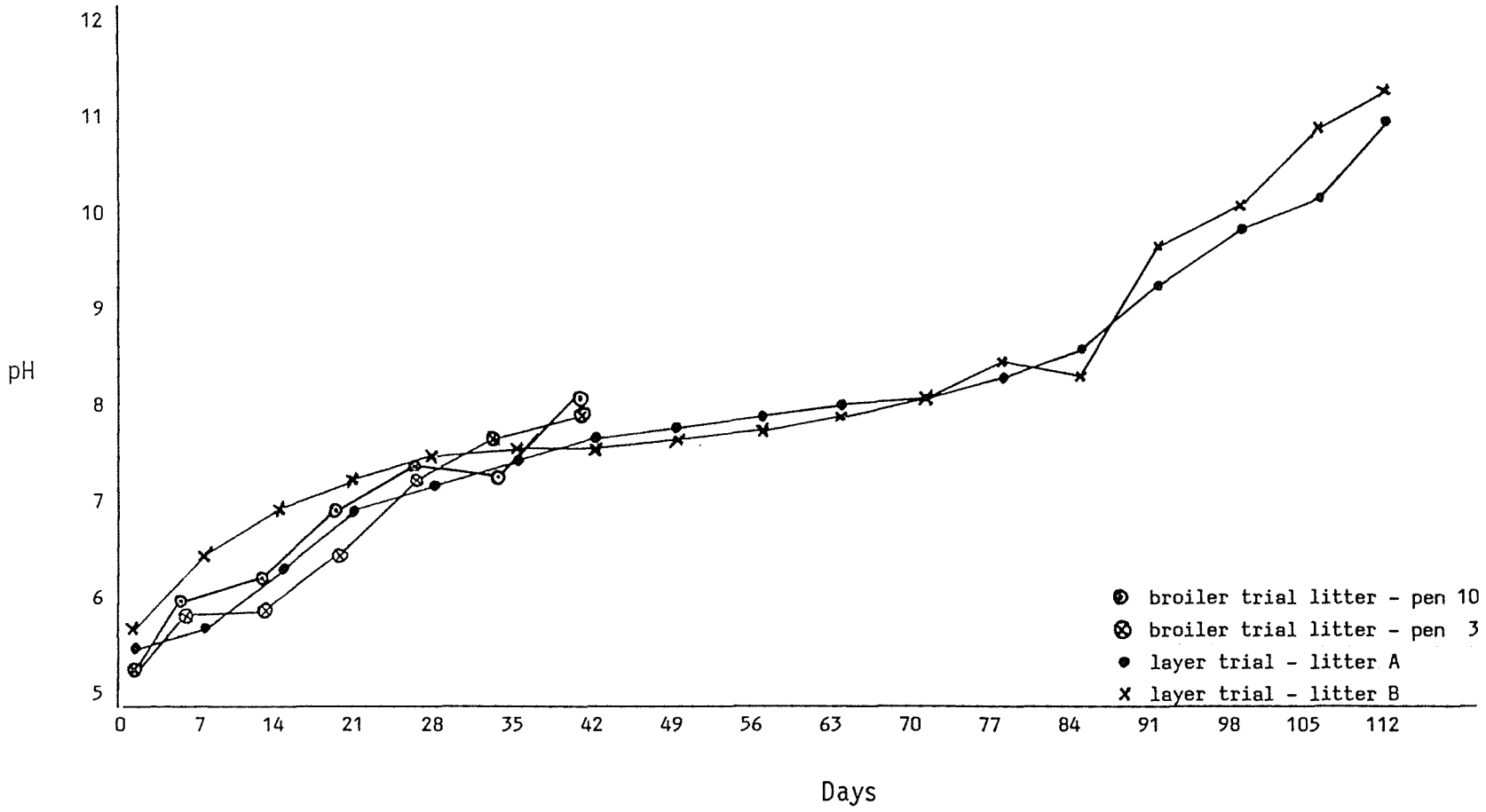
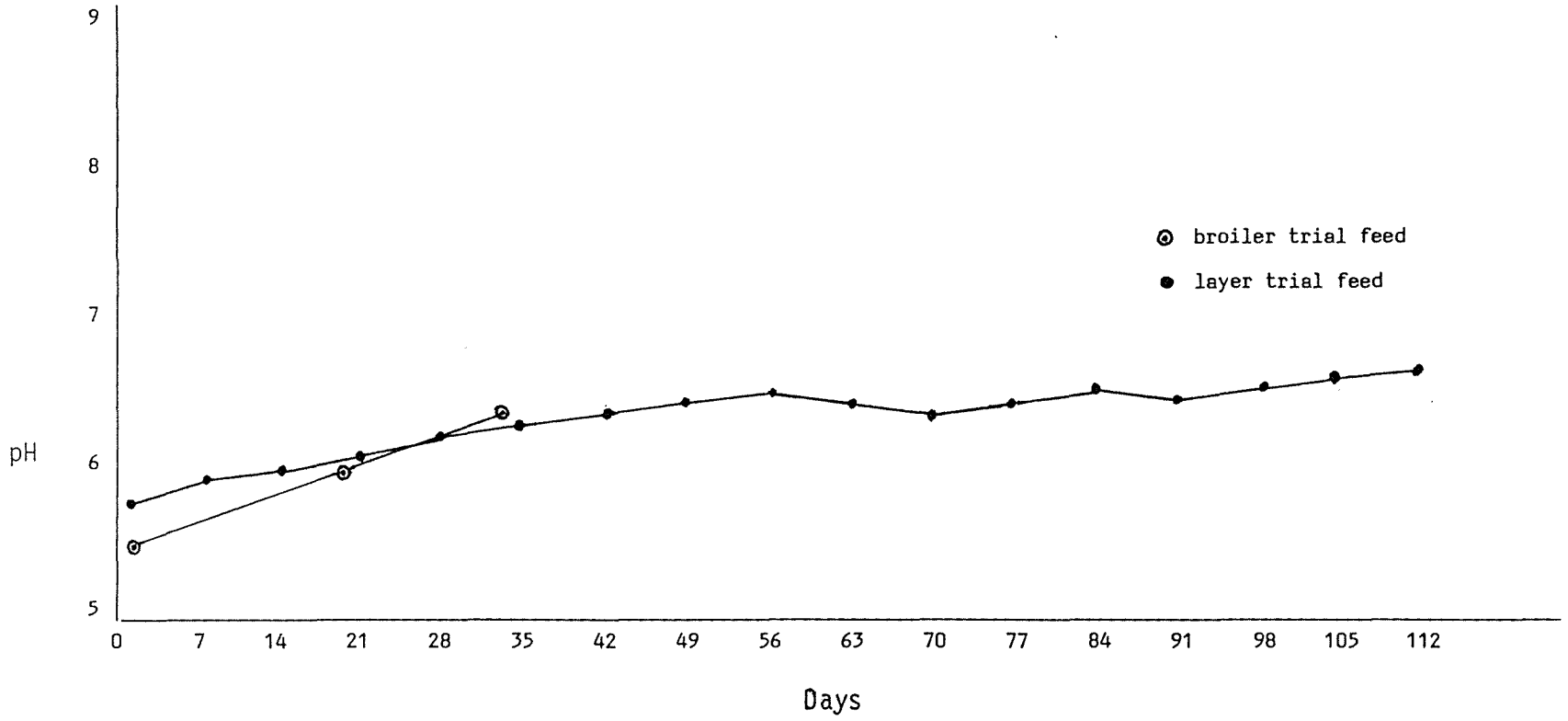


Figure 5. Variations in pH of poultry house feed with time of sampling.



2. TOTAL COUNTS

2.1 Total Counts on PDA at 25°C

2.1.1 Broiler trial

Fluctuations in the overall fungal numbers in the broiler trial litter, feed and air are shown in Figs. 6 and 7. Both litters (pens 10 and 3, Fig. 6) showed a peak in total counts in the middle of the trial, followed by a decline and a subsequent increase to higher levels.

Increases in overall fungal numbers were also evident in the air and feed of the broiler house (Fig. 7).

2.1.2 Layer trial

Weekly changes in the total fungal population of the layer trial are shown in Figs. 8 and 9. Litters A and B showed increases from 52 CFU g^{-1} (average) on day 1 to 642 CFU g^{-1} on day 56, with a subsequent levelling-off of numbers (Fig. 8).

In the feed, numbers increased from 72 CFU g^{-1} to a peak of 348 CFU g^{-1} on day 56 and then decreased (Fig. 9). In the air numbers increased steadily from 14 colonies on day 1 to >400 colonies on day 56 and remained at very high (uncountable) levels (Fig. 9).

Figure 6. Total fungal counts of broiler house litter samples, using PDA, 25°C.

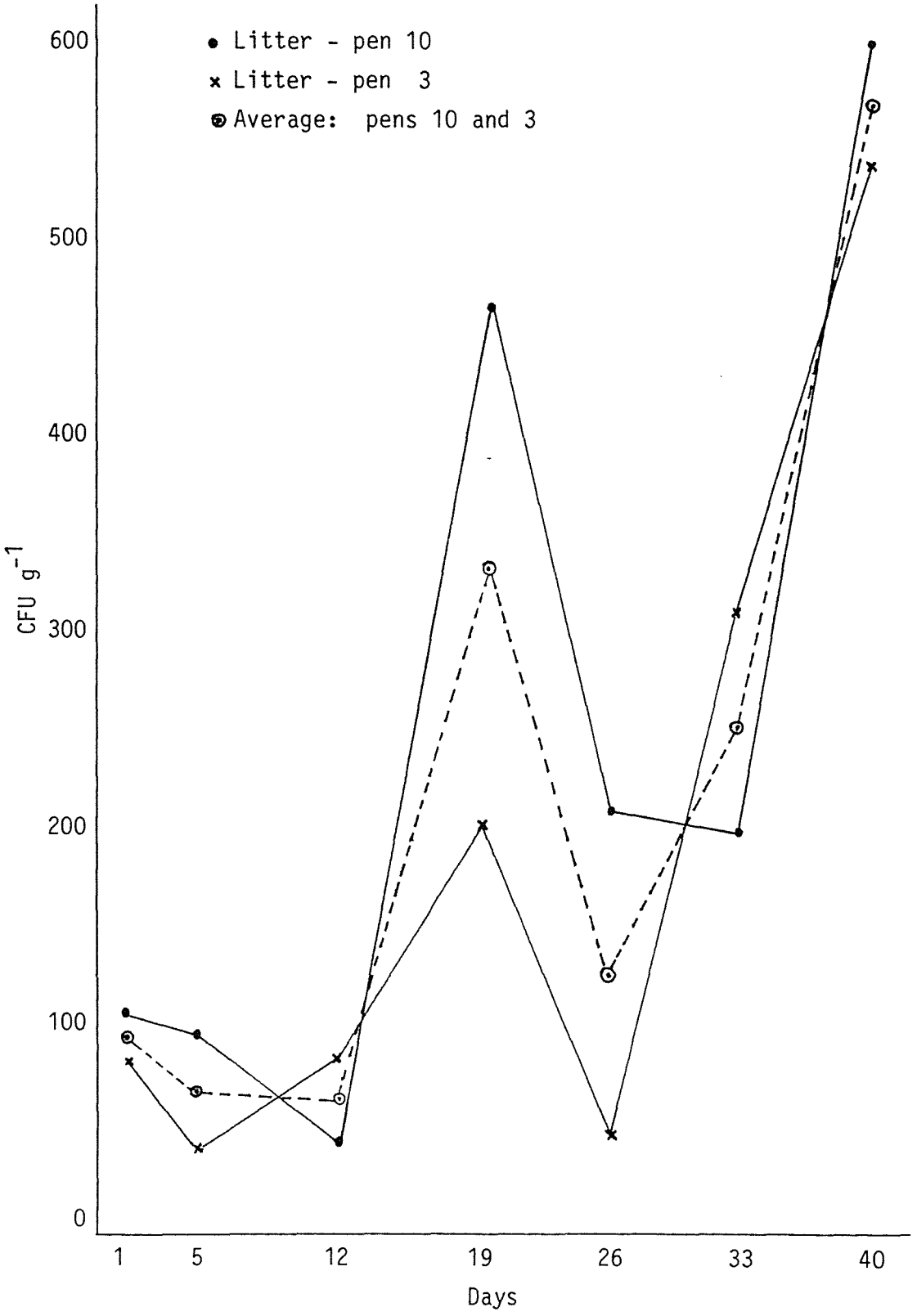


Figure 7. Total fungal counts of broiler house feed and air samples using PDA, 25°.

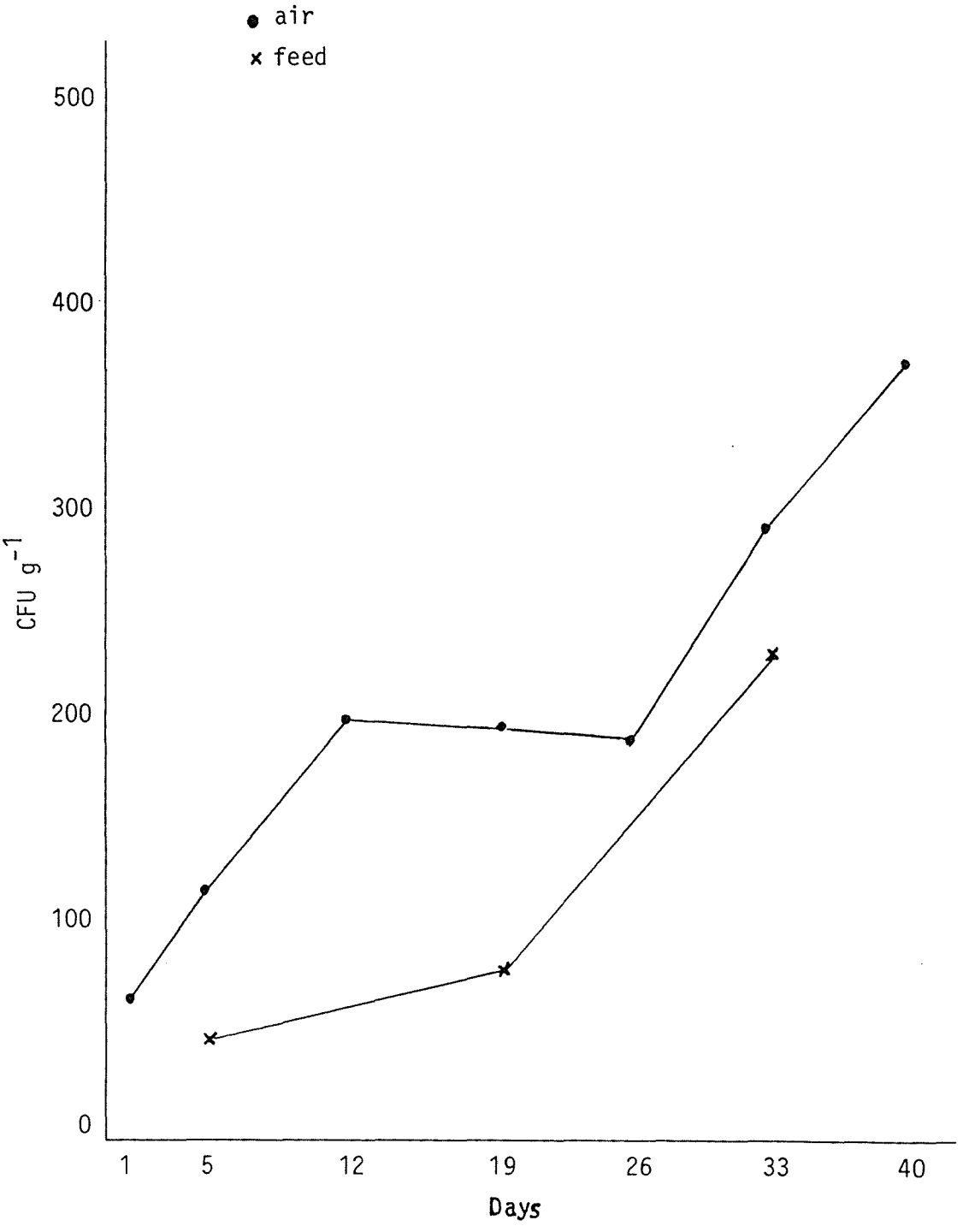


Figure 8. Total fungal counts of layer house litter samples using PDA, 25°C.

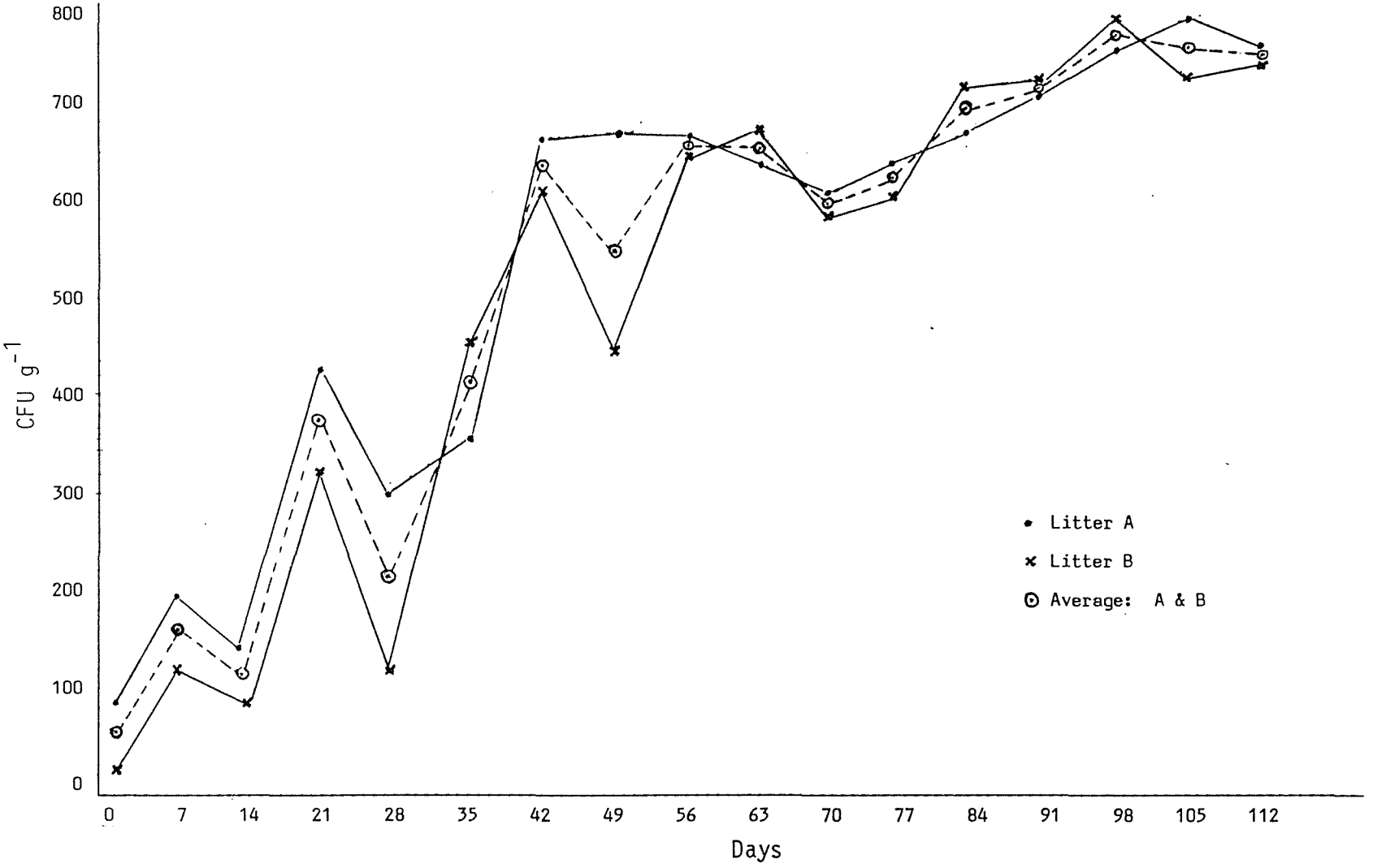
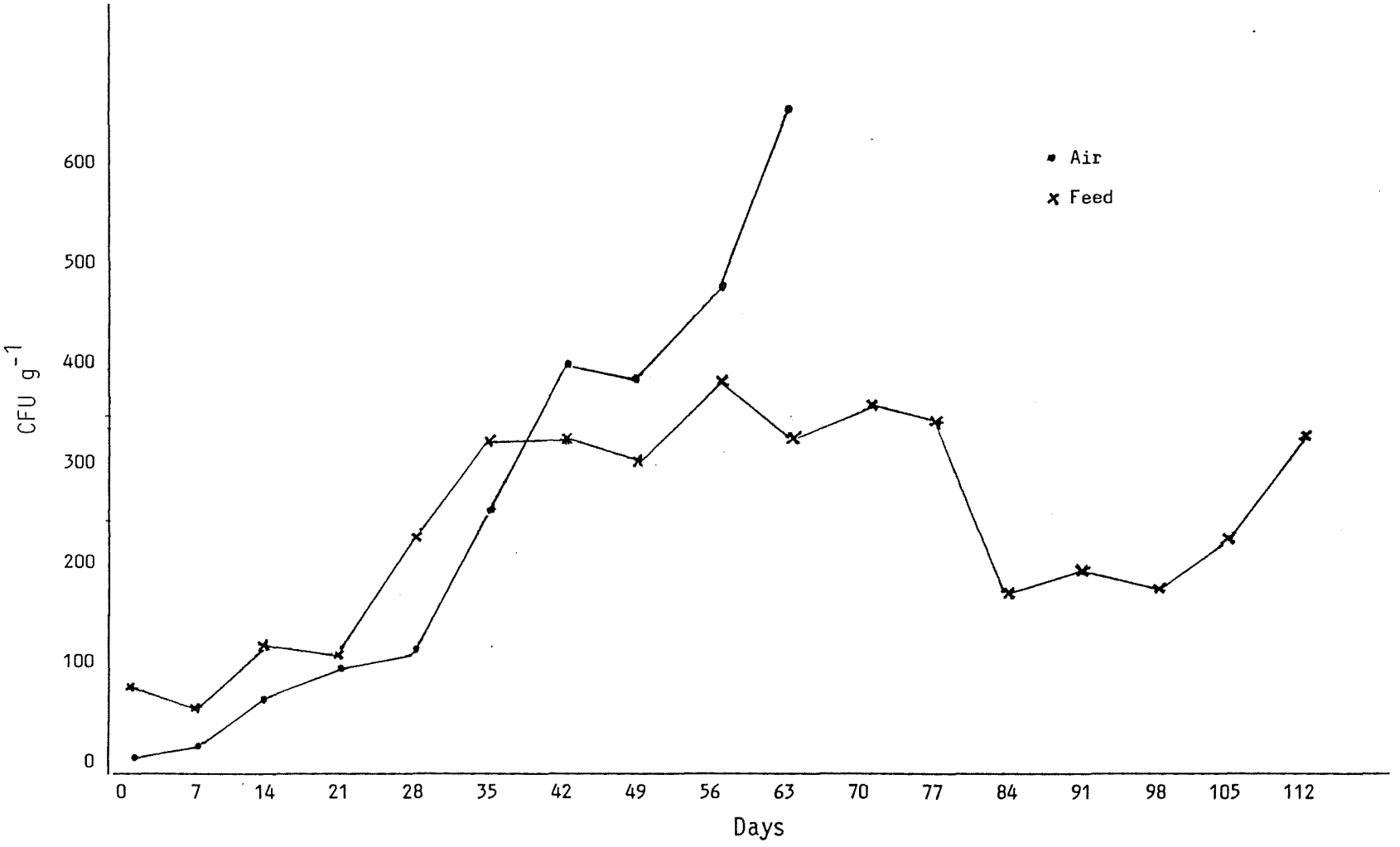


Figure 9. Total fungal counts of layer house feed and air samples using PDA, 25°C.



2.2 Total Counts on ADM at 37°C

2.2.1 Broiler trial

Changes in overall fungal counts are shown in Figs. 10 and 11. In pen 10, a small increase in total counts in litter was observed, numbers remaining below 100 CFU g^{-1}. In pen 3 a peak of 164 CFU g^{-1} was observed in the litter, on day 26, followed by a decline to 62 CFU g^{-1}. Total numbers were low in the feed (Fig. 10) but showed a slight increase with time. The total counts in air increased steadily to >400 colonies at the end of the trial (Fig. 11).

2.2.2 Layer trial

Total fungal numbers of litter, feed and air counted on ADM are shown in Figs. 12 and 13.

The overall fungal counts on ADM at 37°C followed a similar pattern to those on PDA 25°C, for litter, feed and air. Again, numbers were lower in the feed than in the litter.

Figure 10. Total fungal counts of broiler house litter samples using ADM, 37°C.

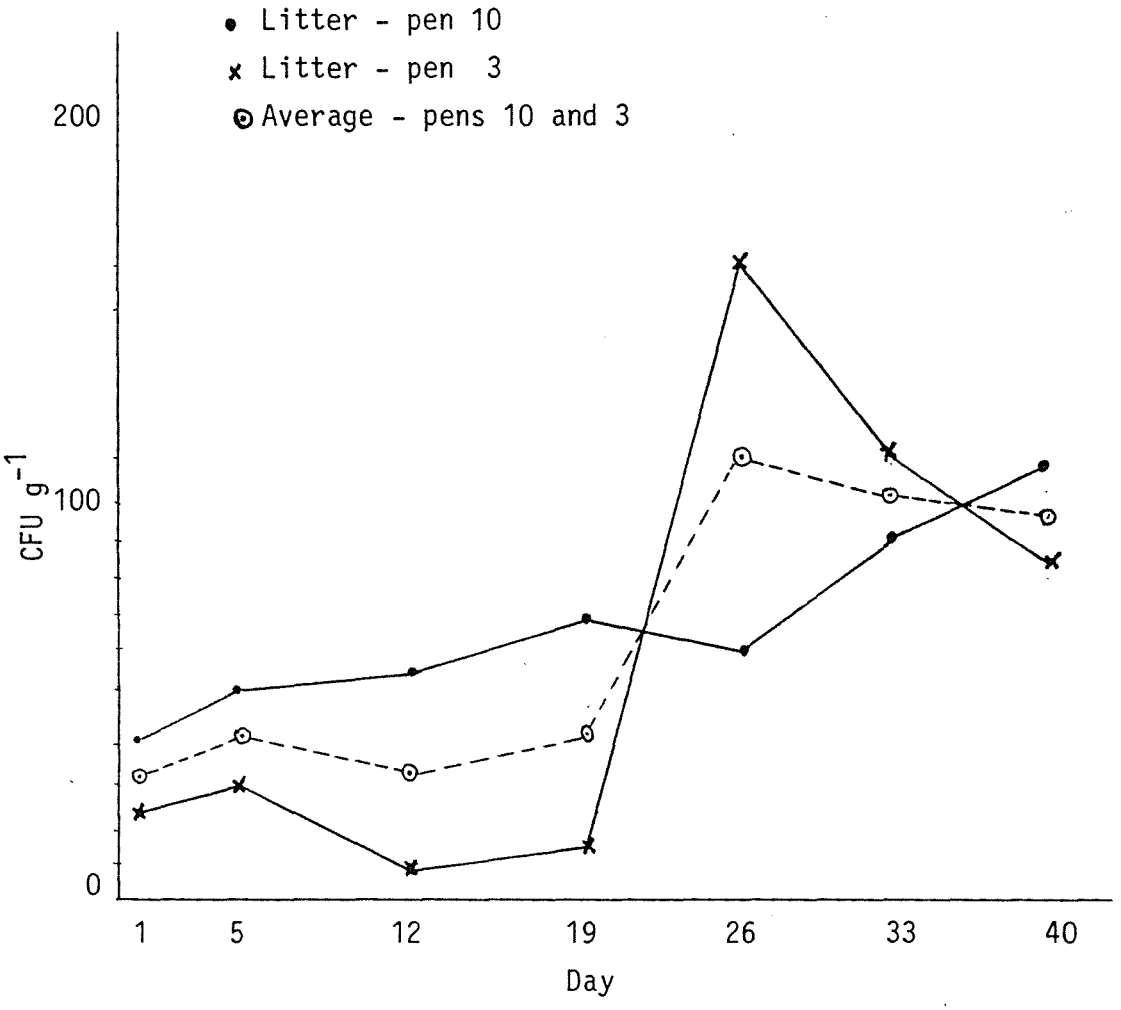


Figure 11. Total fungal counts of broiler house feed and air samples using ADM, 37°C.

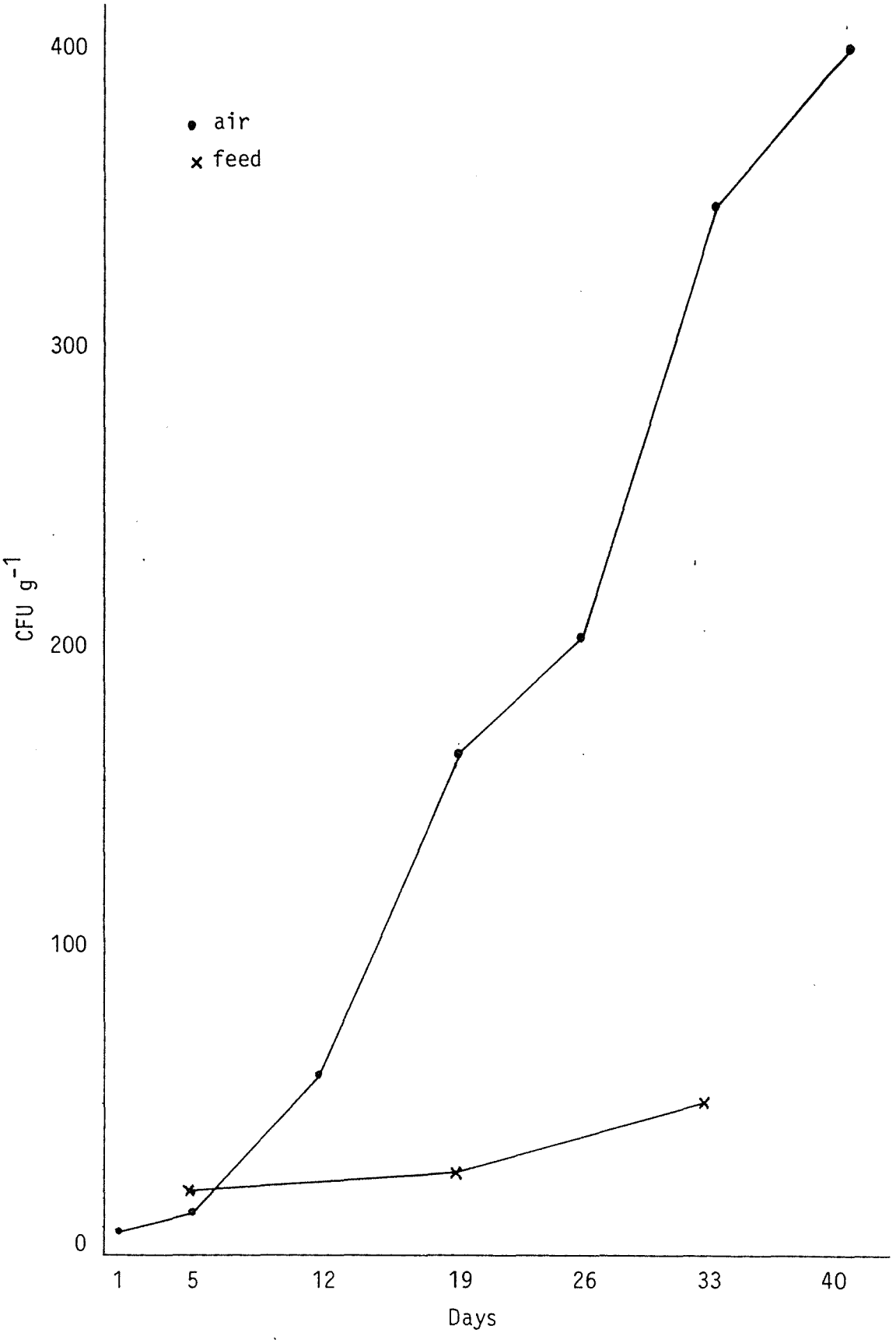


Figure 12. Total fungal counts of layer house litter samples using ADM 37°C.

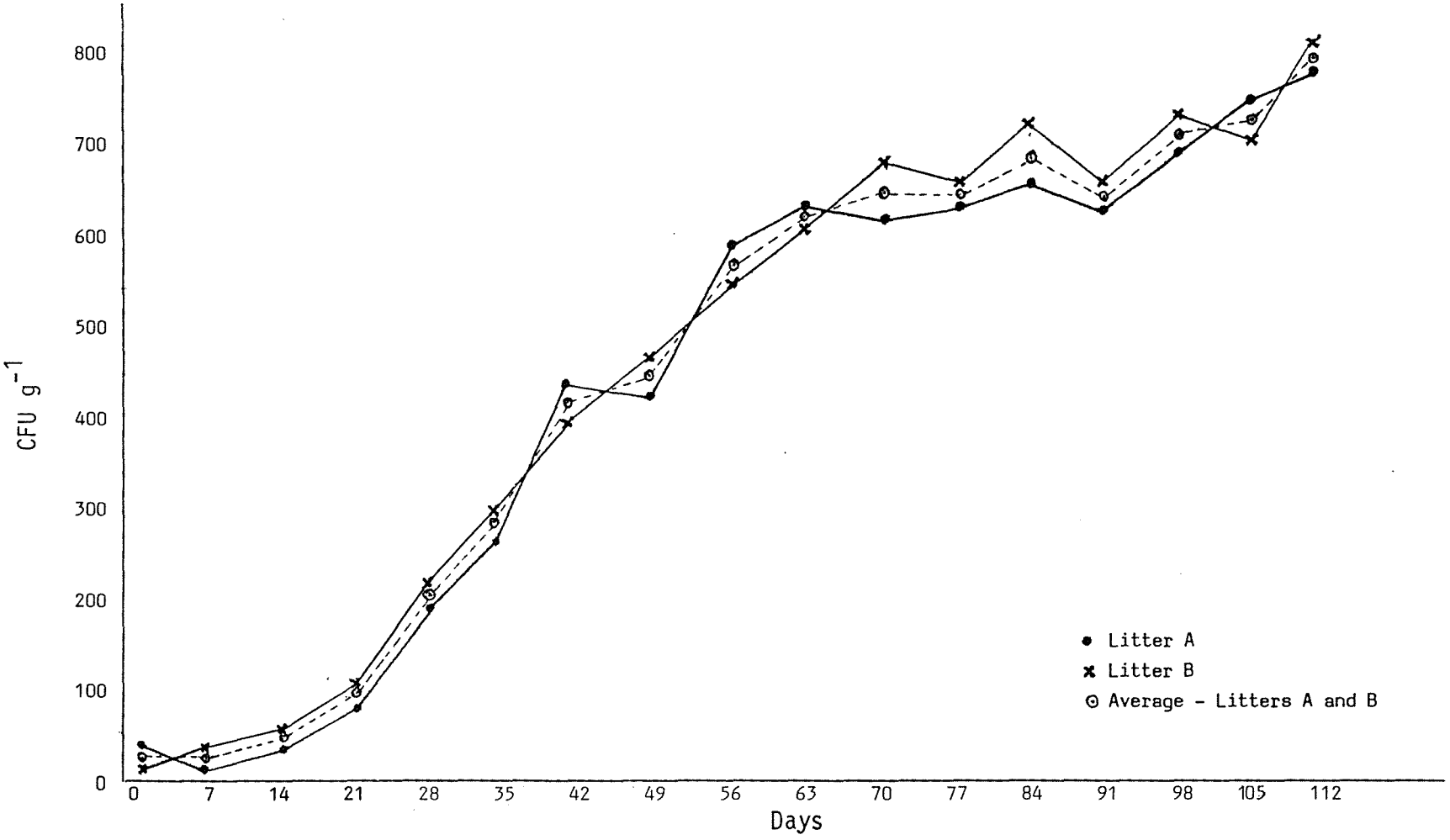
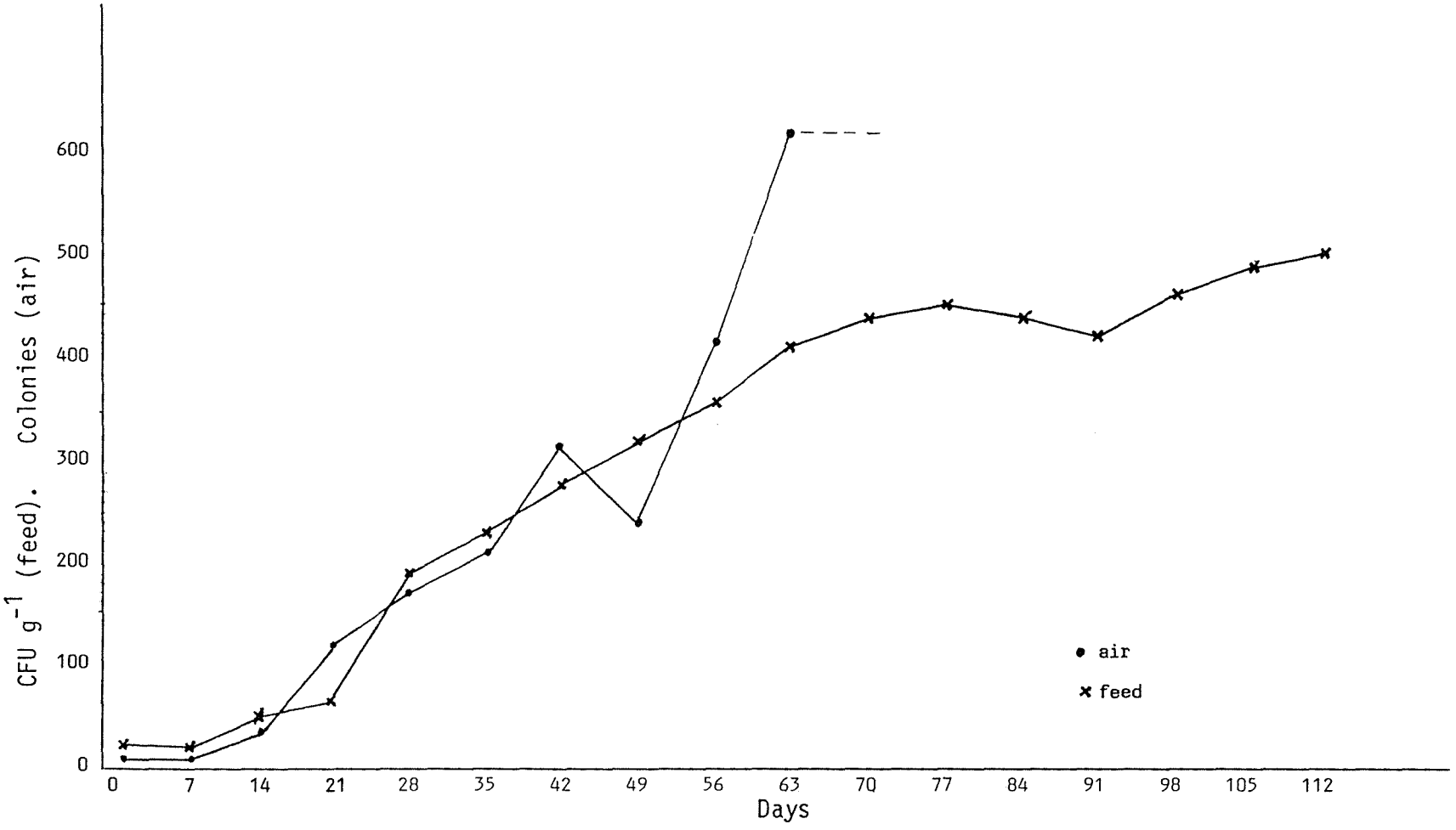


Figure 13. Total fungal counts of layer house feed and air samples using ADM 37°C.



2.3 Relationship between Sample pH and Total Mould Counts

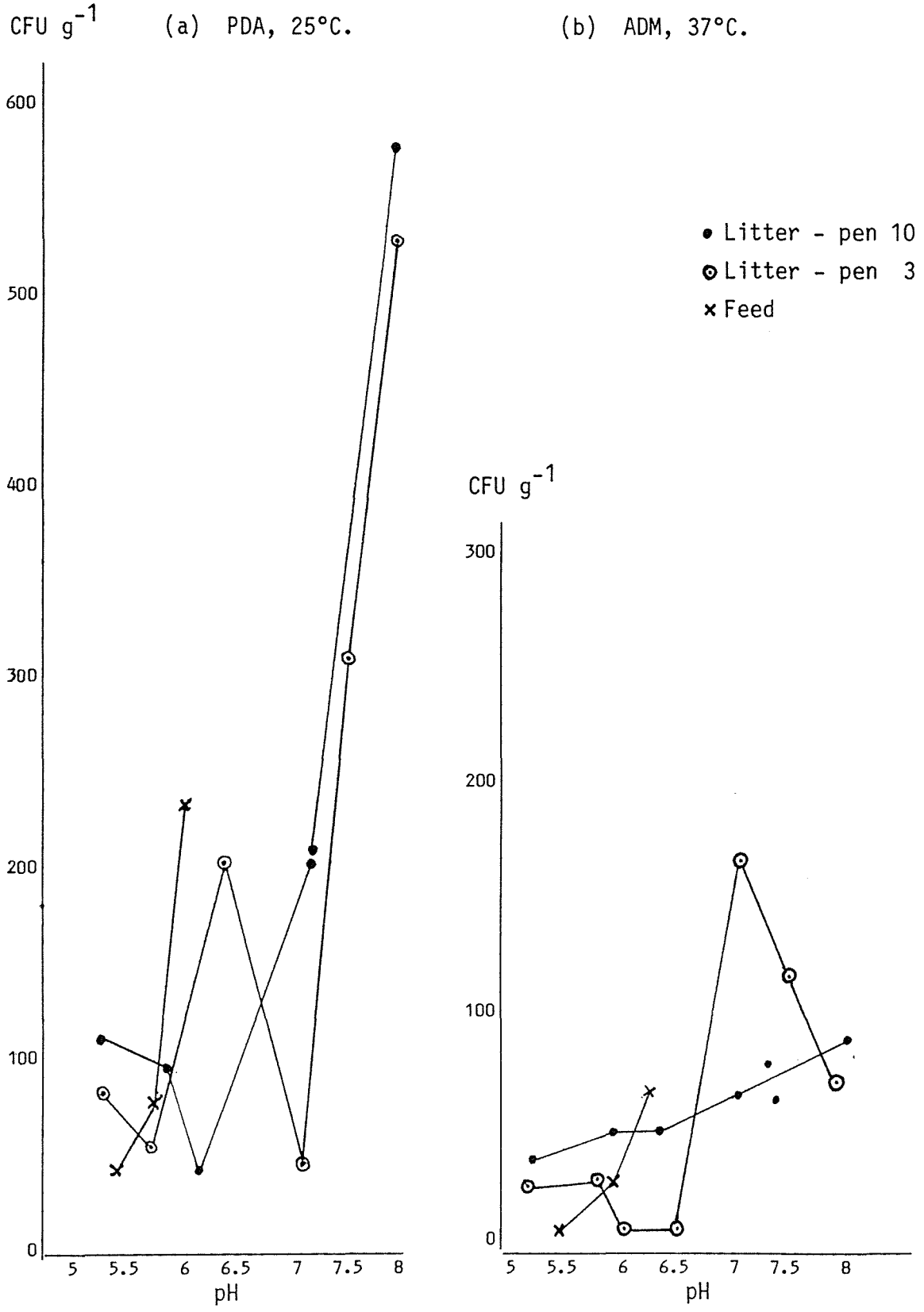
2.3.1 Broiler trial

Fig. 14 (a and b) shows the relationship between total mould counts obtained on PDA 25°C and ADM 37°C, and the pH of litter and feed samples.

PDA 25°C (Fig. 14[a]): In litter of pen 10, total counts increased greatly after a level pH 6.0 was attained. In pen 3, counts were at a medium level at pH 6.5, then declined and increased to high levels beyond pH 7.0. In feed, a gradual increase was observed in total counts, in relation to sample pH.

ADM, 37°C (Fig. 14[b]): In pen 10 litter total counts increased gradually in relation to pH; in pen 3 levels remained steady up to pH 6.5 but then an increase was observed up to pH 7.1 followed by a decline. A slight increase in total numbers was observed in the feed.

Figure 14. Relationship between total counts and pH of broiler house litter and feed samples.



2.3.2 Layer trial

Figs. 15 and 16 show the relationship between total mould counts obtained on PDA 25°C and ADM 37°C, and the pH of the feed and litter samples from the layer house.

PDA, 25°C (Fig. 15): In litter A, total counts increased up to pH 8.5 to a peak of 624 CFU g^{-1}, then decreased and increased again after pH 9. Thus the general trend was an increase in total numbers in relation to increase in pH. This trend was followed by litter B up to 648 CFU g^{-1} at pH 7.8 after which there was a small drop in total counts, followed by an increase and a fairly constant level after pH 8.5. In feed, a sharp rise in total numbers was seen between pH 6.0 and 6.2 after which there was a drop and a subsequent small rise. The total numbers in feed stayed relatively low (<320 colonies).

ADM, 37°C (Fig. 16): In both litters, total numbers rose to high levels in relation to the increase in pH values, showing very similar trends to those on PDA. As with PDA results, feed numbers remained lower than litters.

Figure 15. Relationship between total counts using PDA 25°C and pH of layer house litter and feed samples.

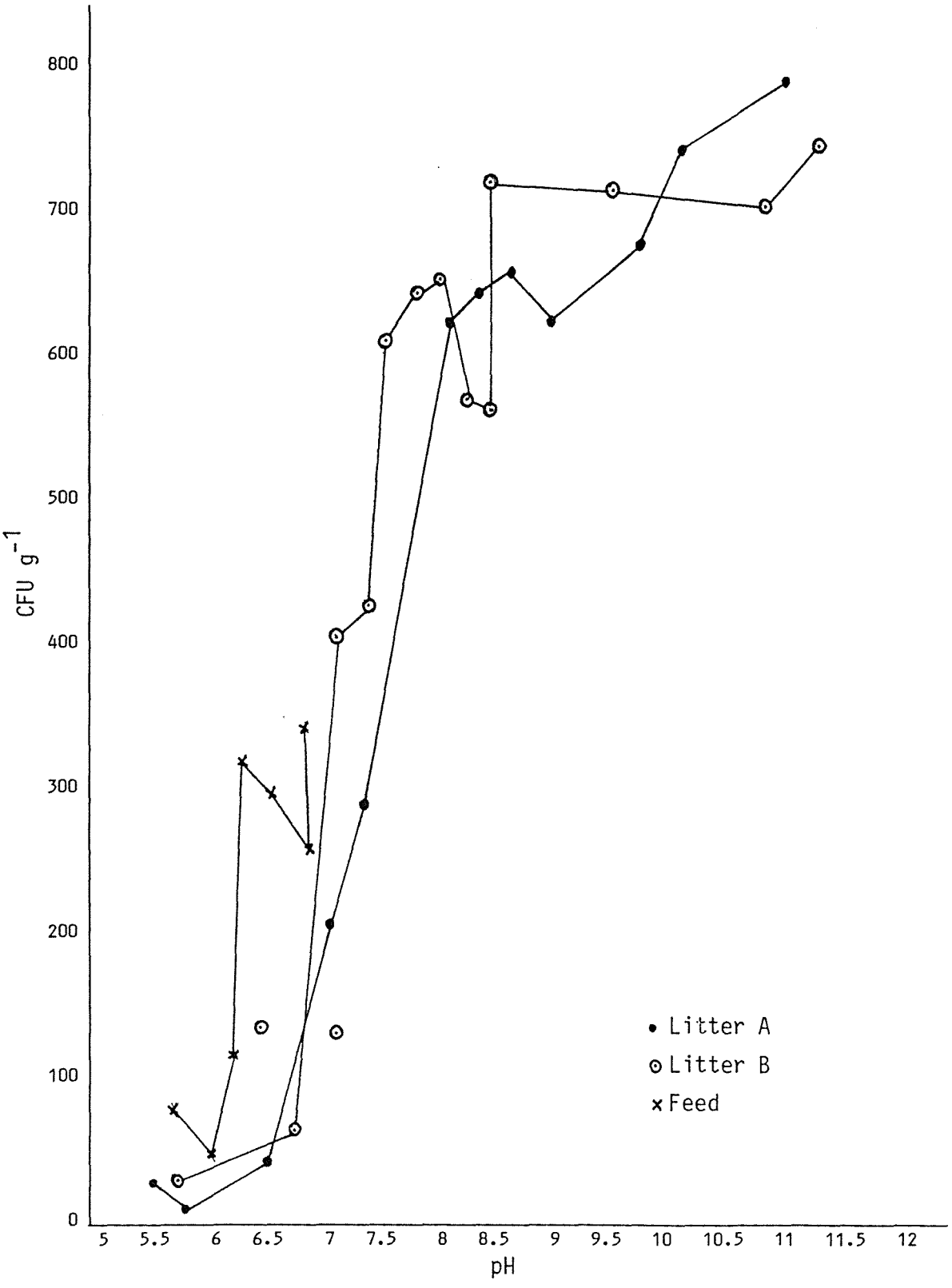
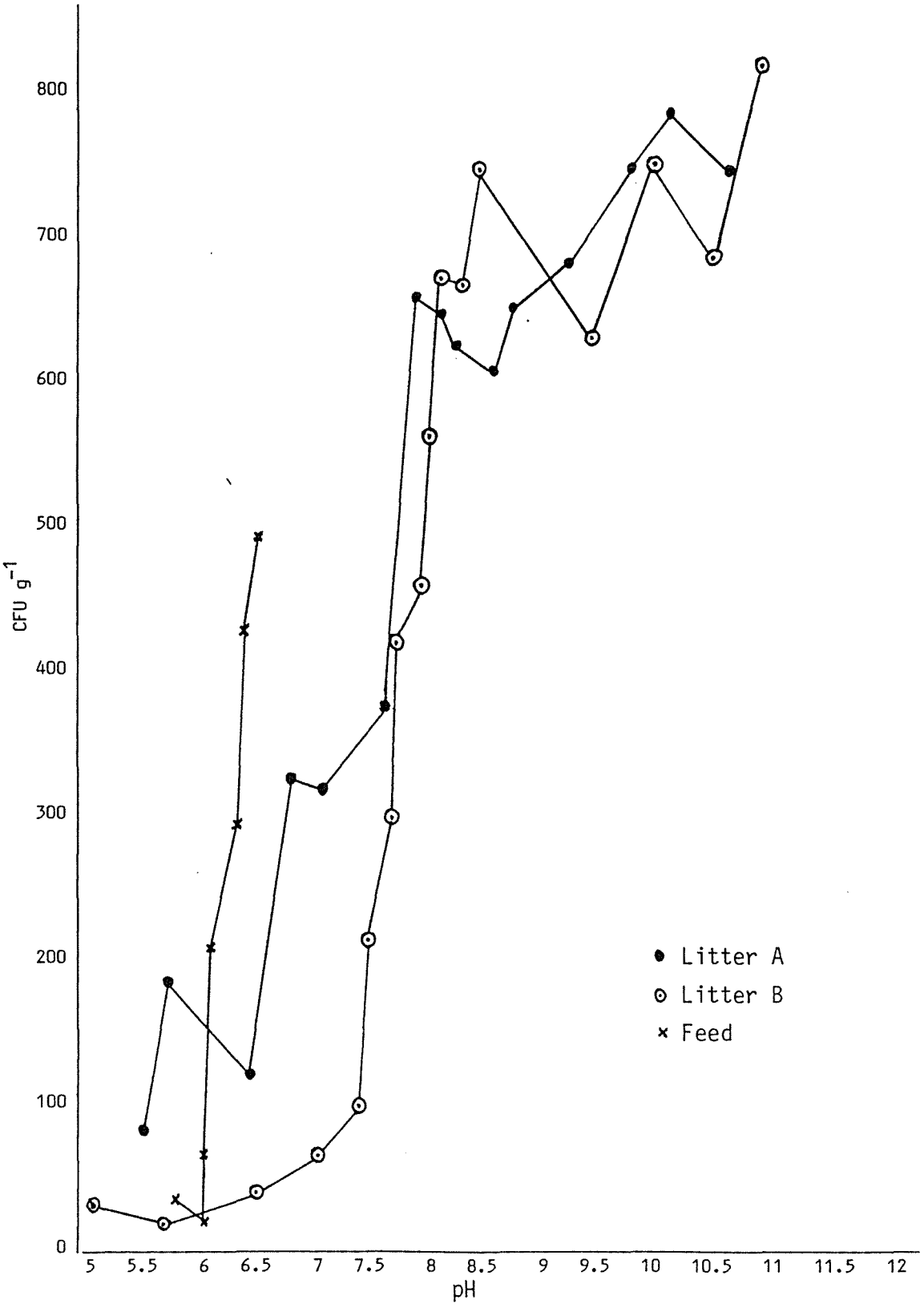


Figure 16. Relationship between total counts using ADM 37°C and pH of layer house litter and feed samples.



2.4 Relationship between Sample Moisture Content and Total Mould Counts

2.4.1 Broiler trial

Figures 17 and 18 show the relationship between total mould counts obtained on PDA 25°C and ADM 37°C, and the m.c. of litter and feed samples.

PDA, 25°C (Fig. 17): In pen 10 litter, low to medium counts were observed except for a peak to 200 CFU g^{-1} between 8% and 9% m.c. Numbers increased after m.c. exceeded 10.5%. A similar pattern was seen in the litter of pen 3. In feed, the low total counts (<100 CFU g^{-1}) corresponded to low m.c.'s (<6.5%).

ADM, 37°C (Fig. 18): Numbers in pen 10 litter increased gradually, whereas those of pen 3 declined after peaking at 9.4% m.c. Feed numbers were low, as were m.c.'s.

2.4.2 Layer trial

Figs. 19 and 20 show the relationship between total mould counts obtained on PDA 25°C and ADM 37°C, and the m.c. of layer house litter and feed samples.

PDA, 25°C (Fig. 19): In both litters, a general increase in total counts accompanied the rise in m.c. 18% and 19% m.c. corresponded with counts of 790-800 CFU g^{-1}. In feed, lower total counts (<350 CFU g^{-1}) corresponded with low m.c.'s (<8%).

ADM, 37°C (Fig. 20): the total counts in both litters increased (with small fluctuations) as m.c. increased. Low m.c.'s (7%) at the beginning corresponded with low (<50 CFU g^{-1}) total counts. Higher m.c.'s (>17%) accompanied high total counts (>650 CFU g^{-1}). Feed m.c.'s were lower (<8%) and so were total counts (<500 CFU g^{-1}).

Figure 17. Relationship between total counts using PDA 25°C and m.c. of broiler house litter and feed samples.

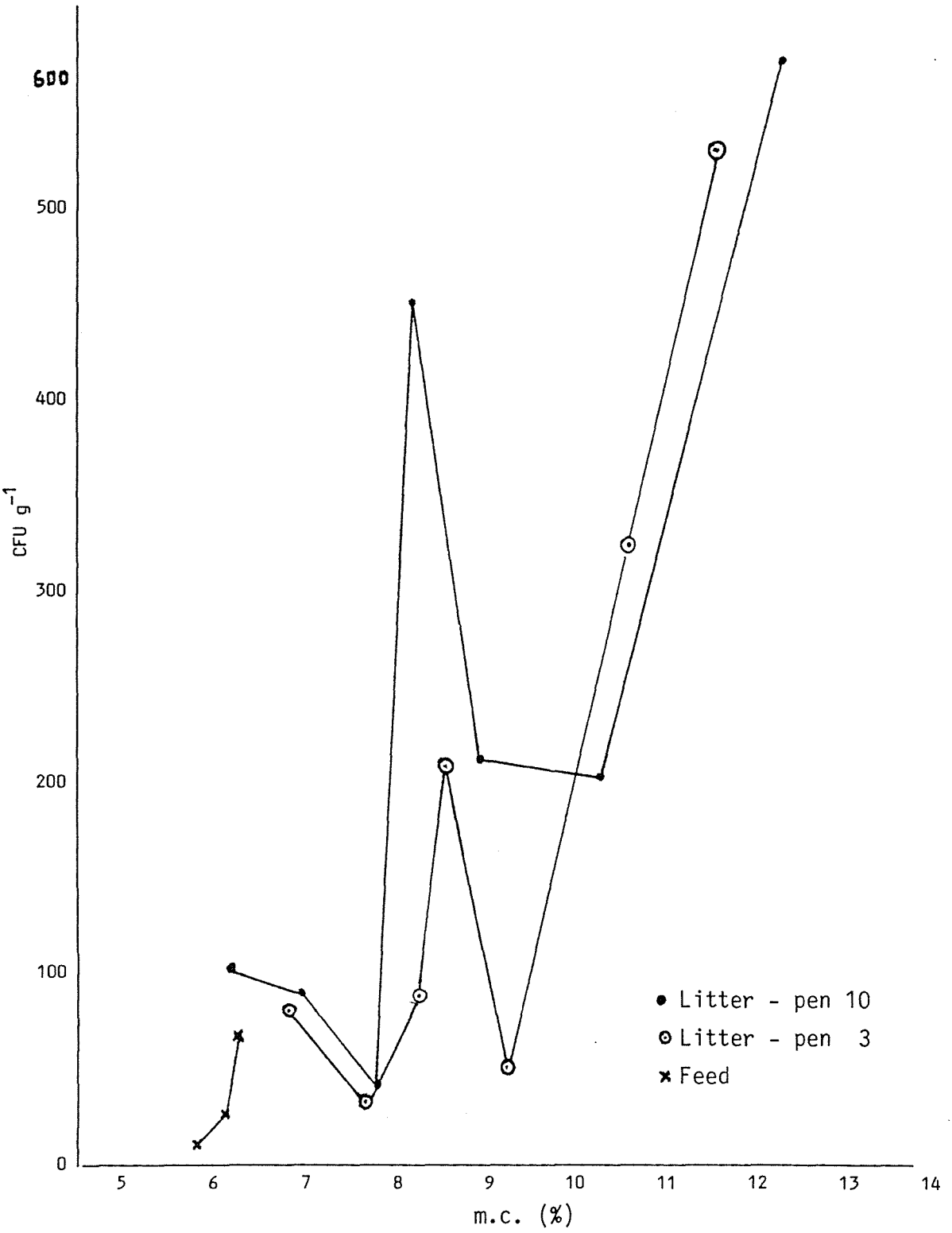


Figure 18. Relationship between total counts using ADM 37°C and m.c. of broiler house litter and feed samples.

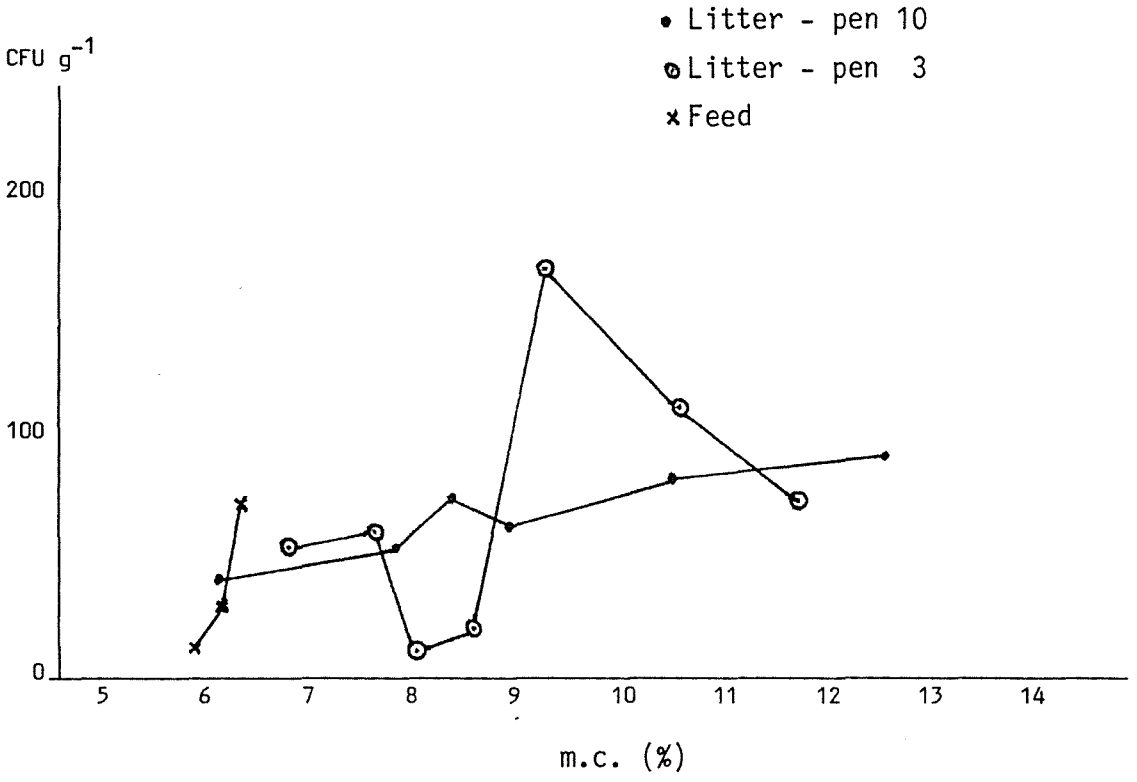


Figure 19. Relationship between total counts using PDA 25°C and m.c. of layer house litter and feed samples.

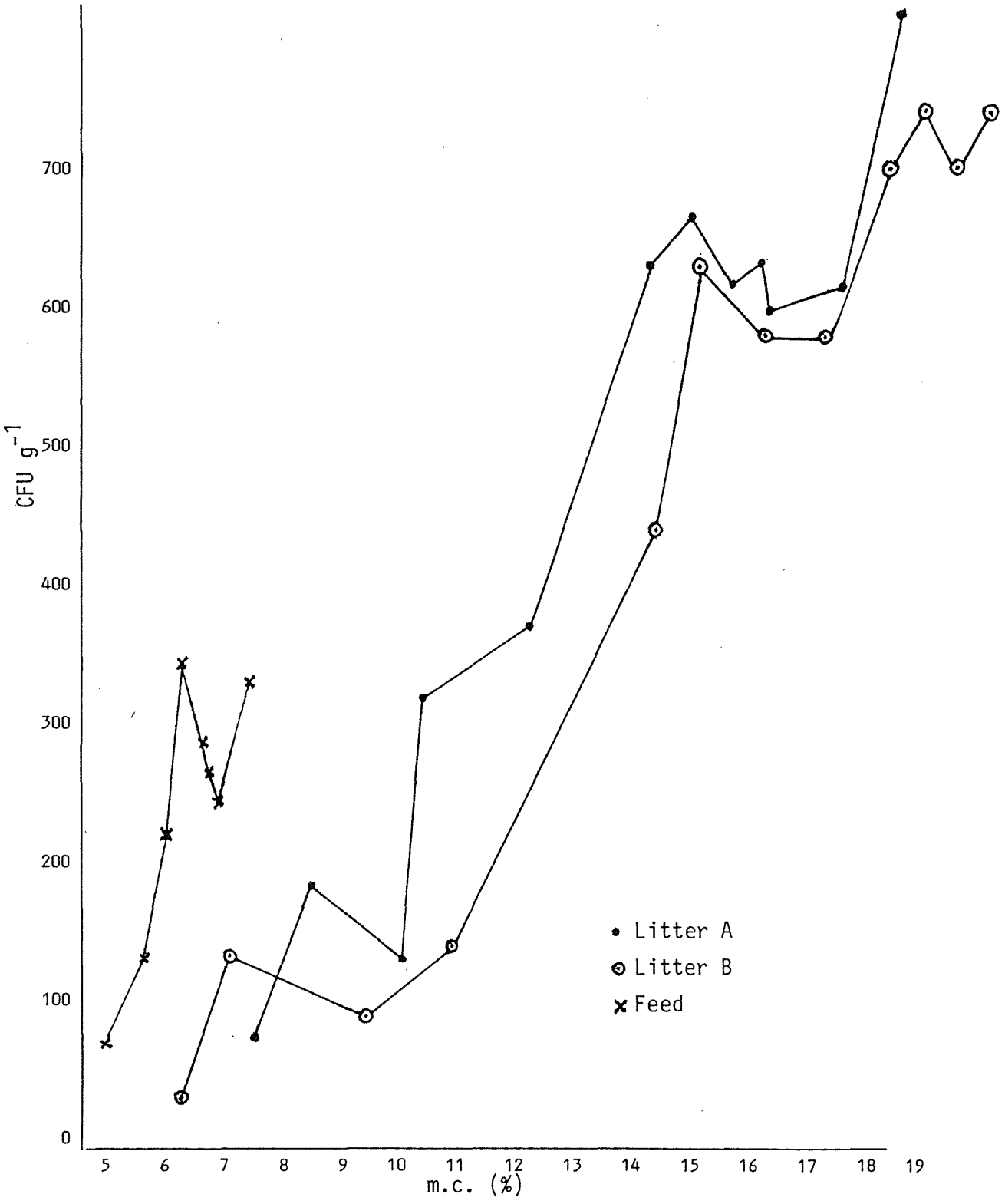
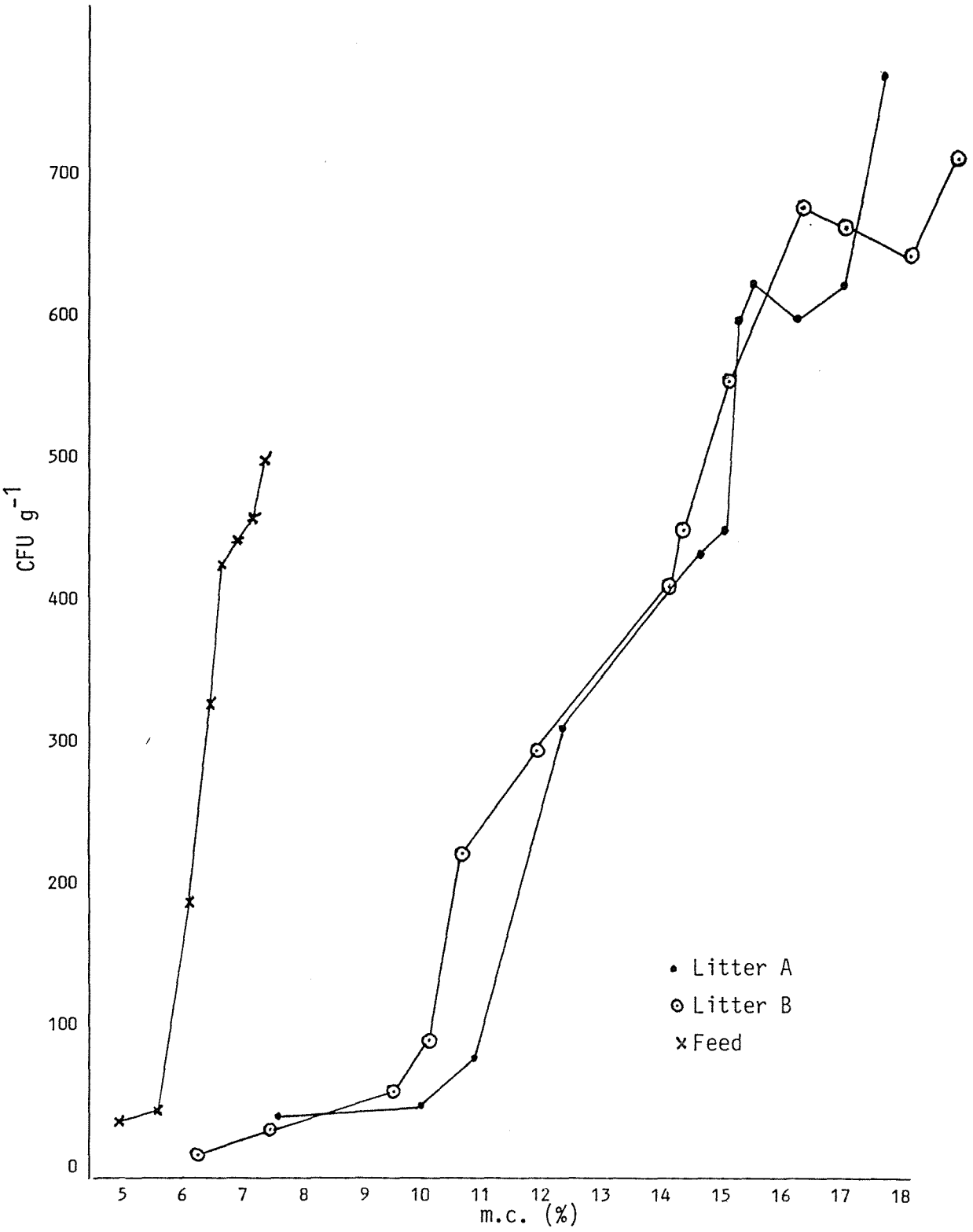


Figure 20. Relationship between total counts using ADM 37°C and m.c. of layer house litter and feed samples.



3. SUCCESSION OF GENERA

In both trials, the 10^{-1} dilution gave the greatest variety of fungal isolations, in countable numbers. Differential counts of genera were determined on PDA at 25°C and on ADM at 37°C.

3.1 Broiler House Samples

3.1.1 Litter

PDA, 25°C (Fig. 21): Levels of Penicillium spp. varied from 40 to 210 CFU g^{-1} during the sampling period. A. fumigatus was present at high levels on days 19 and 40 only. Scopulariopsis appeared only towards the end of the period (days 33 and 40). A. flavus was only found in high numbers on days 26 and 33, although this species was detected in small numbers on days 1 and 5. Cladosporium was isolated in small numbers only up to day 19.

ADM, 37°C (Fig. 22): Numbers were lower under these incubation conditions, than on PDA, 25°C. Scopulariopsis increased gradually from day 26 to day 40. A. fumigatus was only seen at the end. No definite trend was observed with Penicillium, Paecilomyces and Rhizopus. Trichosporon cutaneum increased in numbers between days 12 and 26, but then declined. A. flavus first occurred at day 26, increased to 60 and 64 CFU g^{-1} in pens 10 and 3 respectively at day 33, and then disappeared.

3.1.2 Feed

PDA, 25°C (Fig. 23): Penicillium (P. chrysogenum) was found in medium levels in all 3 samples. Scopulariopsis and A. fumigatus were at high levels at day 33 only. Low numbers of Cladosporium were found on days 5 and 33.

ADM, 37°C (Fig. 24): P. chrysogenum increased from 10 to 60 CFU g^{-1} between days 5 and 33. T. cutaneum and A. fumigatus were found in low numbers.

Figure 21. Changes in the levels of fungal genera on PDA 25°C in broiler house litter samples with age.

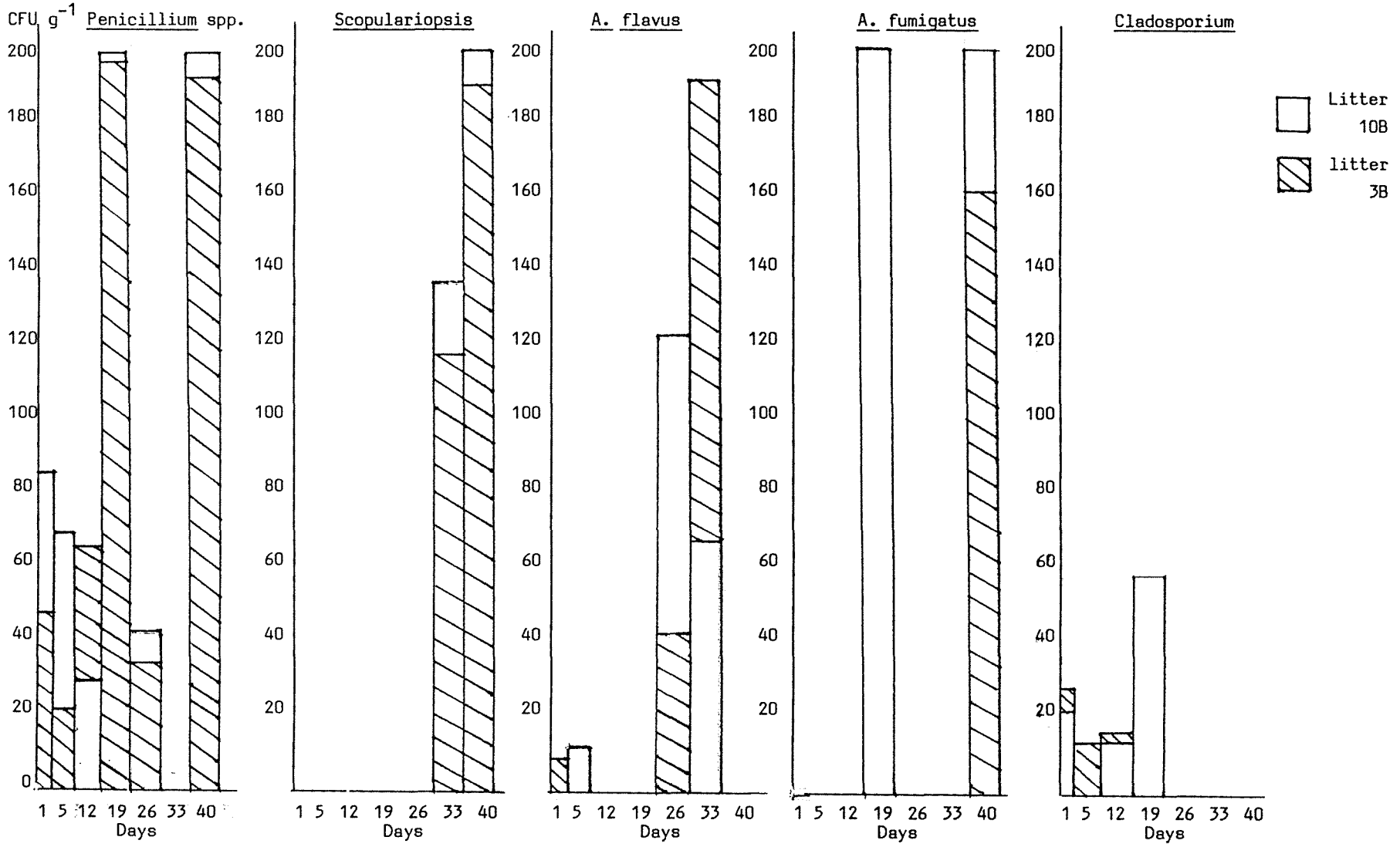


Figure 22. Changes in the levels of fungal genera on ADM 37°C in broiler house litter samples with age.

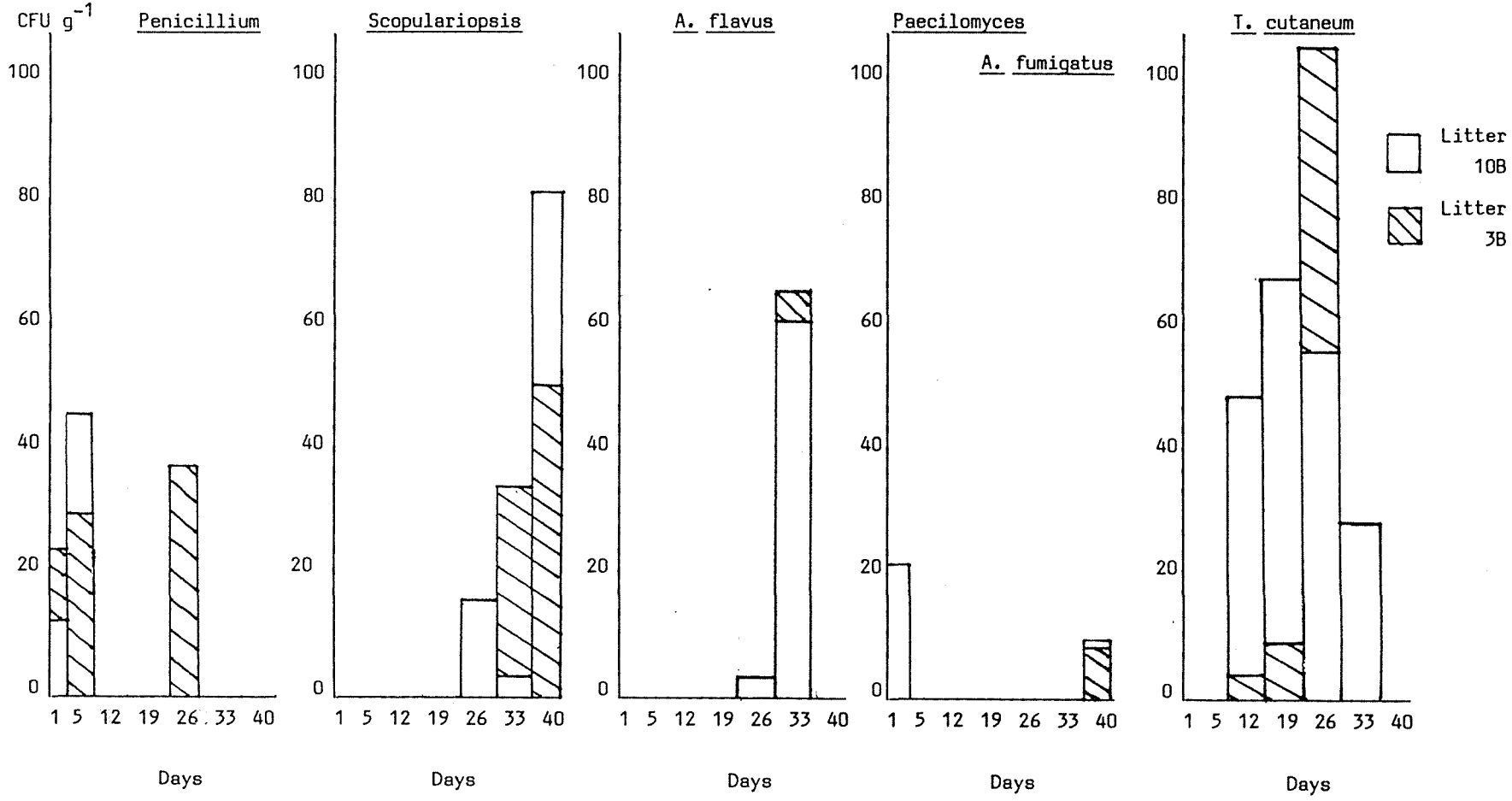


Figure 23. Changes in the levels of fungal genera on PDA 25°C in broiler house feed samples with age.

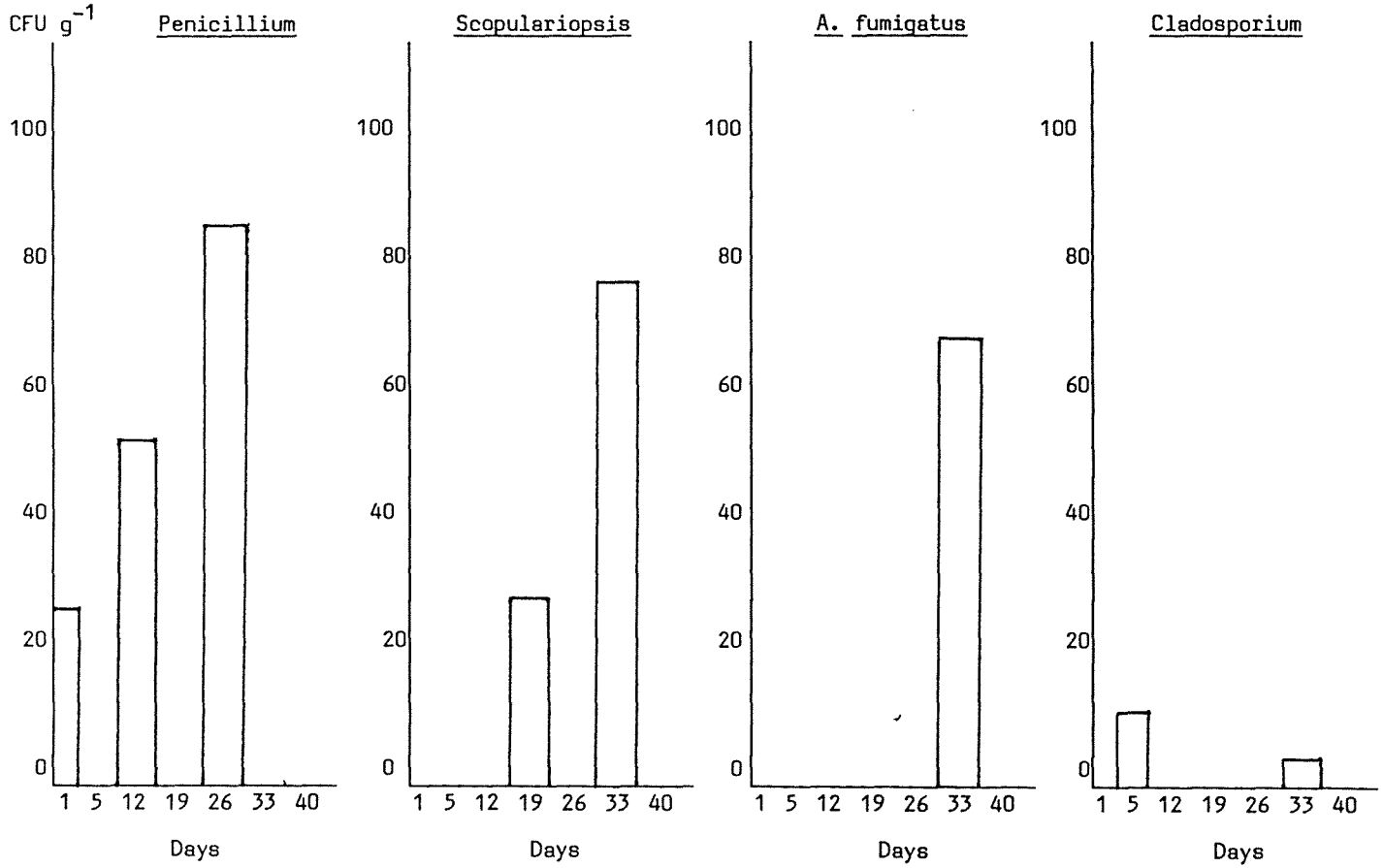
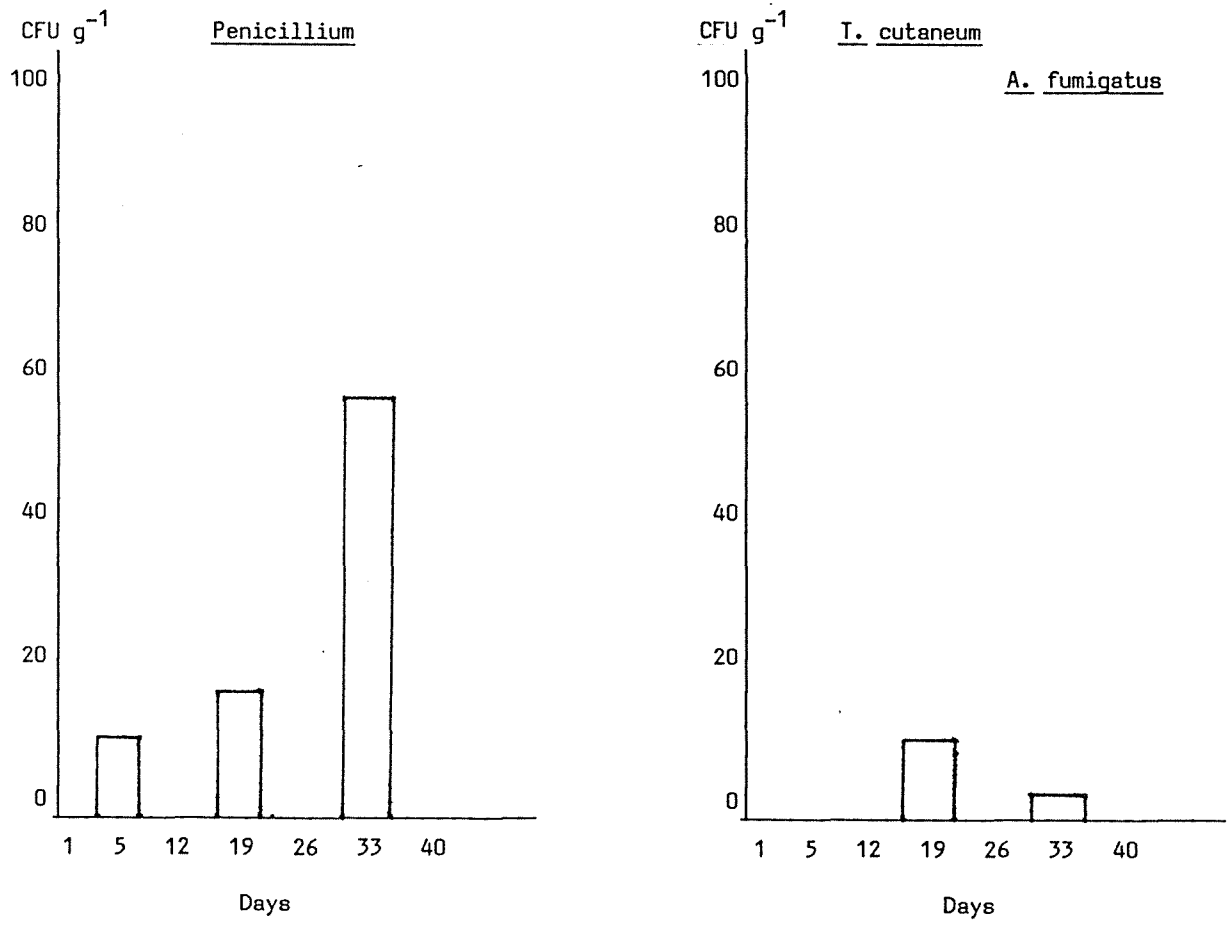


Figure 24. Changes in the levels of fungal genera on ADM 37°C in broiler house feed samples with age.



3.1.3 Air samples

Patterns of fungal succession wre also observed in the air/dust of the poultry shed. As with the dilution plating results for feed and litter, the greatest variety in air counts was obtained with PDA at 25°C and ADM at 37°C.

Patterns of fungal succession in the air of the broiler house are shown in Figs. 25 and 26.

PDA, 25°C (Fig. 25): P. chrysogenum, A. flavus, A. fumigatus and Scopulariopsis showed a marked increase in the air, as the trial progressed. Penicillium was in fairly high levels throughout. A. terreus showed a slight peak at day 12. Cladosporium and T. cutaneum were isolated in comparatively low numbers.

ADM, 37°C (Fig. 26): All moulds isolated increased during the trial except T. cutaneum which showed a decrease after day 26. Penicillium showed a peak on day 19, then declined and showed a subsequent increase. A. terreus was found only at the beginning, on days 5 and 12.

Figure 25. Changes in the levels of fungal genera in broiler house air with time, using PDA 25°C.

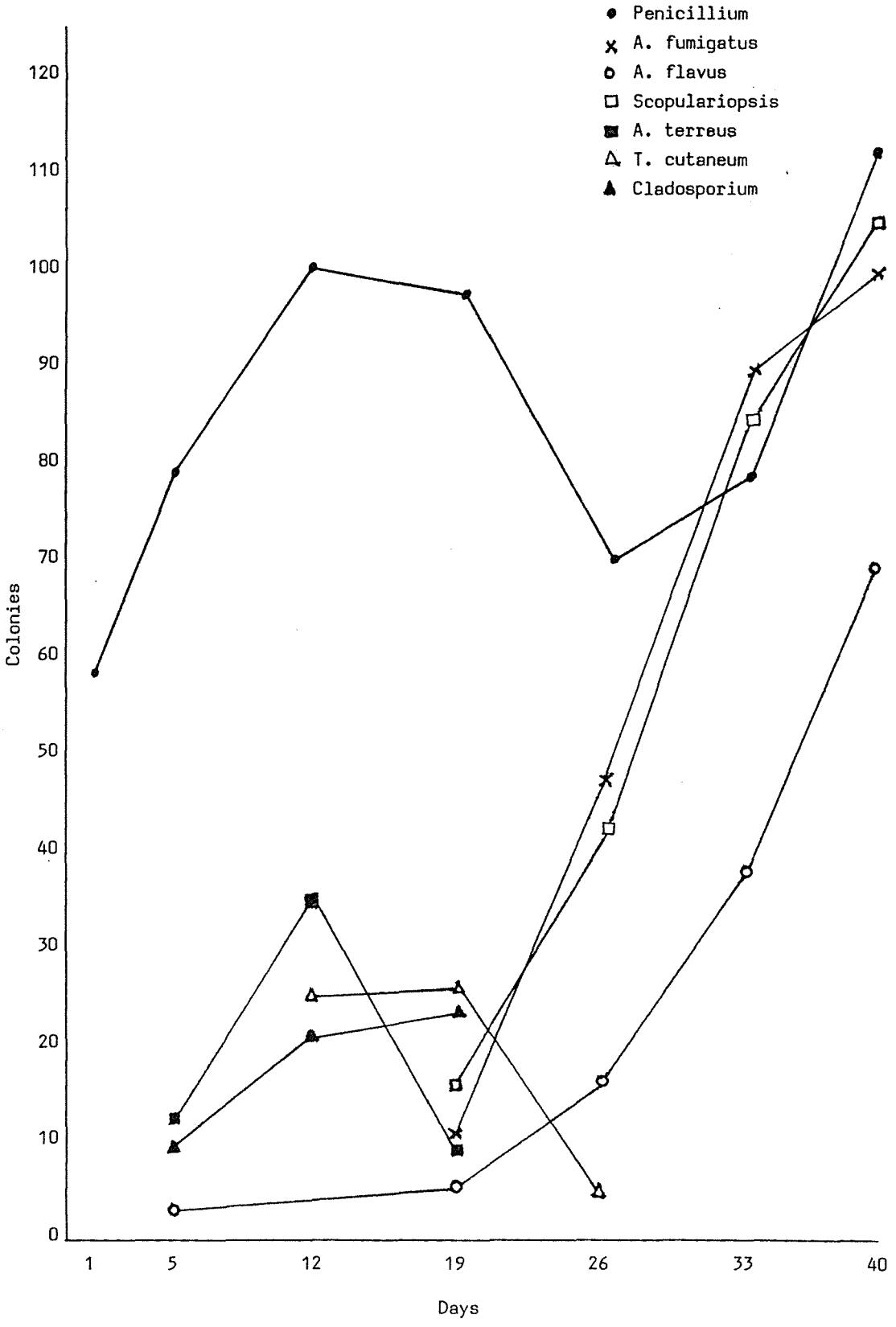
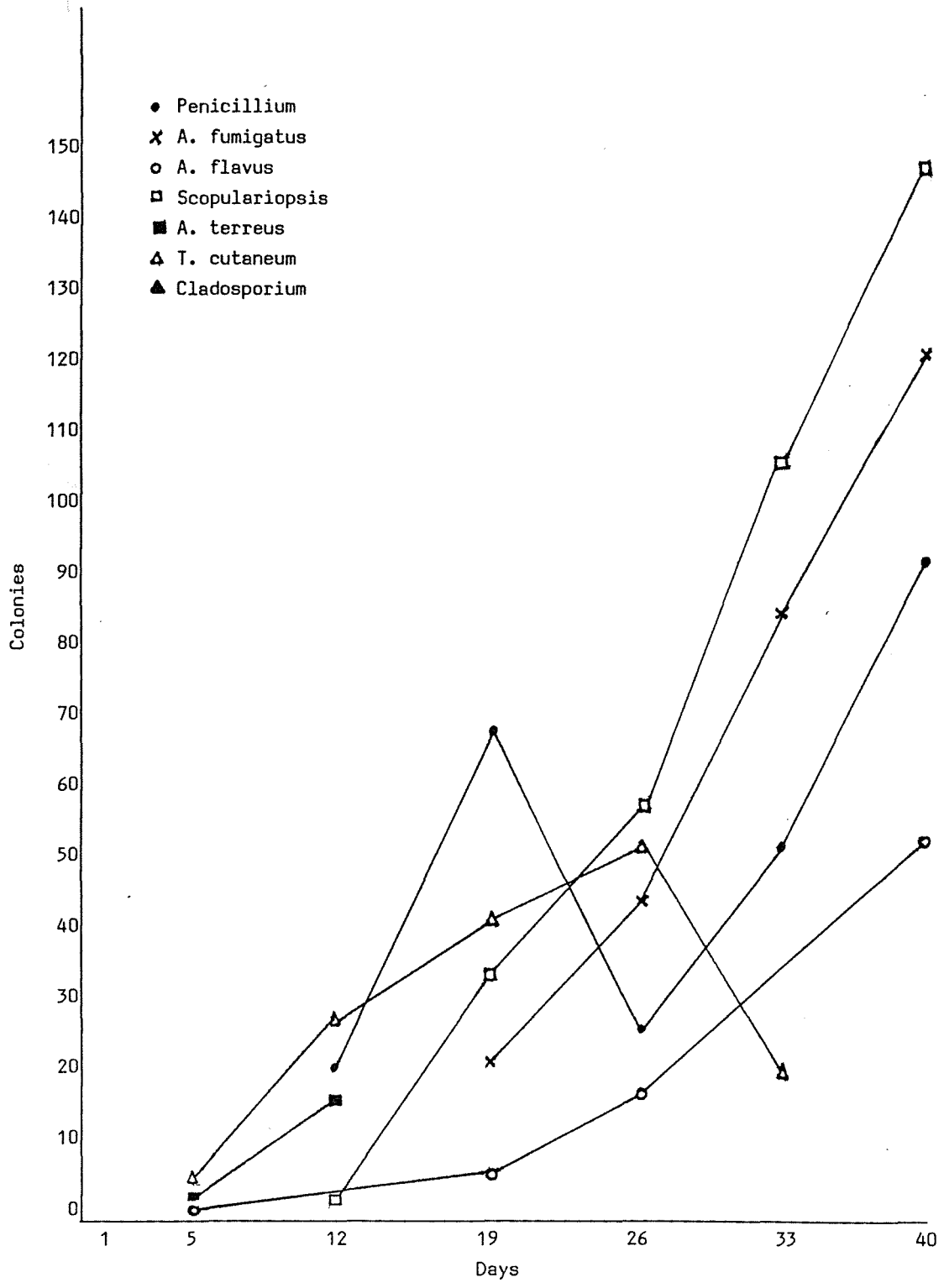


Figure 26. Changes in the levels of fungal genera in broiler house air using ADM 37°C.



3.2 Layer House Samples

3.2.1 Litter

PDA, 25°C (Fig. 27): Paecilomyces was isolated only on day 1. Except for days 1, 7, 14 and 28, levels of Penicillium (mainly P. chrysogenum and some P. variabile) were high (190 - 230 CFU g^{-1}) throughout the sampling period. A. flavus was first detected on day 35 and reached moderate (60 - 90 CFU g^{-1}) levels towards the end of the trial. A. fumigatus showed a gradual increase from day 28 to day 42 (160 - 200 CFU g^{-1}) and fluctuated only slightly from this level thereafter. Scopulariopsis appeared first on day 21 and increased to day 42 (230 CFU g^{-1}) and maintained that level for the rest of the period. Geotrichum decreased steadily from a peak of 110 CFU g^{-1} (average) at day 7 to 0 CFU g^{-1} at day 77, but increased again after day 84 to maintain a level of 60 CFU g^{-1} (average).

A. terreus was isolated from both pens in low numbers in the last four weeks. Levels of Cladosporium and Mucor were very low.

ADM 37°C (Fig. 28): P. chrysogenum increased in both pens up to day 63 and then decreased steadily to 135 CFU g^{-1} (average) at day 112. A. flavus showed a gradual increase to high levels after day 63, reaching 175 CFU g^{-1} (average) on day 112. A. fumigatus was found at a level of 170 CFU g^{-1} (average) on day 63, reducing to between 120 and 130 CFU g^{-1} between days 77 and 91, and then increasing to levels between 150 and 180 CFU g^{-1} after day 98.

Scopulariopsis was found at levels above 160 CFU g^{-1} for most of the trial. Geotrichum was isolated in low levels towards the end only. Paecilomyces was isolated on day 1 only. A. terreus was isolated up to day 56, with a small peak at day 35. Low levels of Mucor and Trichosporon were found on certain days.

3.2.2 Feed

PDA, 25°C (Fig. 29): P. chrysogenum increased to a peak (130 - 140 CFU g^{-1}) between days 35 and 49, then decreased to levels of 35 - 45 CFU g^{-1}. A. flavus and A. fumigatus were found in low levels (<55 CFU g^{-1}) mainly at the end. Scopulariopsis increased up to

Figure 27. Changes in the levels of fungal genera on PDA 25°C in layer house litter samples with age.

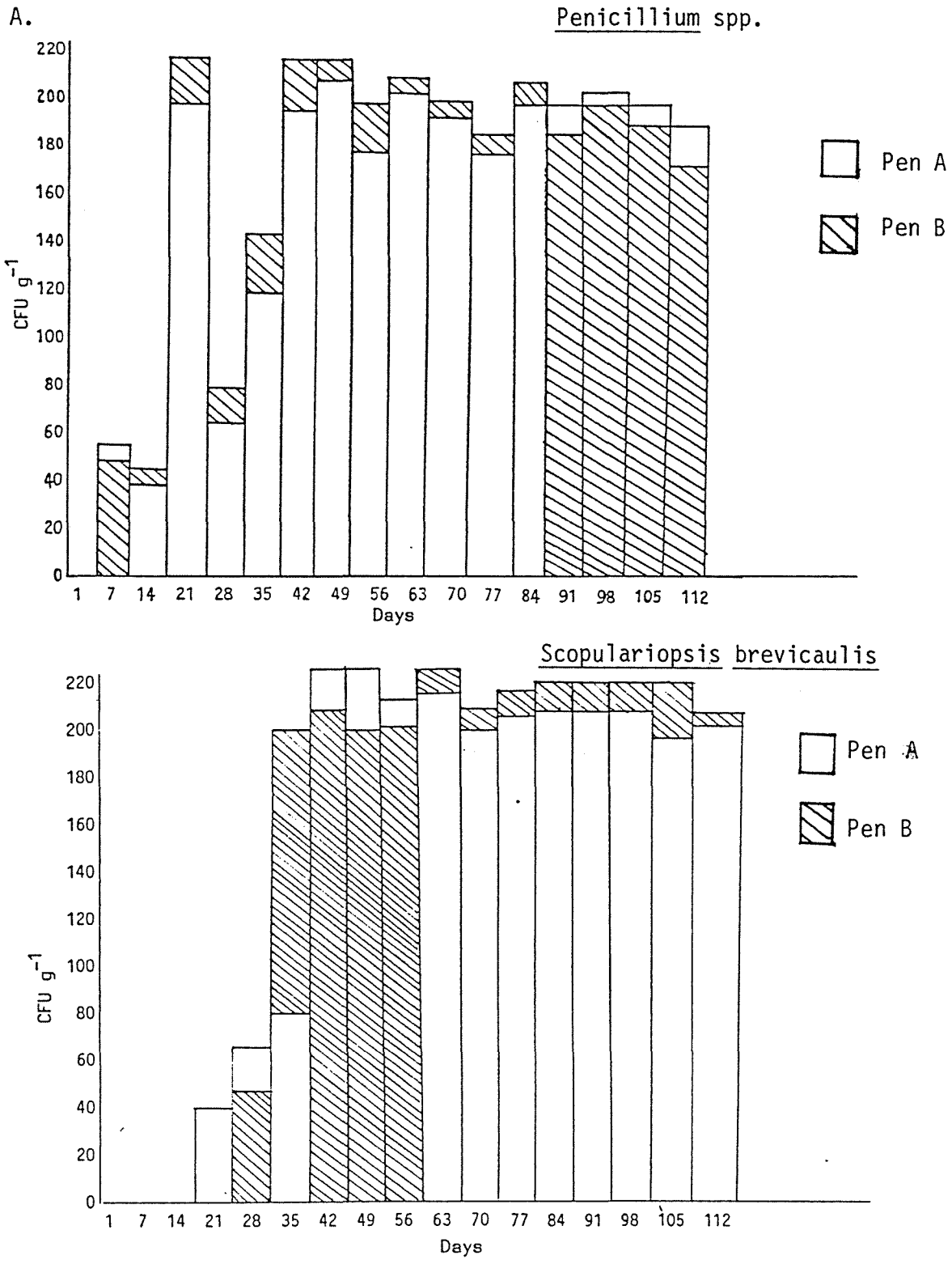
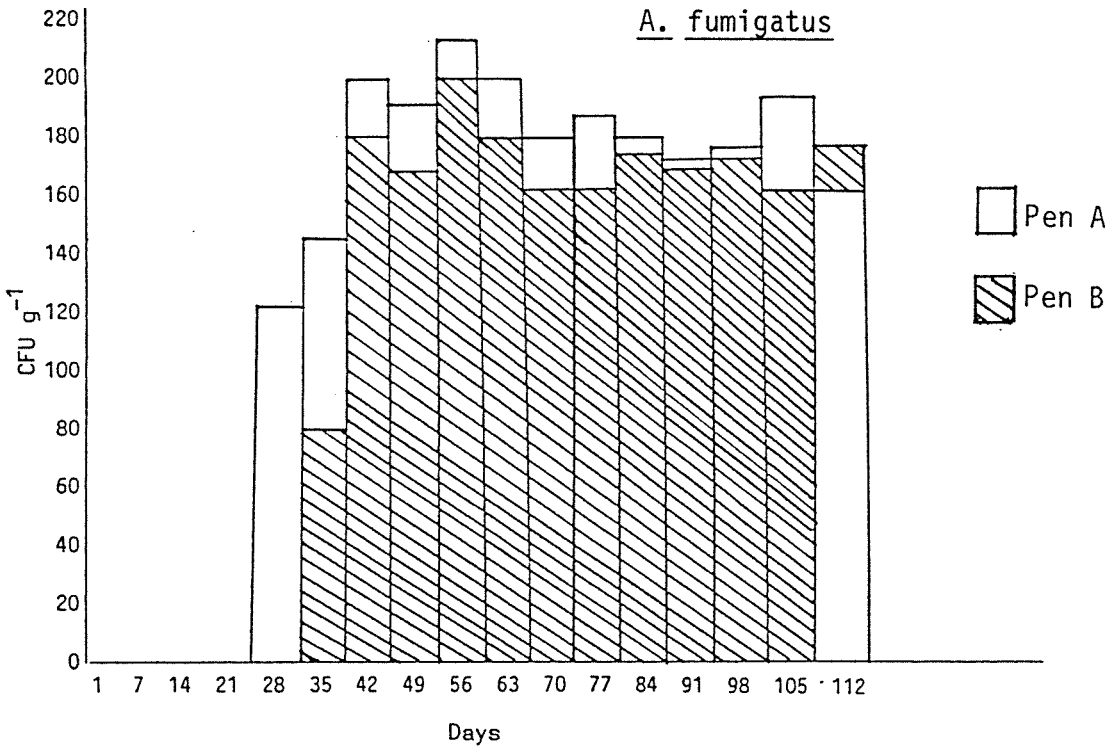


Figure 27. B.


A. fumigatus
CFU g-1
220
200
180
160
140
120
100
80
60
40
20
0
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days
Pen A
Pen B

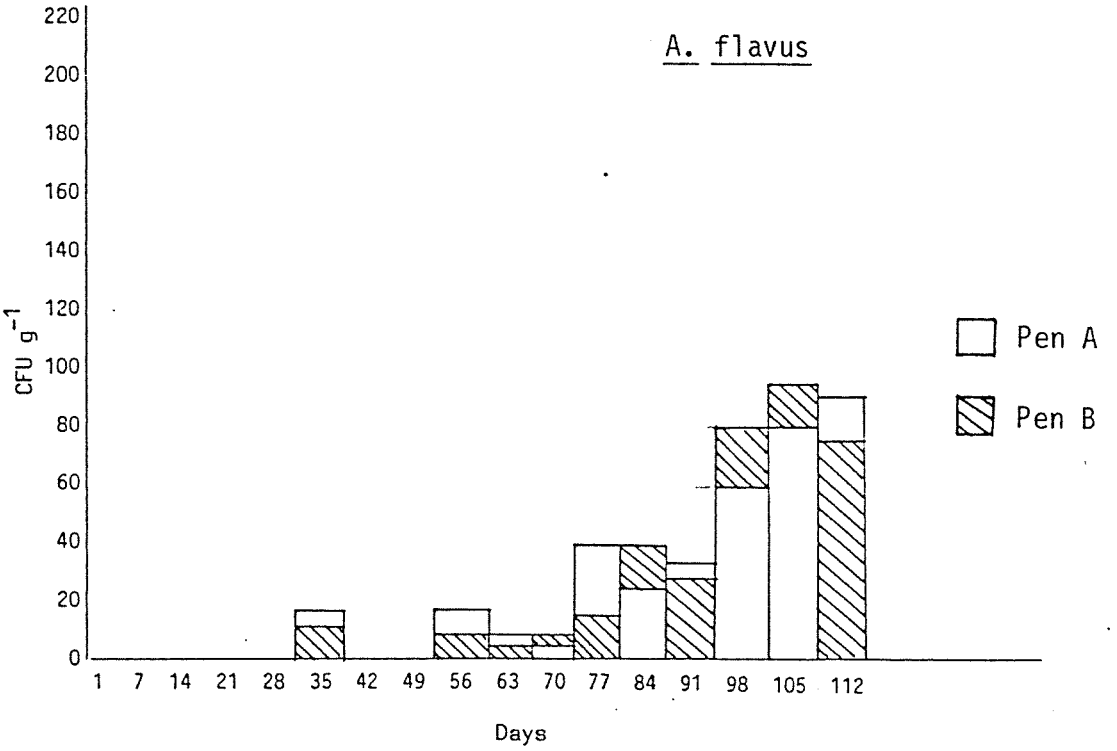
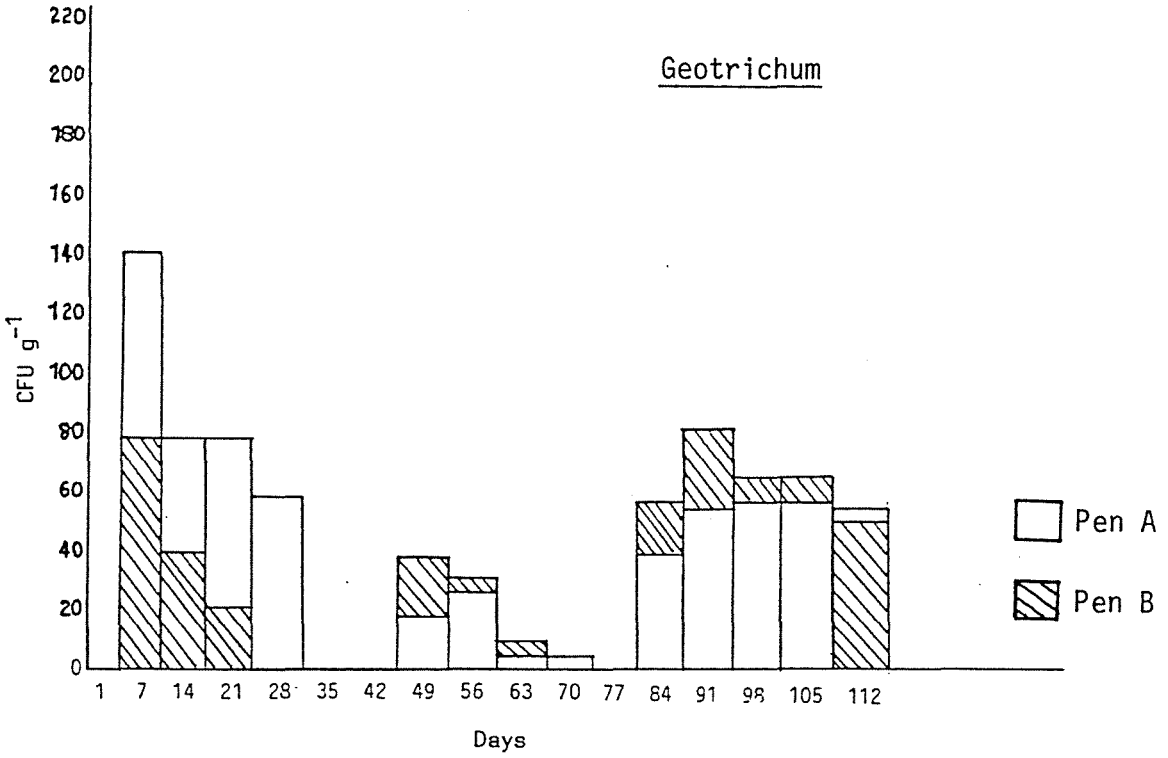

A. flavus
CFU g-1
220
200
180
160
140
120
100
80
60
40
20
0
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days
Pen A
Pen B

Figure 27. C.


Geotrichum
CFU g^{-1}
220
200
180
160
140
120
100
80
60
40
20
0
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days
Pen A
Pen B

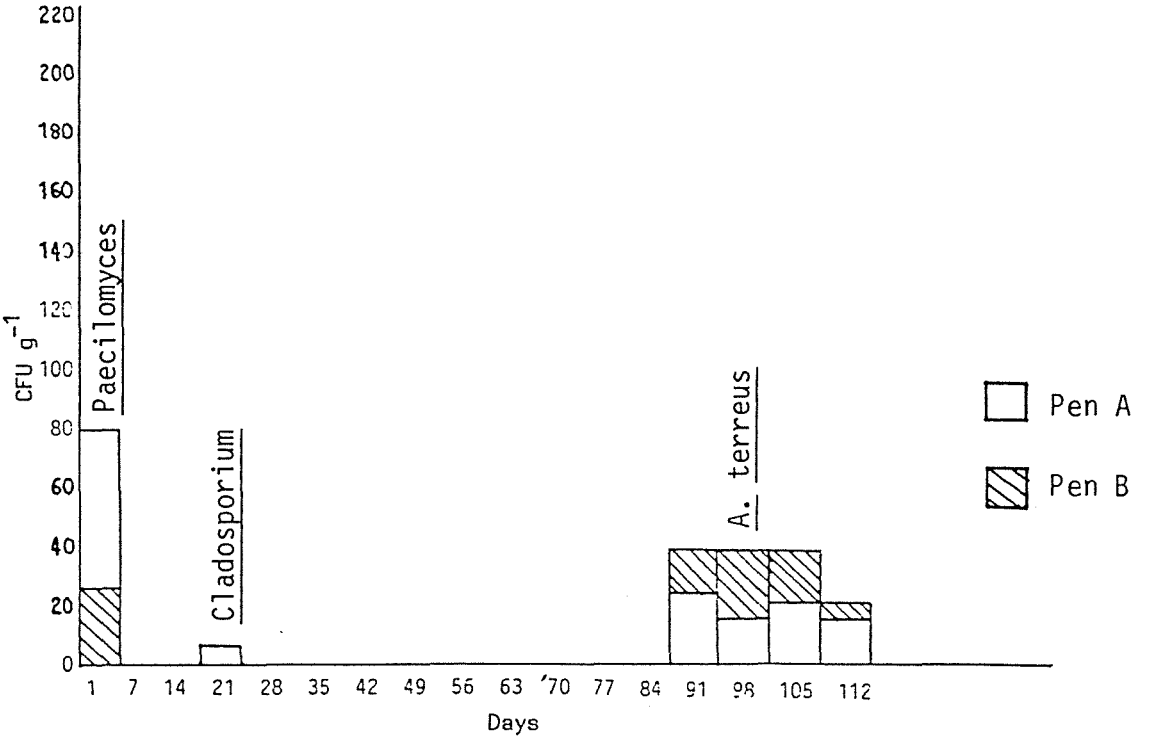

Paecilomyces
Cladosporium
A. terreus
CFU g^{-1}
220
200
180
160
140
120
100
80
60
40
20
0
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days
Pen A
Pen B

Figure 28. Changes in the levels of fungal genera on ADM 37°C in layer house litter samples with age.

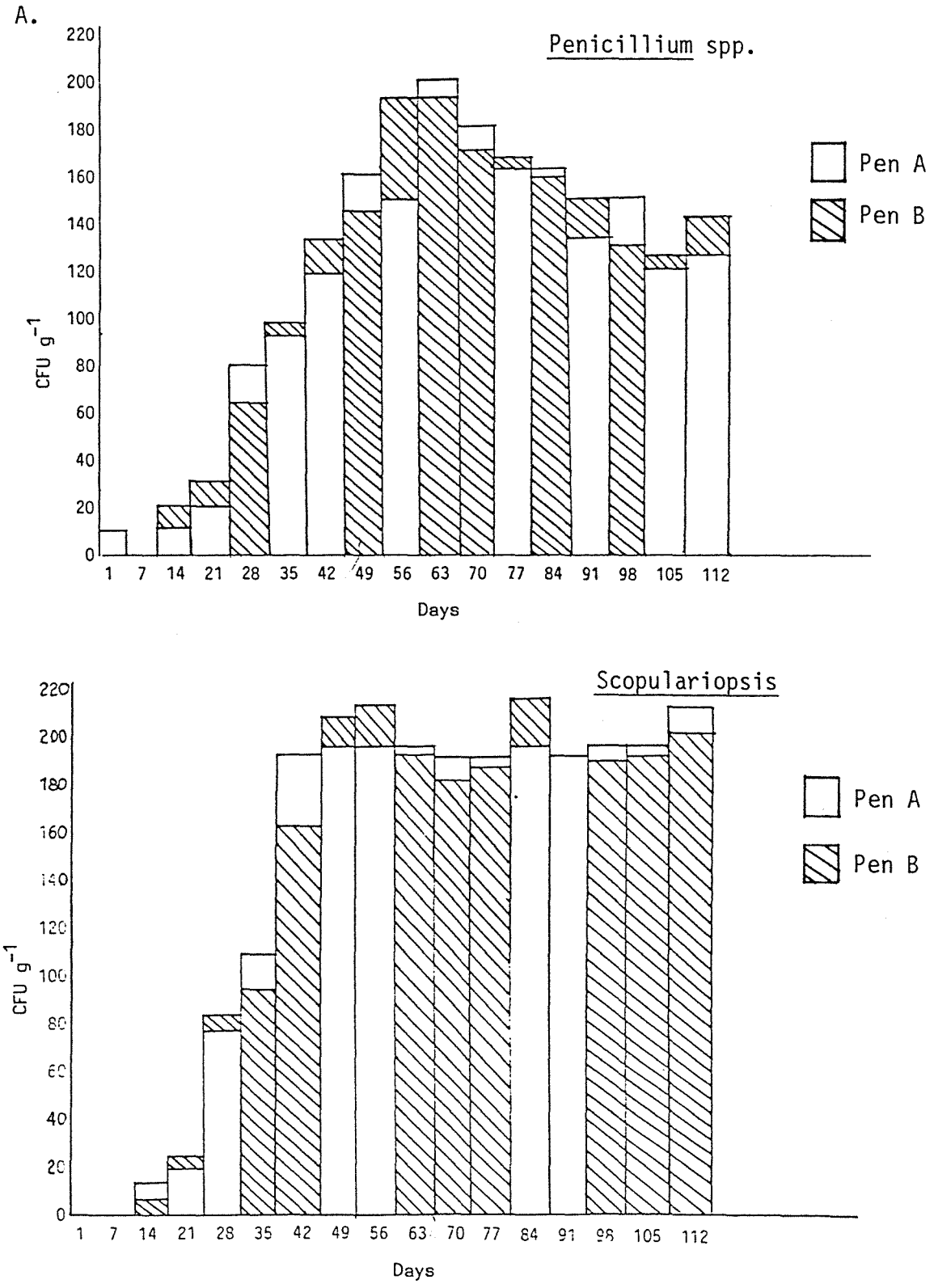
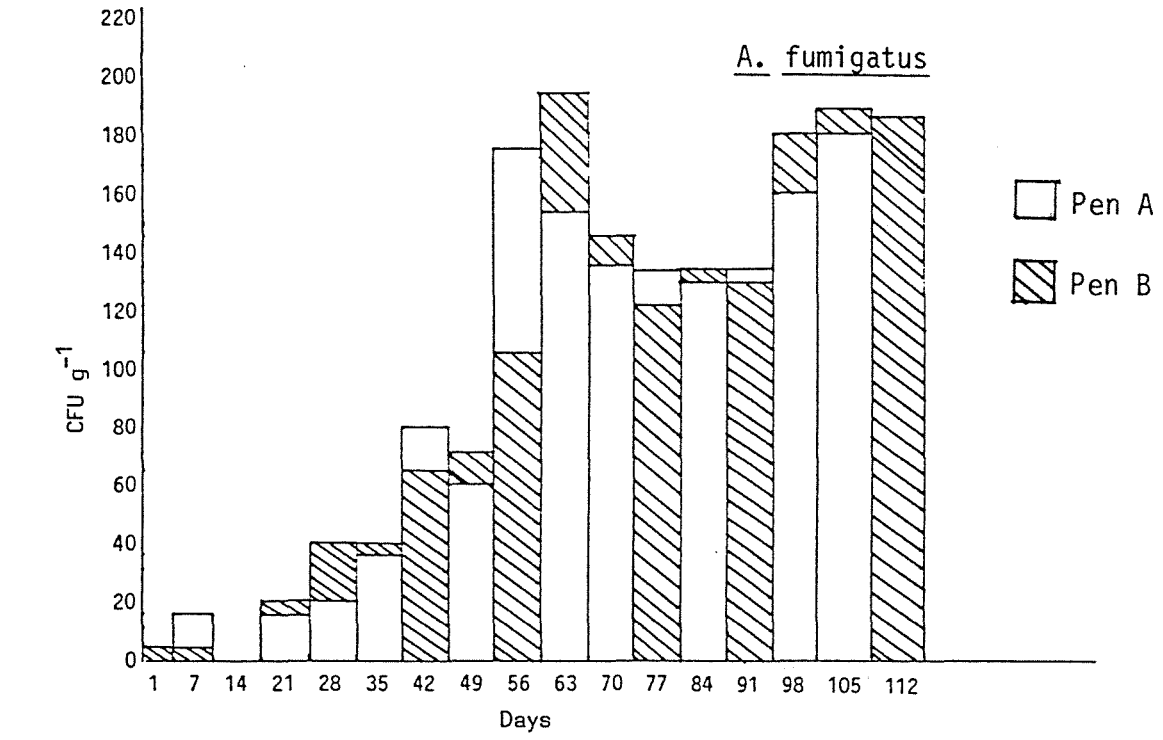


Figure 28. B.


A. fumigatus
CFU g-1
220
200
180
160
140
120
100
80
60
40
20
0
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days
Pen A
Pen B

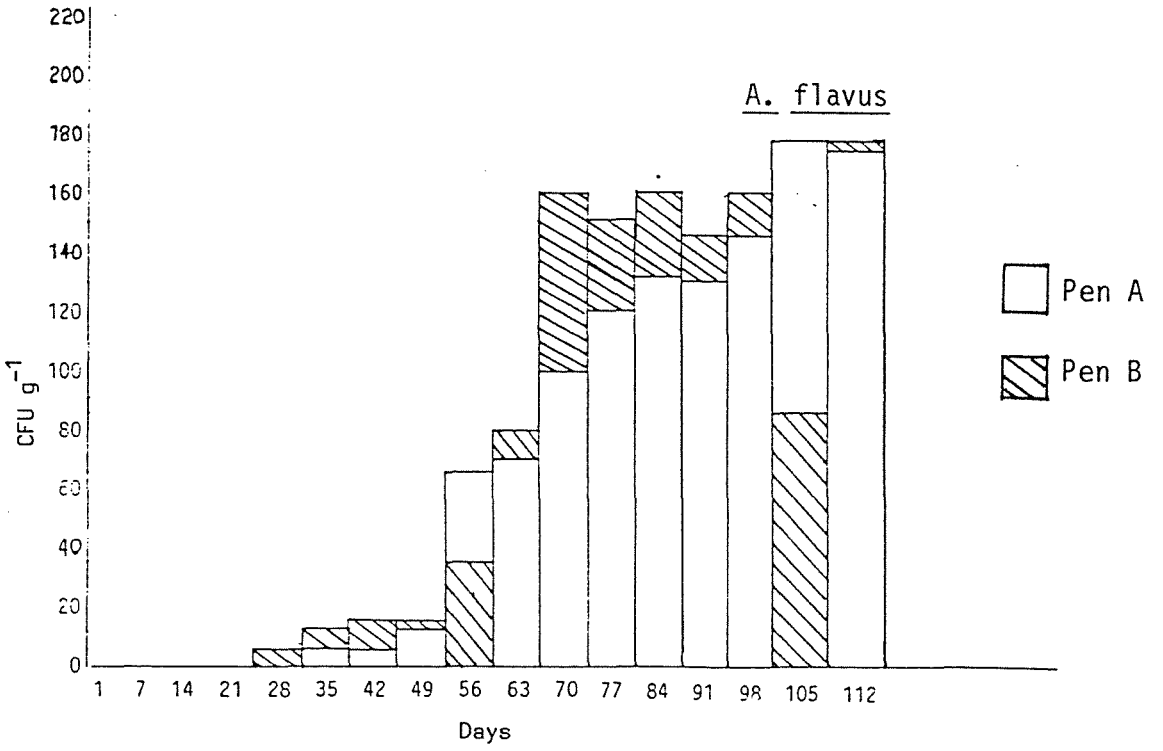

A. flavus
CFU g-1
220
200
180
160
140
120
100
80
60
40
20
0
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days
Pen A
Pen B

Figure 28. C.

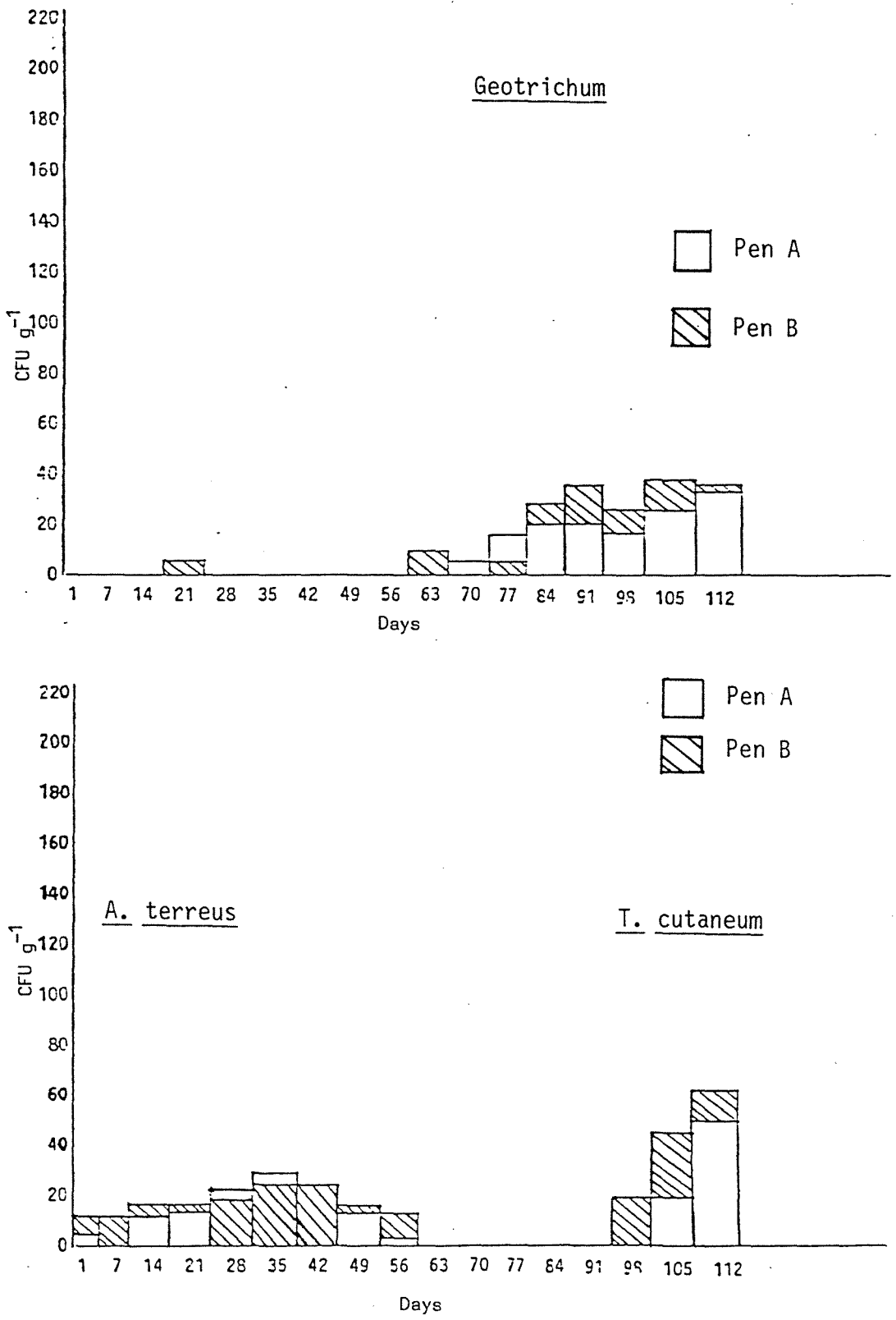
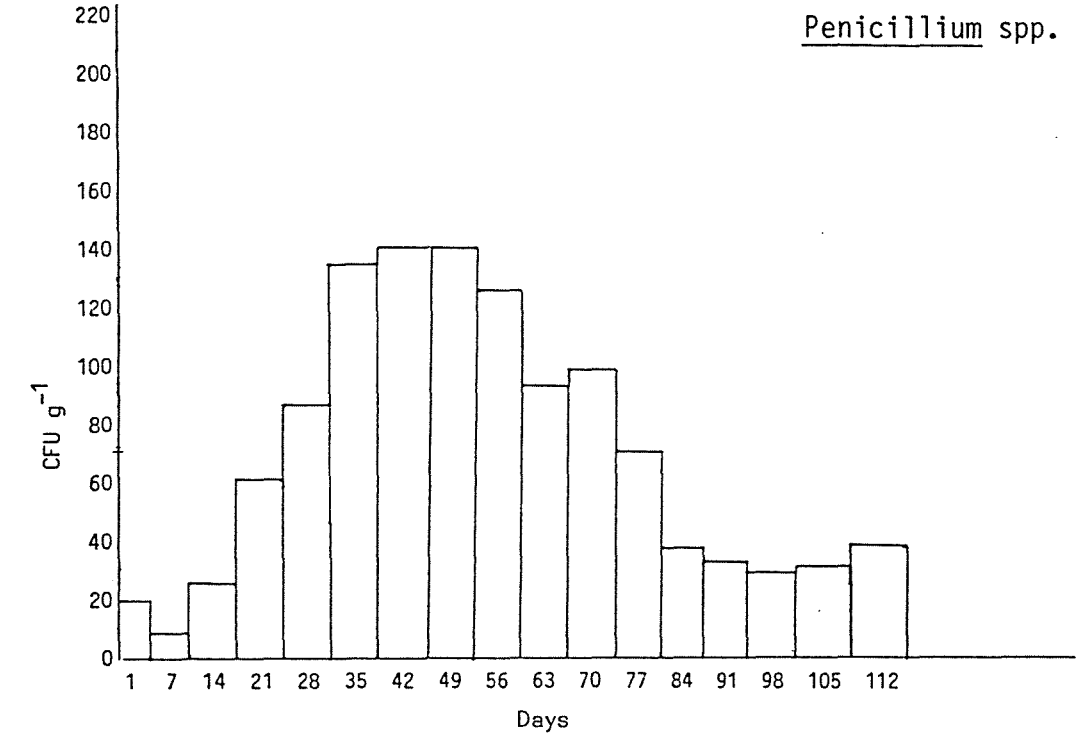

Geotrichum
Pen A
Pen B
CFU g⁻¹
220
200
180
160
140
120
100
80
60
40
20
0
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days
A. terreus
T. cutaneum
Pen A
Pen B
CFU g⁻¹
220
200
180
160
140
120
100
80
60
40
20
0
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days

Figure 29. Changes in the levels of fungal genera on PDA 25°C in layer house feed samples with age.

A.



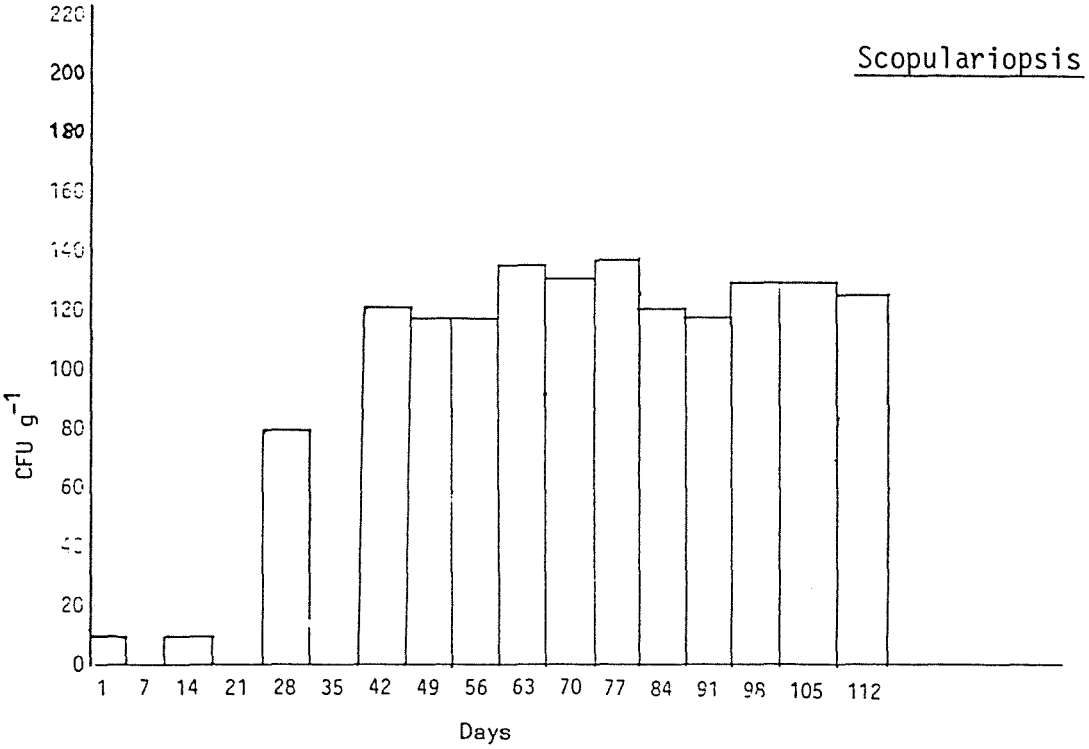
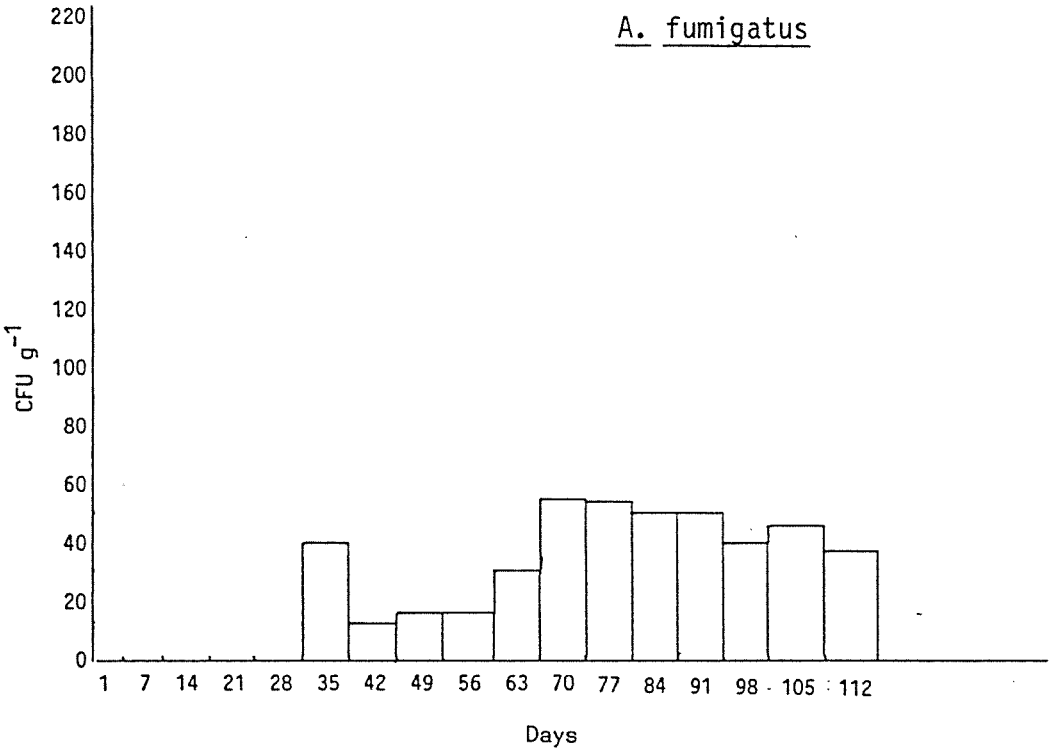


Figure 29. B.


A. fumigatus
CFU g^{-1}
220
200
180
160
140
120
100
80
60
40
20
0
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days

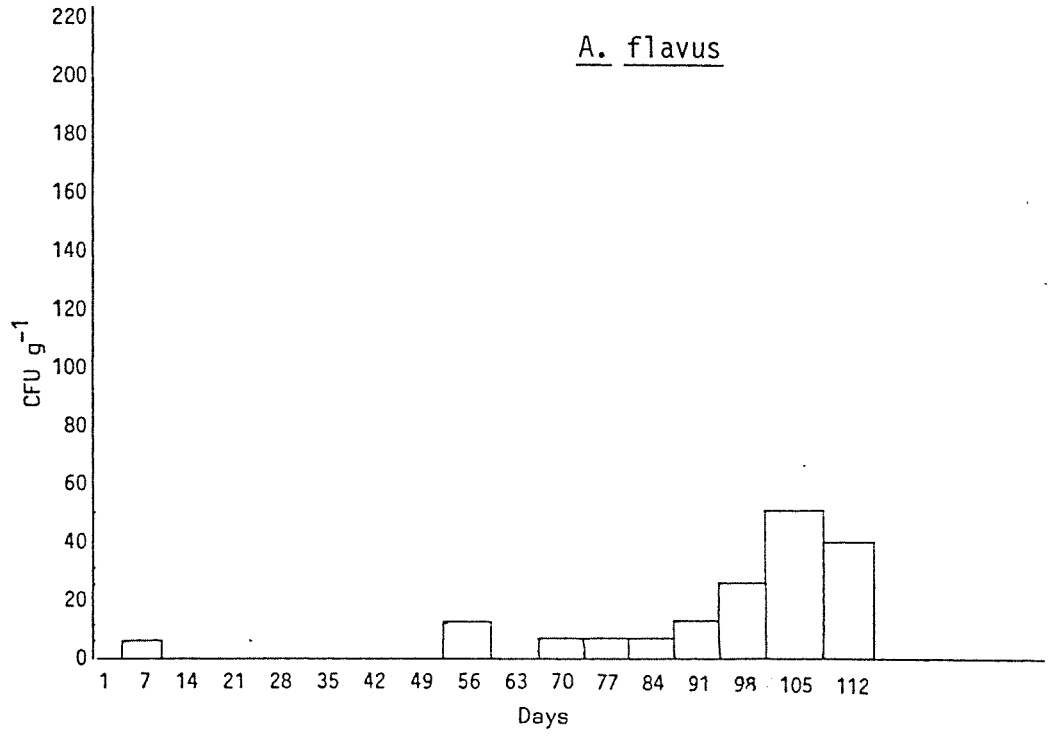
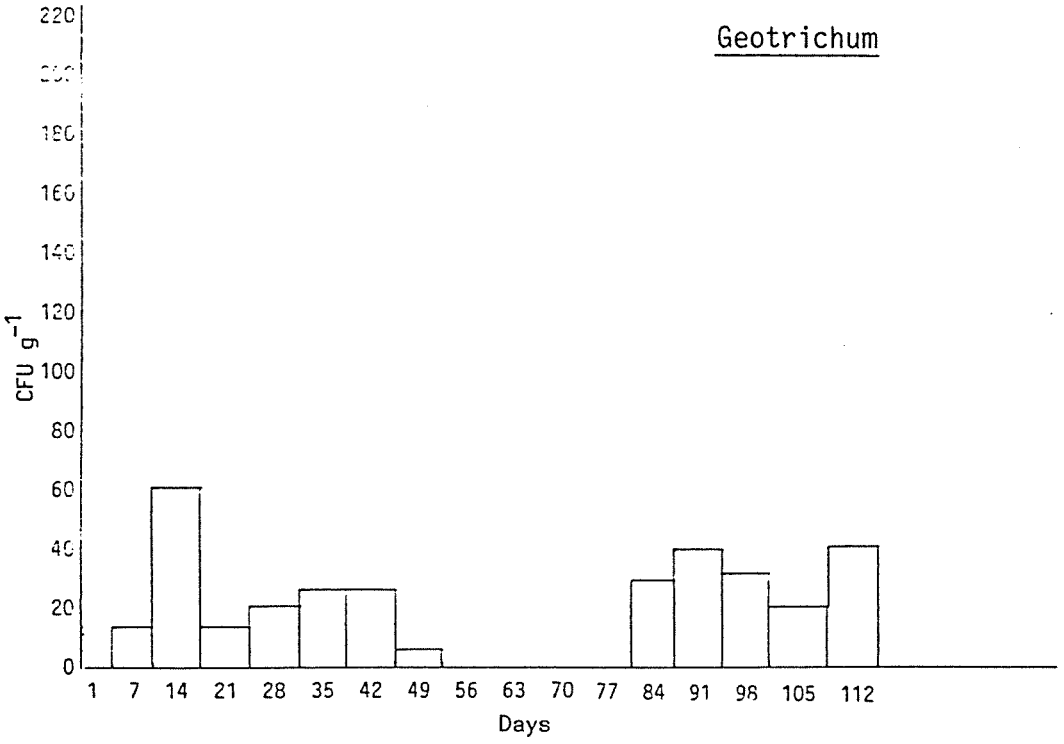

A. flavus
CFU g^{-1}
220
200
180
160
140
120
100
80
60
40
20
0
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days

Figure 29. C.


Geotrichum
CFU g^{-1}
220
200
180
160
140
120
100
80
60
40
20
0
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days

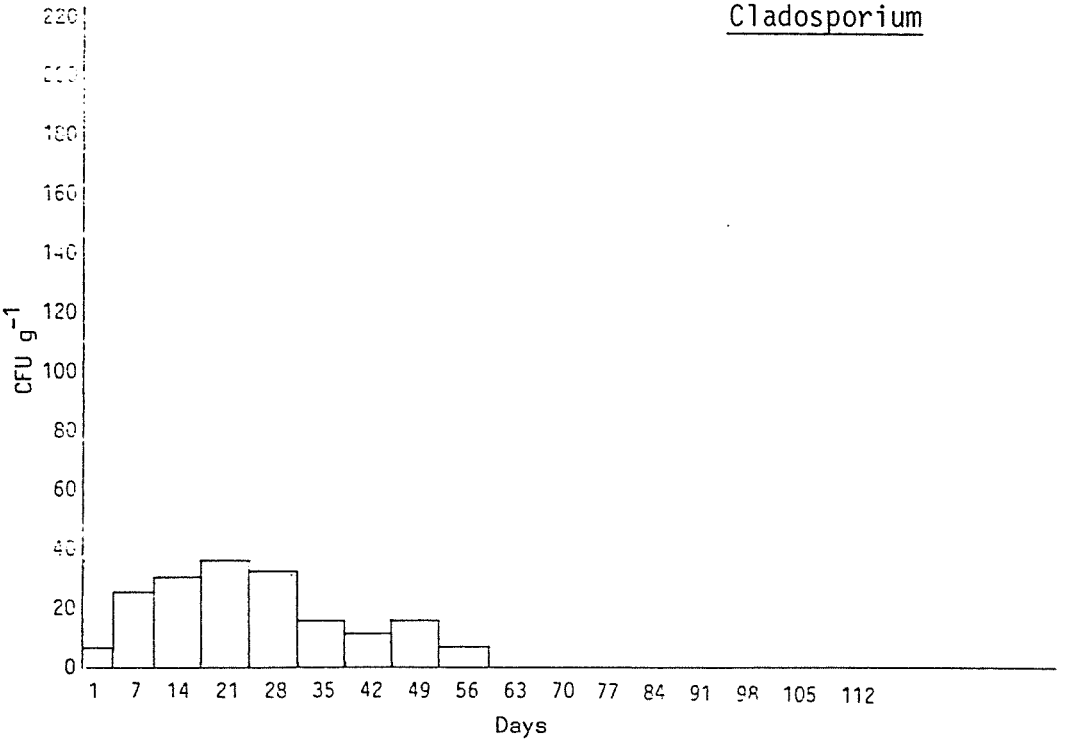
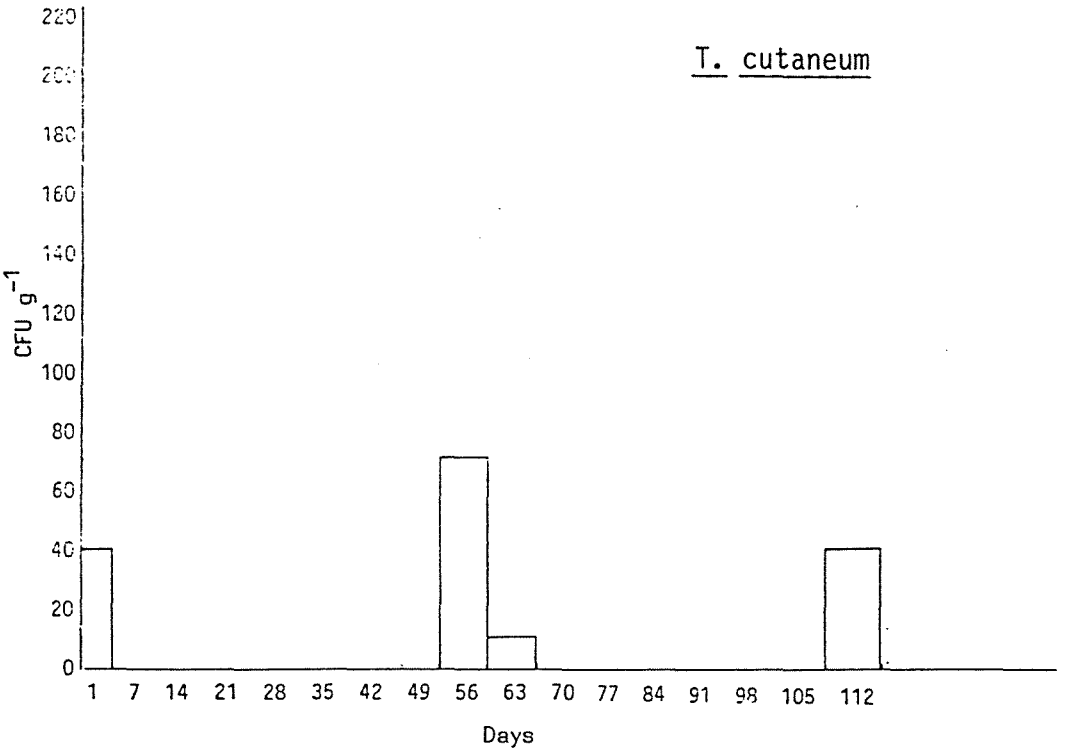

Cladosporium
CFU g^{-1}
220
200
180
160
140
120
100
80
60
40
20
0
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days

Figure 29. D.


T. cutaneum
CFU g⁻¹
220
200
180
160
140
120
100
80
60
40
20
0
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days

110 CFU g^{-1} (average) on day 42 and then remained more or less constant. Geotrichum had a small peak of 60 CFU g^{-1} at day 14, then decreased beyond day 77. Cladosporium was isolated between days 1 and 56, at levels below 40 CFU g^{-1}. T. cutaneum showed a peak of 70 CFU g^{-1} at day 56 and was also found on days 1, 63 and 112.

ADM, 37°C (Fig. 30): Penicillium increased to a peak of 140 CFU g^{-1} on day 63 and then decreased slightly, increasing to almost 140 CFU g^{-1} on days 105 and 112. Scopulariopsis was found in moderately high levels (100 - 130 CFU g^{-1}) on day 42 and thereafter. Levels of A. fumigatus were fairly low (<80 CFU g^{-1}). Low levels of A. terreus (<40 CFU g^{-1}) were found, up to day 56. Trichosporon and Cladosporium were scarce, being isolated only occasionally.

3.2.3 Air samples

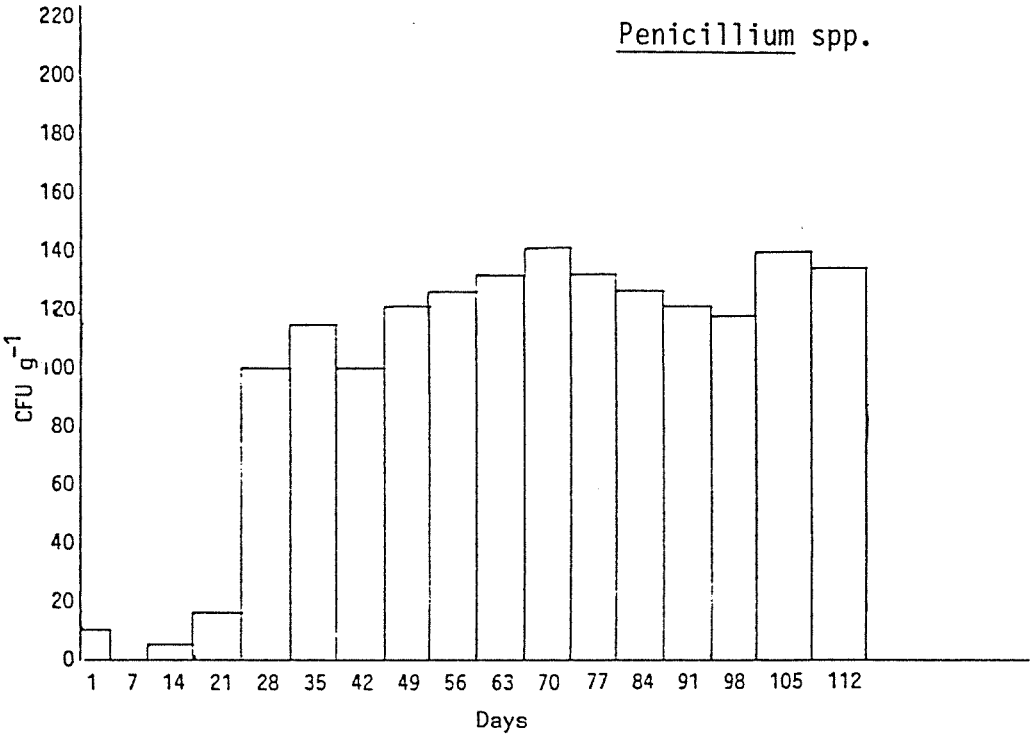
Patterns of fungal succession in the air of the layer house are shown in Figs. 31 and 32.

PDA, 25°C (Fig. 31): A. flavus increased up to day 70 and remained at medium levels thereafter. A. fumigatus and Scopulariopsis reached very high levels. Penicillium reached a peak between days 70 and 84 and reduced later. A. terreus was isolated in low numbers in the early and late parts of the trial. Cladosporium was found around the middle of the 18-week trial. Geotrichum increased, although at a low level.

ADM, 37°C (Fig. 32): A. flavus showed a greater increase than on PDA. Penicillium increased and showed a more marked decrease than on PDA. A. fumigatus and Scopulariopsis increased and remained high. A. terreus increased towards the end, as did Geotrichum.

Figure 30. Changes in the levels of fungal genera on ADM 37°C in layer house feed samples with age.

A.



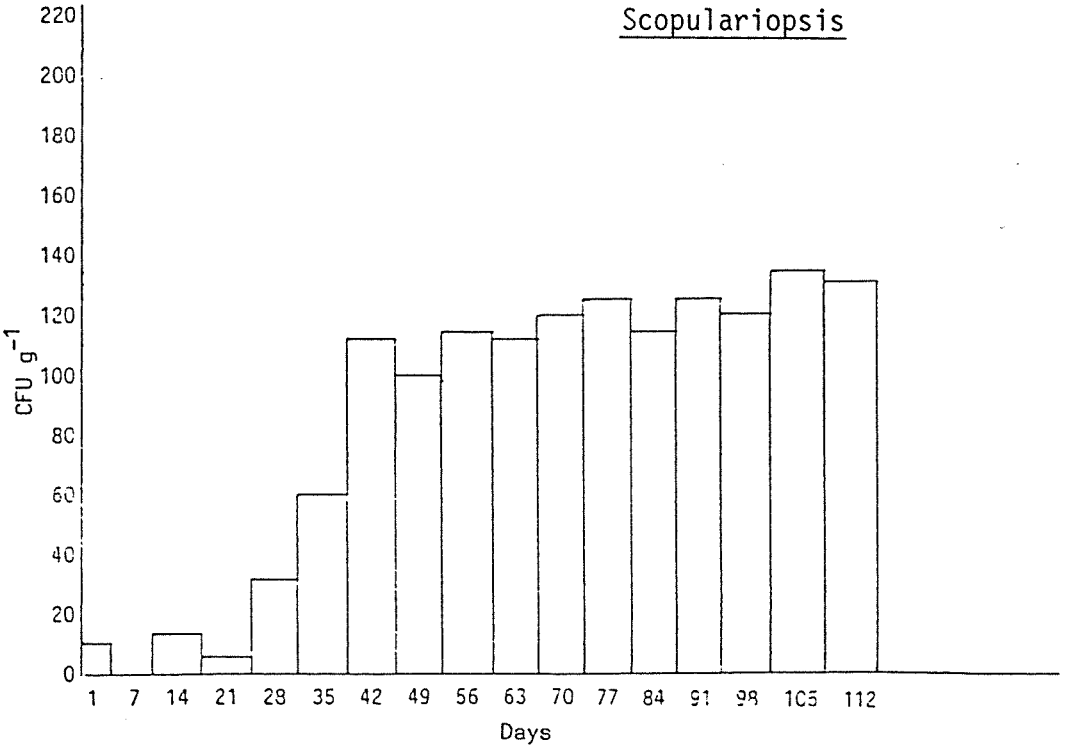


Figure 30. B.

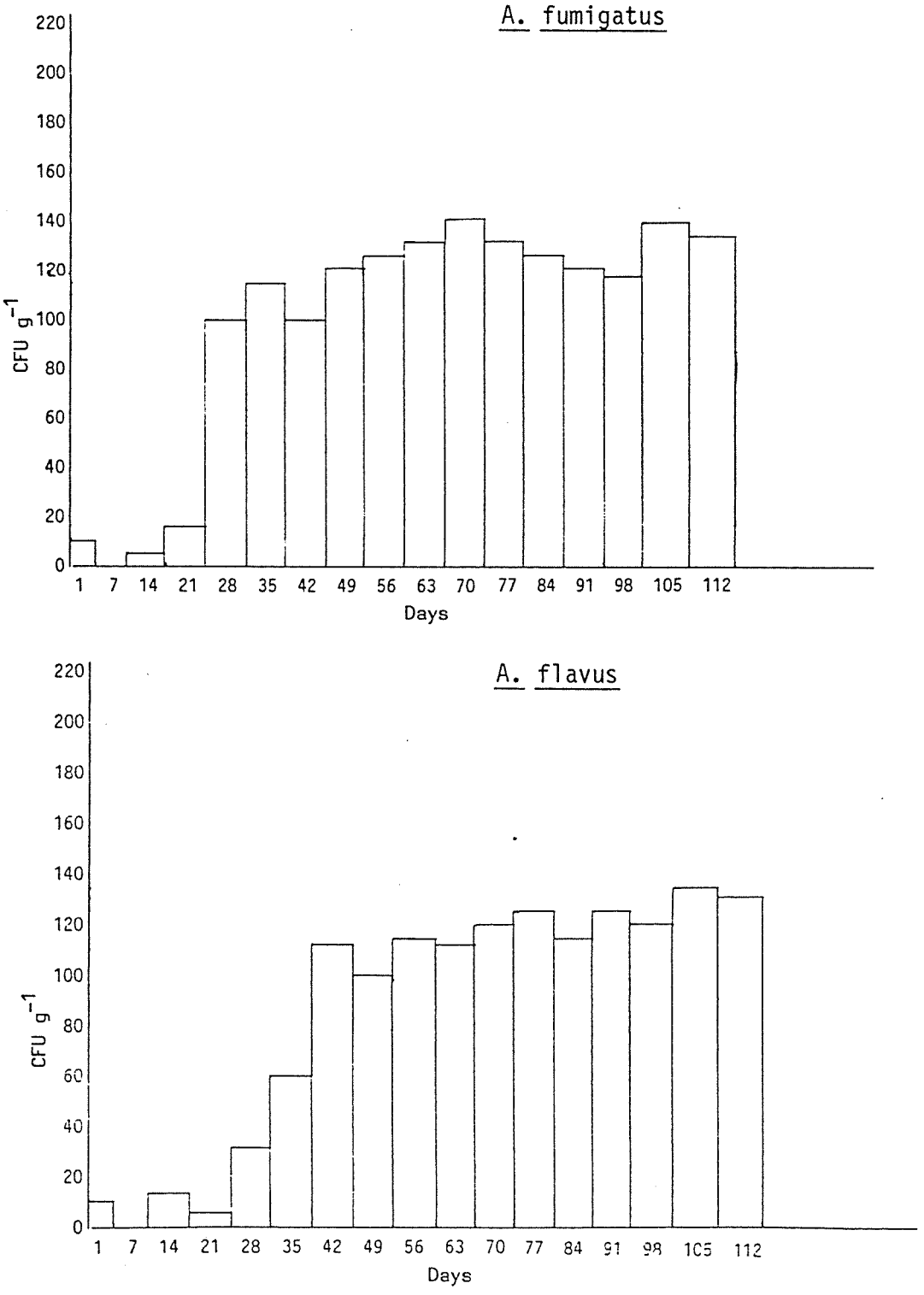
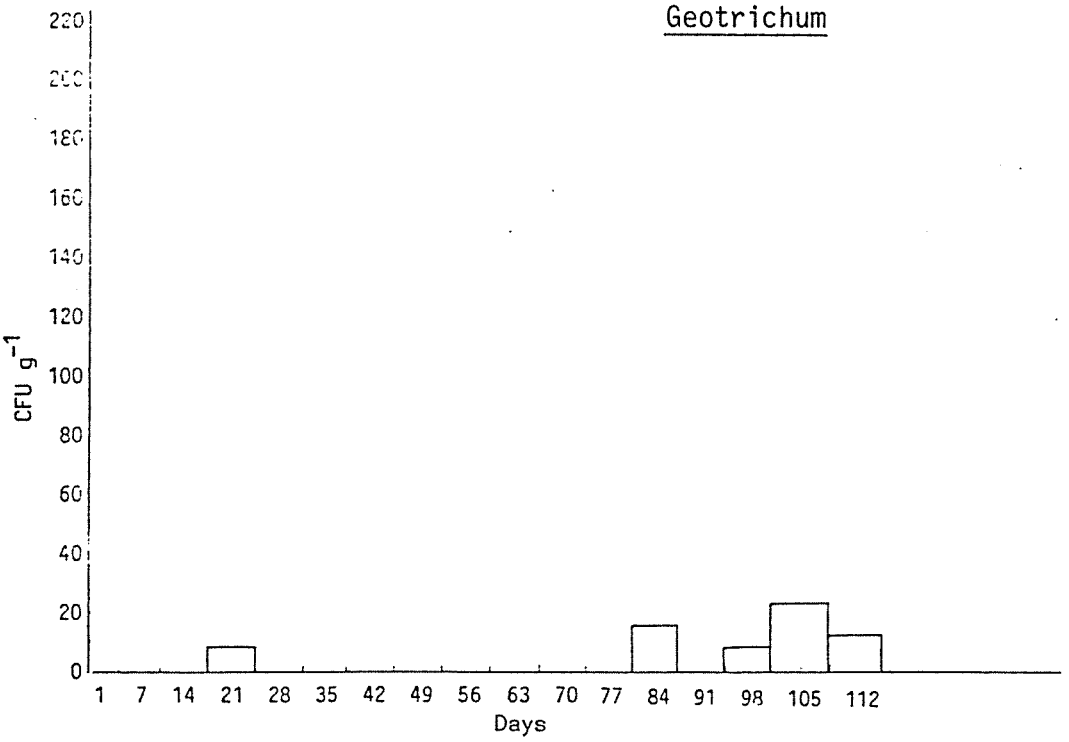

A. fumigatus
CFU g⁻¹
220
200
180
160
140
120
100
80
60
40
20
0
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days
A. flavus
CFU g⁻¹
220
200
180
160
140
120
100
80
60
40
20
0
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days

Figure 30. C.


Geotrichum
CFU g^{-1}
0
20
40
60
80
100
120
140
160
180
200
220
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days

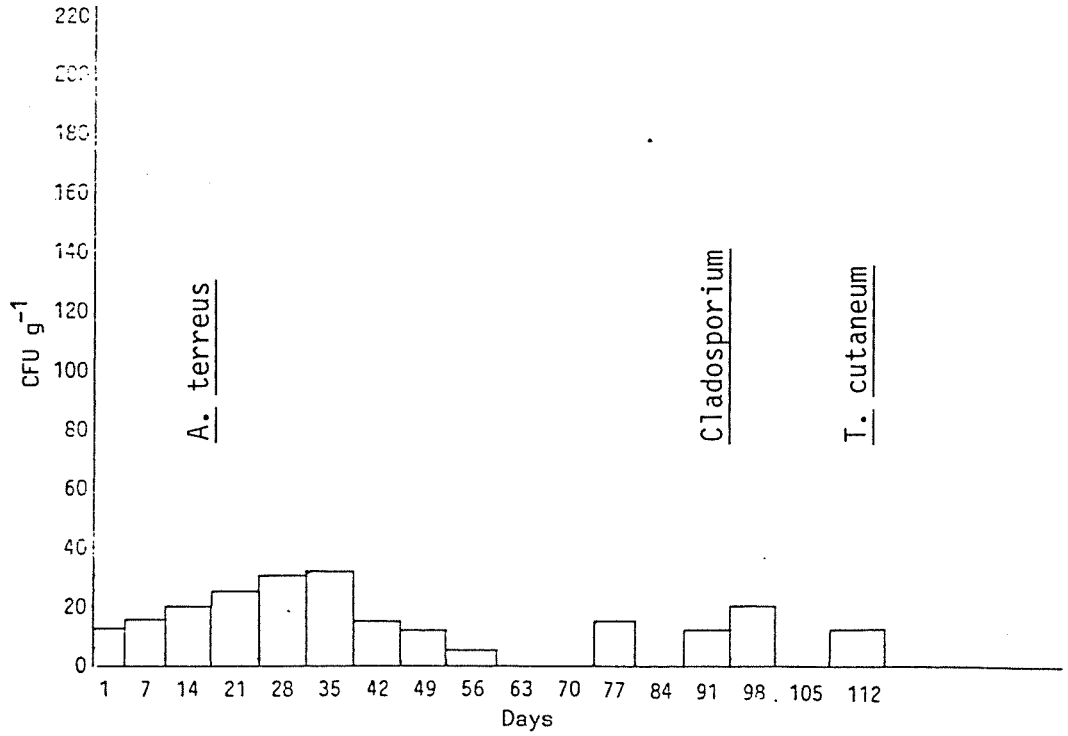

A. terreus
Cladosporium
T. cutaneum
CFU g^{-1}
0
20
40
60
80
100
120
140
160
180
200
220
1 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105 112
Days

Figure 31. Changes in the levels of fungal genera in layer house air with time using PDA 25°C.

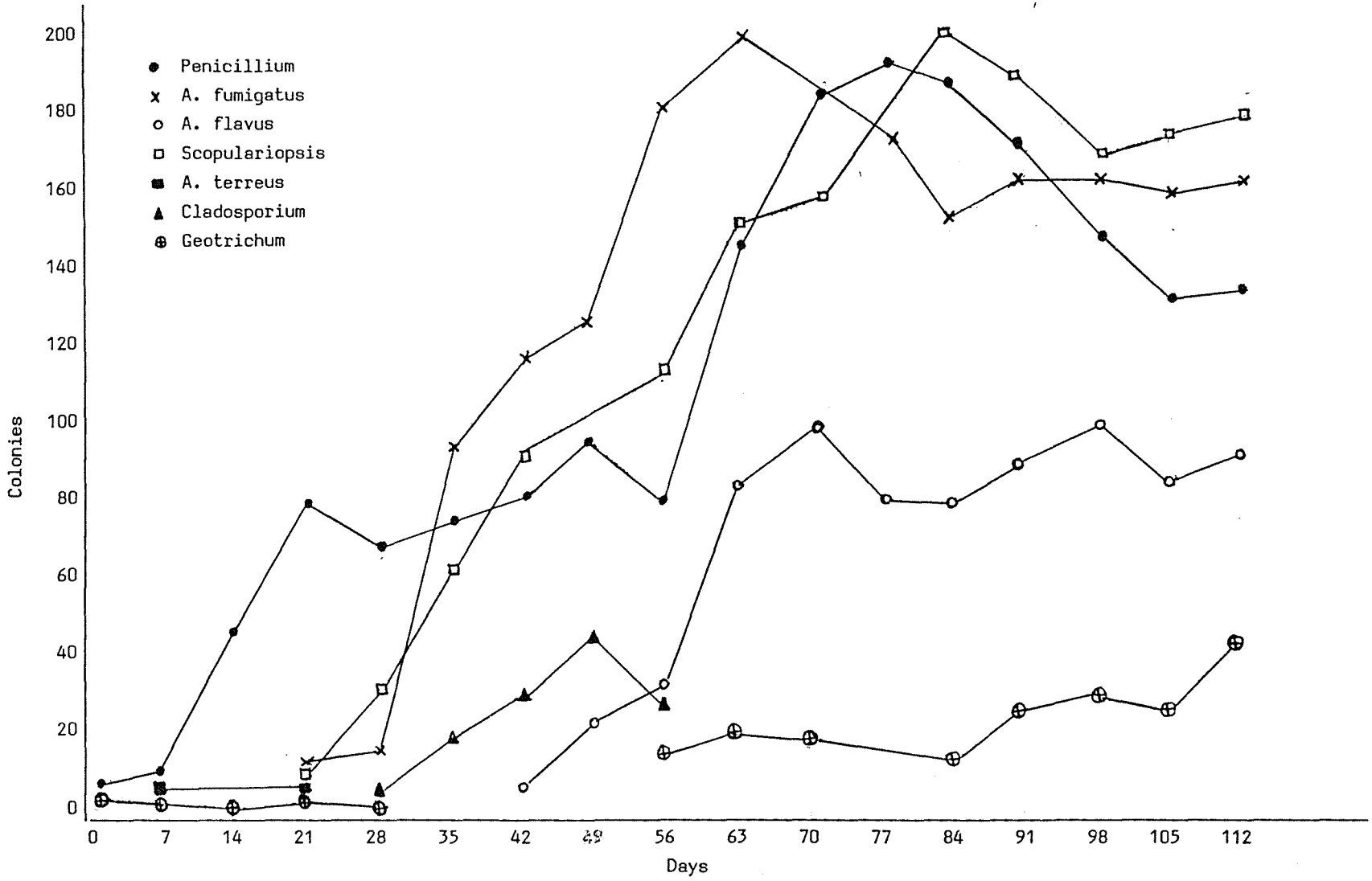
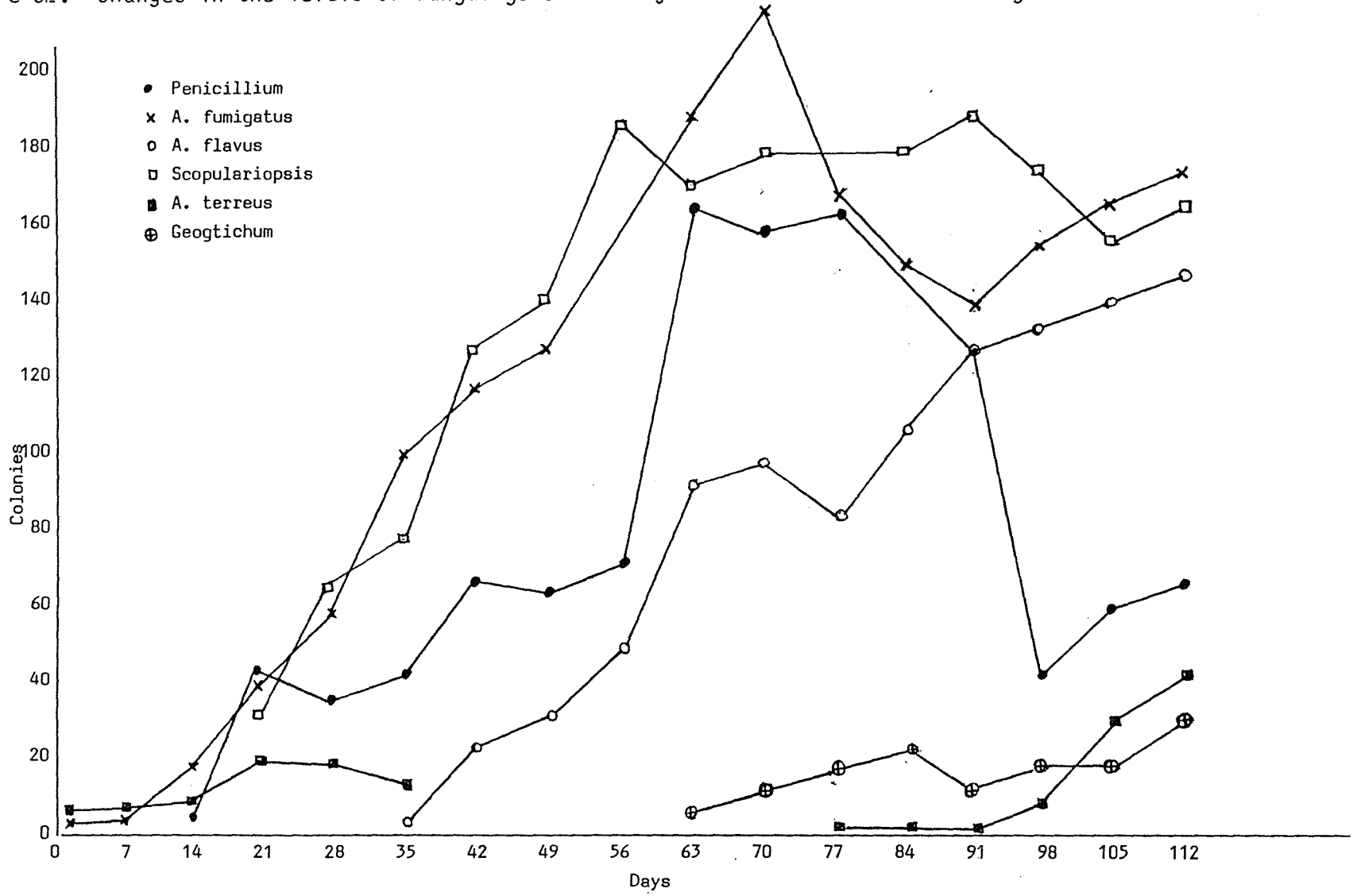


Figure 32. Changes in the levels of fungal genera in layer house air with time using ADM 37°C.



4. STRIP-BAIT TECHNIQUE

4.1 Strip Bait Effectiveness

The following results were obtained in preliminary experiments conducted to investigate the conditions needed for the strip-bait technique to function most efficiently:

4.1.1 Use of the membrane filter in the centre of the bait.

In comparisons of the use of a membrane filter to that of a nutrient-soaked filter paper as the centre square in the stack of five squares as used in the strip-bait technique (Methods 3.3.1a), it was found that the membrane filter (m.f.) and the filter paper (f.p.) gave almost identical results. The same fungal genera were isolated from the soil specimens, and in similar numbers (Plate 1).

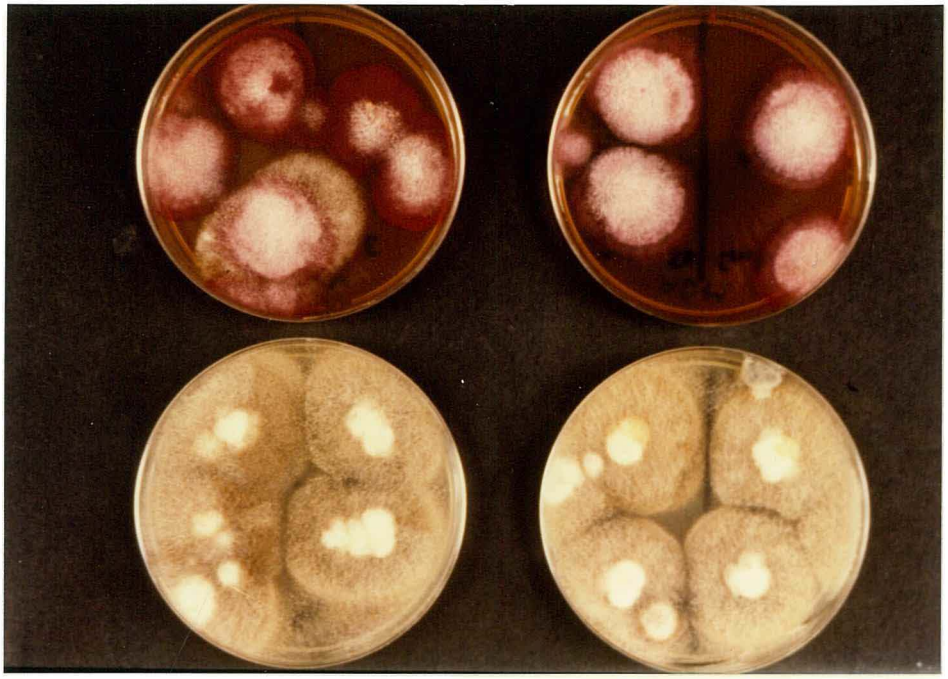
4.1.2 Nutrient solutions, incubation temperature and culture media

M.f.'s positioned between f.p. squares previously soaked in nutrient solutions A, B, C or D (Methods, 2.1) were cultured after baits had been embedded in the soil for 8 days (Methods 3.3.1b). Both PDA and ADM were used, and the cultures incubated at 25°C and 37°C. The isolation of species of Aspergillus and Penicillium from the soil appeared most favourable on ADM, at 37°C incubation temperature. The nutrient solutions giving the greatest number of subsequent isolations were solutions B (Dextrose-peptone-yeast extract made with 2% dextrose + 2% tannic acid) and D (DPY with 4% dextrose + 2% tannic acid) (Table 3).

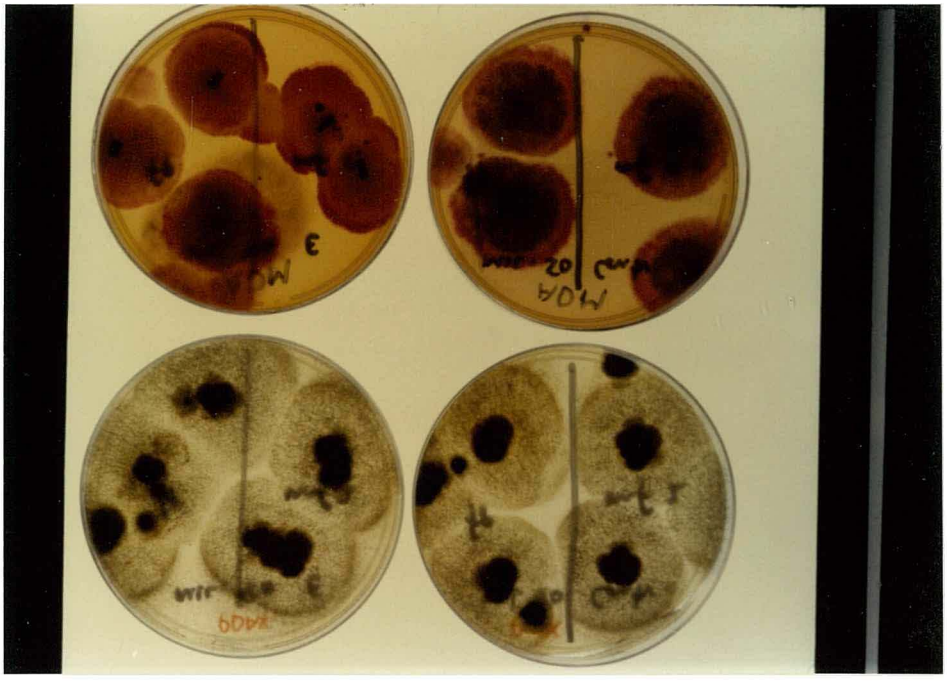
4.1.3 Substrate moisture content

M.f.'s were removed from baits at 5, 8 and 14 days after being in soils of varying moisture content (5%, 10%, 20%, 50%; Methods 3.3.1c). It was found that with the m.f.'s removed at 5 days, only 20% substrate m.c. allowed isolation of Penicillium and Aspergillus. At 8 days, 10% and 20% gave favourable results and at 14 days 5%, 10% and 20% gave adequate isolations of these genera. There were no isolations from baits incubated in soil at 50% m.c.

Plate 1. STrip-bait technique: Comparison of the use of a membrane filter square and a filter paper square as centre cultured bait.



Surface view. Membrane filter left, filter paper right. ADM upper, PDA lower.



Reverse view. Membrane filter left, filter paper right. ADM upper, PDA lower.

Table 3. Effect of various conditions on the efficiency of the strip-baits.

Nutrient Solution	Temp. of Incubation	Culture Medium PDA	Culture Medium ADM
A	25°C	N.G.	1 Penicillium
	37°C	1 Penicillium	3 Aspergillus 2 Penicillium
B	25°C	N.G.	3 Aspergillus 3 Penicillium
	37°C	1 Aspergillus 2 Penicillium	9 Aspergillus 6 Penicillium 2 Fusarium
C	25°C	N.G.	2 Aspergillus
	37°C	3 Rhizopus	1 Penicillium 1 Penicillium 2 Aspergillus
D	25°C	1 Rhizopus	2 Penicillium 5 Aspergillus
	37°C	2 Rhizopus 3 Aspergillus 2 Penicillium	15 Aspergillus 12 Penicillium 1 Fusarium 1 Cladosporium

N.G. = No Growth.

4.1.4 Fungal isolation in proportion to numbers of propagules in substrate.

Determination of the efficiency of strip baits to detect Aspergillus and Penicillium in soil when the density of the fungal populations varied (Methods 3.3.1d) indicated that the technique was adequate at all levels tested (Table 4).

Table 4. Ability of strip-bait to isolate fungi in proportion to numbers present in substrate.

Ratio Aspergillus:Penicillium	Nutrient soln. used	Incubation temp. (ADM) 25°C	37°C
1:1	B	2 Aspergillus 2 Penicillium	3 Aspergillus 3 Penicillium
	D	2 Aspergillus 2 Penicillium	3 Aspergillus 3 Penicillium
2:1	B	5 Aspergillus 3 Penicillium	6 Aspergillus 3 Penicillium
	D	4 Aspergillus 2 Penicillium	6 Aspergillus 3 Penicillium
3:1	B	5 Aspergillus	4 Aspergillus 1 Penicillium
	D	8 Aspergillus 3 Penicillium	9 Aspergillus 3 Penicillium

In experiments where the reverse, i.e. a fixed level of Aspergillus and varying levels of Penicillium were inoculated into the soil, it was again found that isolations made were in direct proportion to the level of each genus isolated.

4.1.5 Microscopic examination of membrane filters

Microscopic examination of the stained m.f.'s showed a network of narrow, septate hyphae traversing the membrane filters. Conidial heads bearing the distinctive phialides and conidia of Aspergillus and Penicillium species could be clearly seen. These genera were distinguished easily in the early parts of the trials when fungal densities in the substrates were relatively, but after 8 days' incubation the abundance of hyphae concealed finer details.

4.2 Use of Strip-baits to Study the Succession of Fungi in Litter and Feed

Types of fungi isolated from the membrane filters in the centre of the strip-baits were counted and recorded as number of colonies grown from each fragment. In the results, fungal growth was recorded as follows:-

+	:	1-5 colonies of a fungus growing from one fragment cultured
++	:	6-10 colonies " " " " " "
+++	:	11-15 colonies " " " " " "
++++	:	>15 colonies " " " " " "

Results of the two nutrient solutions used to soak filter papers and of the unadjusted and adjusted substrate m.c.'s (Methods 3.3.2) are combined in the tables of results (Tables 5-7).

4.2.1 Broiler trial

Litter (Tables 5 & 6): P. chrysogenum was found only up to day 26. A. flavus was isolated in low numbers, on most days. A. fumigatus appeared on day 19 and on subsequent days in pen 10 and on day 26, and thereafter in pen 3. Scopulariopsis was found from day 19 onwards in both pens. Paecilomyces and A. terreus were isolated in low numbers, on occasional days.

Feed (Table 7): P. chrysogenum and A. terreus were found only on day 1. A. flavus was isolated on all 3 sampling days. A. fumigatus was found only on day 33, and Scopulariopsis was found on days 19 and 33.

Tables 5-7: Succession of fungal genera in the broiler house litter and feed samples using strip-baits.

Table 5. Litter, pen 10.

	Day						
	1	5	12	19	26	33	40
Penicillium sp.	+	+	++	+++			
Paecilomyces				+			
A. flavus	+	+	+	+		+	+
A. fumigatus				+	+	+	++
A. terreus	+						
Scopulariopsis				+	++	++	++

Table 6. Litter, pen 3.

	Day						
	1	5	12	19	26	33	40
Penicillium	+	+	+	++	+		
Paecilomyces				+			
A. flavus	+	+	+	+	+	+	+
A. fumigatus					+	+	+
A. terreus	+						
Scopulariopsis				+	++	++	++

Table 7. Feed.

	Day 5	Day 19	Day 33
Penicillium	+		
A. flavus	+	+	+
A. fumigatus			+
A. terreus	+		
Scopulariopsis		+	+

4.2.2 Layer trial

Litter (Tables 8 and 9): Penicillium spp. were present throughout the whole trial, with highest levels between days 42 and 77. A slight reduction was found after day 84. A. flavus first appeared on day 21 in litter A and on day 14 in litter B. A gradual increase was observed, with high levels after day 56. A. fumigatus appeared early (day 7) at low levels, and then showed a steady increase throughout the trials, with highest levels after day 56. A. flavus was found regularly after day 21 in litter A and day 28 in litter B. Levels were high after day 56 with a slight drop on day 70 in both pens. Geotrichum was mainly found in low levels up to day 28 and then again at the end, after day 77. A. terreus was found on occasional days up to day 21, then disappeared and re-appeared again on occasional days after day 70 in litter A and after day 77 in litter B. T. cutaneum was found on day 7, and then at the end (days 105 and 112 in litter A and only on day 112 in litter B). Cladosporium was found on rare occasions. Scopulariopsis appeared first at day 21 and increased gradually, with high levels after day 56.

Feed (Table 10): In general, colony numbers were lower than in litter strip-baits. Penicillium was found throughout the period but reduced after day 63. Highest levels were found on days 49-63. A. flavus was isolated on all days except days 7, 14 and 28. High

Tables 8-10: Succession of fungal genera in layer house litter and feed samples, using strip-baits.

Table 8. Litter A.

	Days																
	1	7	14	21	28	35	42	49	56	63	70	77	84	91	98	105	112
Penicillium	+	+	++	++	++	+++	+++	++++	++++	++++	++++	+++	++	+	++	+	+
Cladosporium			+							+		+					
A. flavus	+			+	+	+	+	++	++++	++++	+++	++++	++++	++++	++++	++++	++++
A. terreus	+	+		++								+	+	+		+	
A. fumigatus		+	+	+	++	++	++	+++	++++	++++	+++	++++	++++	++++	++++	++++	
Scopulariopsis				+	+	++	++	+++	++++	++++	++++	++++	++++	+++	++++	++++	++++
Geotrichum	+	+		+	+							+	++	+	+	+	+
Trichosporon		+														+	+

Table 9. Litter B.

	Days																
	1	7	14	21	28	35	42	49	56	63	70	77	84	91	98	105	112
Penicillium	+	+	+	++	++	++	+++	++++	++++	++++	++++	+++	++	+	+	++	+
Cladosporium				+													
A. flavus			+		+	+	+	+	+++	++++	+++	++++	++++	++++	++++	++++	++++
A. terreus	+	+	+										++	+	+		
A. fumigatus		+	+	+	++	++	++	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Scopulariopsis				+	+	++	++	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Geotrichum	+		+	+	+				+				+	++	++	++	+
Trichosporon		+															+

Table 10. Feed.

	Days																
	1	7	14	21	28	35	42	49	56	63	70	77	84	91	98	105	112
Penicillium	++	+	+	++	++	++	++	++++	++++	++++	++	+	+	+	+	+	+
Cladosporium		+			+	+		++									
A. flavus	+			+		+	+	++++	++++	++++	++++	++++	++	+++	+++	+++	+++
A. terreus		+	+		++	++	++								+		
A. fumigatus	+	+	+	+	++	++	+	+++	+++	+++	+++	+++	+++	++	++	+++	+++
Scopulariopsis			+	+	+	++	++	++++	++++	++++	++++	++++	+++	+++	+++	+++	+++
Geotrichum														+			
Paecilomyces	+	+							+	++				+	+	++	
Trichosporon		+													+	+	

levels occurred between days 49 and 77, with slightly lower levels thereafter. A. fumigatus showed a general increase throughout. Scopulariopsis was first isolated in feed on day 14, increased to high levels between days 49 and 77, and then reduced slightly on and after day 84. Geotrichum was isolated only once in feed, on day 91. Paecilomyces was found on certain days at the beginning (days 1 and 7), in the middle (56 and 63) and at the end (91-105). A. terreus occurred on most days up to day 42, then disappeared and was found once again at day 98. Cladosporium was found on occasional days, only up to day 49. T. cutaneum occurred at the beginning (day 7) and at the end (days 98 and 105).

5. MYCOTOXIN PRODUCTION

5.1 Aflatoxin Production by A. flavus Isolates

5.1.1 Coconut agar

130 A. flavus isolates from litter and 189 A. flavus isolates from feed were tested for the production of aflatoxin on coconut agar, during the layer trial. This procedure is described in Methods 5.1.1.

Aflatoxin (AT)-positive isolates gave a blue-green fluorescence in the surrounding agar medium when examined under long-wave (365 nm) U.V. light. In most AT-positive isolates fluorescence became apparent within 3 days of incubation at 25^{o}C, but in some the fluorescence was not apparent until 7 days and in others, until 12 days.

Table 11 shows the frequency of occurrence of AT-positive isolates among those tested. It is apparent from these results that on most sampling days this frequency was between 20 and 30%.

The table shows that in total, 26.01% of all A. flavus isolates tested on coconut agar were AT-positive. Approximately 20% were strongly positive (strong fluorescence, first appearing at 3 or 7 days) and approximately 6% were weakly positive (weak fluorescence becoming evident at 12 days).

In all, 130 litter isolates and 189 feed isolates were tested. Of the 130 litter isolates, 28 (21.5%) were AT-positive (all strong). Of the 189 feed isolates, 54 were positive (36 strong [19%] and 18 [9.5%] weak).

Table 11. Frequency of AT-positive A. flavus isolates.

Sampling day	No. isolates tested	No. positive		Frequency of +ve isolates %		
		Strong	Weak	Strong	Weak	Total
7	5	1	-	20%	-	20
14	2	0	0	0	0	0
28	2	0	0	0	0	0
35	8	2	-	25	-	25
42	9	2	-	22	0	22
49	9	1	1	12.5	12.5	25
56	15	2	1	13	7	20
63	26	6	-	23	-	23
70	29	6	1	21	3	24
77	30	8	-	26	-	26
84	30	6	3	20	10	30
91	30	7	3	23	10	33
98	30	5	3	16	10	26
105	45	7	4	15	8	24
112	50	11	3	22	6	28
Total	319	64	19	20.06	5.95	26.01

5.1.2 Aflatoxin production on semisynthetic liquid medium (SMKY)

15 isolates of A. flavus from the feed and litter of the layer trial were grown in 50 cm^3 SMKY culture medium (Methods 5.1.2).

The isolates tested included:

5 isolates which were strongly AT-positive on coconut agar at 3 days;

5 isolates weakly positive at 12 days

and 5 isolates negative on coconut agar.

TLC plates spotted with culture extracts of these isolates and developed (Methods 5.3) were examined under long-wave U.V. light. The results are shown in Table 12.

Table 12. Aflatoxin production on SMKY stationary medium.

				TLC analysis			
				B1	B2	G1	G2
	Sigma Standard Aflatoxin			+	+	+	+
	Day 35	A. flavus	1	+	+	+	+
Strongly +ve on	Day 63	"	5	+	+	+	+
coconut agar	Day 63	"	19	+	+	+	+
at 3 days	Day 84	"	5	+	+	+	+
	Day 91	"	6	+	+	+	+
	Day 56	"	5	-	-	-	-
Weakly +ve on	Day 70	"	9	-	-	-	-
coconut agar	Day 84	"	19	-	-	-	-
at 12 days	Day 84	"	24	-	-	-	-
	Day 91	"	27	-	-	-	-
	Day 56	"	3	-	-	-	-
-ve on	Day 63	"	14	-	-	-	-
coconut agar	Day 77	"	10	-	-	-	-
	Day 91	"	23	-	-	-	-
	Day 105	"	10	-	-	-	-

Aflatoxins (B1, B2, G1 and G2) were isolated from A. flavus isolates which were strongly positive on coconut agar, but not from isolates which were weakly fluorescent or negative on coconut agar.

5.2 Multimycotoxin Analysis of Litter/Feed Extracts

10 litter samples and 6 feed samples randomly selected from the layer trial were analysed from the presence of different mycotoxins (Methods 5.2).

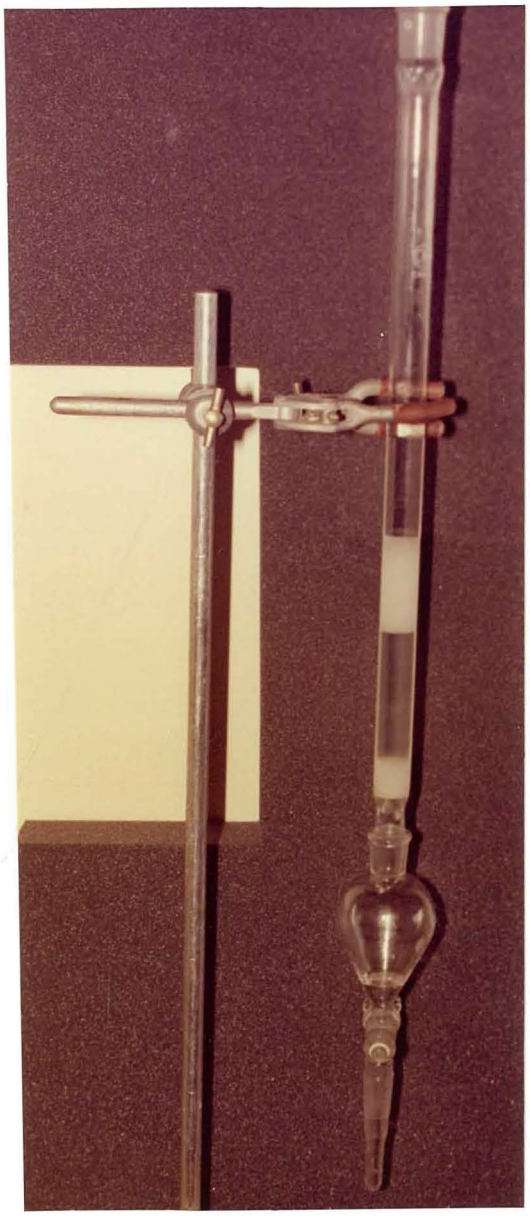
Chloroform extracts of various samples were eluted through a silica gel column with different solvent mixtures, and the eluates spotted onto TLC plates, developed and viewed under long-wave and short-wave U.V. light.

Plate 2 shows the silica gel column. The two white layers are the layers of anhydrous sodium sulphate, and the clearer area in the middle is the silica gel component of the column. The solvent (chloroform) used to make up the column has been drawn off to the top of the upper sodium sulphate layer. Table 13 lists the samples tested by the multi-mycotoxin method for aflatoxin, ochratoxin, zearalenone and T-2 toxin. All sample extracts were negative for the 4 mycotoxins when compared to the respective standards on the TLC plates under the U.V. light of the appropriate wavelength. A few unidentified spots were observed in the day-35 litter and feed sample eluates. These did not correspond to any of the fluorescent spots of the standards.

Table 13. Litter and feed samples tested for the presence of mycotoxins.

Day 14	Litter A	Day 77	Litter A
Day 14	Feed	Day 77	Litter B
Day 35	Litter A	Day 84	Litter A
Day 35	Feed	Day 84	Feed
Day 49	Litter B	Day 91	Litter A
Day 56	Feed	Day 91	Feed
Day 63	Litter B	Day 98	Litter B
Day 63	Feed	Day 112	Litter A

Plate 2. Silica gel column.



DISCUSSION

The environment within a poultry rearing shed plays an important role in the health and productivity of the growing chickens. That environment is influenced by many factors, both physical and biological, including temperature, humidity, air movement within the shed, the nature and composition of feed and litter, the number of birds housed and the microbial flora present. Of these factors, it is the microbial component of the environment that has the most direct effect on the health of the birds, but the microbial population is in turn influenced by all the other factors. In the present investigation, just one of the groups of microorganisms in that microbial population, the fungi, has been studied.

Two methods, dilution plating and strip-baits, were used for studying the succession of fungi in litter and feed. Most other investigators have also used various dilution plating techniques for fungal isolation in such studies (Dennis and Gee, 1975).

With the dilution plating technique, total counts and counts of individual genera and species were obtained by culturing on potato dextrose agar and Aspergillus differential medium, both media containing phosphon. The addition of phosphon greatly reduced overgrowth by spreading moulds. This is in agreement with King (1979) and Henson (1981) who found that the colony size of such moulds was reduced, enabling enumeration of a wide range of other species and so removing one of the problems often associated with the dilution plating method.

The strip-bait method was also found to be a suitable technique for the demonstration and isolation of fungi from poultry feed and litter. Bacon and Burdick (1977) used a similar method, isolating 18 species of fungi, including all those isolated in the present study. The tannic acid incorporated in the nutrient solutions, and other cultural conditions used, seemed to be useful in encouraging the growth of Penicillium and Aspergillus species.

The use of a membrane filter as the inner square to replace the filter paper disc used by Luttrell (1967) was advantageous as microscopic examination of part of the m.f. could give an immediate indication of the types of fungi present. This could be confirmed by

culturing. Such microscopic examination was particularly useful up to the mid-point of the trials when fungal numbers were relatively low. At these lower levels, hyphae and their relationship to conidial heads were easily established by staining the membrane filter with a light stain (diluted lactophenol cotton blue) prior to microscopic examination. When fungal numbers were high, however, the m.f.'s were too heavily infiltrated with hyphae for easy identification of fungal types.

The patterns of fungal development in the litter and feed recognized by dilution plating and by the strip-bait techniques were similar, and almost all genera found by dilution plating were also isolated from strip-baits. Exceptions were genera such as Rhizopus and Mucor. The growth of zygomycetes is not encouraged by the conditions of the strip-bait technique, and furthermore, it is possible that they are unable to grow through the baits due to the width of their hyphae. The adjustment of m.c. of each sample to a higher level, used in addition to the unadjusted sample, proved to be beneficial in that on days when the m.c. of a sample may have been too low for the isolation of a particular genus present, it was possible to isolate that fungus by raising the m.c. of the substrate.

Patterns of fungal succession were more obvious in the layer trial which extended over a longer time period, than in the broiler trial. Also, correlations between dilution plate results and strip-bait results were more pronounced in the layer trial as also were the relationships between patterns of fungal development in litter, feed and air.

In both poultry trials, the main species of Penicillium isolated was P. chrysogenum. A few isolates of P. variabile and P. oxalicum were also recovered. Penicillium was isolated throughout both trials in litter and feed. In the broiler trial, the day 19 peak of Penicillium seen on PDA, was also noticed in strip-bait results and in air samples. In air, the peak first appeared on day 12. In the layer trial, a similar increase was observed and very high levels were attained in the litter. The patterns of development of Penicillium in litter and feed closely resembled those in air. In air the initial peak was on day 2, as it was in the litter.

In air, Penicillium levels declined after day 63, and in litter, levels declined at a later time (after day 84 in litter). In feed,

numbers declined around the same time as air (day 63). This pattern is similar to that found by Dennis and Gee (1973), who found Penicillium to be the main genus in dust.

A. flavus was isolated in the litter of both trials, with numbers increasing as trials progressed. However, no isolations were obtained from the feed in the broiler trial. In the layer trial, A. flavus levels were low in the litter and feed until day 70, when a peak occurred (ADM results). In air the peak was seen slightly earlier (day 63). After day 70, numbers remained fairly constant, as in litter and feed. A. flavus has been found in the poultry environment by many investigators, such as Bacon and Burdick (1977) who found A. flavus to increase in broiler house litter from beginning to end of the period of housing broilers; Lovett (1972) who isolated it from litter and feed, and Pinello et al. (1977) who isolated it from poultry house air.

A. fumigatus was one of the major species isolated in this investigation. Numbers were higher and occurred earlier in the layer trial than in the broiler trial. In the layer trial, the dilution plating of litters A and B resulted in isolations of A. fumigatus from an early stage. A gradual increase was observed, to fairly high levels on both media used. Similarly, the strip-baits isolated this fungus from litter in low colony numbers at the beginning, increasing to high levels at the end. In the air of the layer house, A. fumigatus was found throughout the trial at levels reflecting its presence in the litter. A. fumigatus levels began to increase around the same time in air (day 35) as in litter and feed. This fungus has been found by many investigators in litter and feed (Bacon and Burdick; 1977, Dennis and Gee, 1973) and in air (Sauter et al., 1981).

Sopulariopsis brevicaulis has been found in practically all investigations of fungi in poultry houses. Dennis and Gee (1973) reported that species of Scopulariopsis predominated in final litter samples, along with species of Aspergillus. Also S. brevicaulis predominated in dust samples. Scopulariopsis dominating in later litters was also noted by Lovett et al. (1971), who correlated this to the high alkalinity of the older litters. In the present study, S. brevicaulis was isolated in high levels in both trials. In the layer trial, levels of S. brevicaulis were much higher, and were found earlier than in the broiler trial In the broiler trial, S. brevicaulis first appeared in litter on day

26 (ADM results), whereas it appeared in air on days prior to this (12 and 19). In the layer trial, Scopulariopsis first appeared in the air at day 21, but occurred slightly earlier in litter and feed. Numbers in the air began to rise from an early stage (day 21) and reached very high levels (as in litter and feed) at the end of the trial.

A. terreus was another species of Aspergillus found. This species was present in much lower levels than the other Aspergillus species. In the broiler trial it was isolated only by strip-baits. A corresponding peak was noted in the air on day 12. Slightly higher levels were found in the layer trial. In this trial, A. terreus increased during the early stages, was absent in the middle stages and was re-isolated during the last 3-4 weeks. This pattern was observed by dilution plating, strip-baits and air sampling. A. terreus has not been reported in many investigations, but other species of Aspergillus, eg. A. repens, A. clavatus and A. candidus, have been found to occur in low levels in the air (Dennis and Gee, 1973) and litter (Bacon et al., 1974).

Paecilomyces sp. has been found by various investigators in poultry litter and feed. Lovett (1972) isolated this genus from feed and litter. Dennis and Gee (1973) noted that Paecilomyces was one of the genera found in fresh litter. In the present study, this genus was found only on the first day of sampling in both trials. Numbers were low in litter and feed, and none was isolated from the air.

Geotrichum was isolated only in the layer trial. The pattern observed was similar to A. terreus. An initial decrease occurred to very low levels, followed by a later increase. Strip-bait and dilution plating results showed similar patters to that in the air. Geotrichum has been reported to occur in poultry litter by Lovett et al. (1971), although no patterns of occurrence have been described.

Low levels of other genera, such as Cladosporium, Rhizopus, Mucor and Trichosporon were also observed in this study. Cladosporium levels in feed and litter corresponded to levels in the air, in the broiler trial. In the layer trial, the peak of T. cutaneum on day 26 in the litter ws preceded by an increase in the air on days 12-19. Lovett et al. (1971) reported the presence of low levels of Cladosporium, Rhizopus and Mucor in poultry feed and litter. Pinello et al. (1977) recovered

Cladosporium, Geotrichum, Mucor, Rhizopus and Paecilomyces from the air and litter of a turkey house.

In both trials the total mould count in air increased with time. Penicillium, Aspergillus and Scopulariopsis showed the highest frequencies of occurrence. This is in agreement with Sauter et al. (1981). As discussed above, levels of fungi in the litter and air are interrelated; eg. Penicillium and A. fumigatus occurred in litter and feed around the same time as in air. In the cases of A. flavus and Scopulariopsis, the peak levels found in air preceded those in litter and feed. Thus the air appears to play a role in influencing the types and relative densities of some fungi found in feed and litter, but high levels of others in feed and litter preceed high levels in the air. This correlation was more obvious in the layer shed than in the broiler shed. Also, in the layer shed, the numbers of fungi isolated from the air, litter and feed were higher than in the broiler shed. This could be due to the fact that the layer trial was much longer (18 weeks) than the broiler trial (40 days), there were more birds per pen, and because layers are much more active birds than broilers. The broilers are fed a richer diet, are larger and are more docile, causing little air movement. Thus in the layer shed, moulds in feed and litter are much more likely to be dispersed in the air.

Dennis and Gee (1973) noted that environmental conditions in poultry houses appeared to favour the development of the organisms present in the dust (Penicillium, Aspergillus, Scopulariopsis) rather than those in initial wood shavings (Paecilomyces, Trichoderma). In this study Penicillium was found in air, litter and feed throughout. Aspergillus and Scoppulariopsis increased in litter and feed mainly towards the middle and end of the trials, and may have been influenced by levels in air. Paecilomyces, which was found in fresh litter, was not isolated in later litters.

In litters, the total counts increased during the trials. Litter of pen 10 of the broiler trial showed higher total counts for most of the trial than litter of pen 3. Pen 10 was one of the pens at the end of the shed, whereas pen 3 was closer to the door. Regular opening and closing of the door of the shed may have had the effect of "airing" pen 3, thereby slightly reducing the total mould counts. In the layer trial, the similarity in total counts may have been due to the relative

closeness of the two pens and their similar surroundings.

In layer trial litters, total counts increased greatly in both tested litters during the trial, reaching about 800 CFU g^{-1} at the end. Litter A was from pen 3 and litter B from pen 6 in the layer house. These pens had similar surroundings and similar bird densities. Each had a fan placed in the wall, and thus would have been equally aerated.

Although litter and feed may contain fungi, the numbers and types present in a poultry house are probably not due entirely to moulds already present. Various factors such as poultry house design (including temperature and humidity), the moisture content and pH of litter/feed are also likely factors in determining the fungal flora present at any one time.

The litter and feed were found to be favourable substrates for fungal growth. Litter is composed of organic materials which may harbour fungi and fungal spores on entry into the shed. Litter, especially when older, has a wide nutrient composition. Kunkle et al (1981) reported that built-up litter could be used as feedstuff for ruminants because of its mixed composition, and Kennard and Chamberlain (1948, 1951) reported it to be beneficial to chickens.

However, because of its rich nutrient composition, ageing litter becomes an increasingly favourable substrate for fungal proliferation. Ross and Miyahara (1971) have reported that old litter may perpetuate disease organisms and suggested that fumigation with methyl bromide is necessary if old litter is to be re-used. In the poultry trials studied in the present investigation it is significant that potentially disease-causing organisms increase as the litter ages, perhaps encouraged by the increasing nutrient content.

Feed and feed ingredients are also known to harbour microorganisms. In this study, the various fungi isolated from litter were also isolated from feed, although in lower numbers. Tabib et al. (1981) found feeds to contain high mould counts (5 - 10^6 g^{-1}). Certain fungi, eg. A.flavus, can survive in feed ingredients for many years (Hesseltine and Rogers, 1982). The feed used at the poultry research centre is unpelleted. Tabib, Jones and Hamilton (1984) reported that pelleted feeds have lower mould counts.

Many of the feed ingredients are nutritious to moulds, eg. Apex meal, one of the feed components used at the P.R.C., is a blood and bone meal made from the effluent of the floor of a freezing works. The effluent is collected and allowed to settle; the upper fat layer is drained, and the lower protein component is concentrated and purified, and subsequently incorporated into poultry diets, to supply energy.

Fungal numbers in feed from the broiler house were lower than in the litters. Feed in this trial was collected from the pan base of a feed trough in pen 10. In these feed troughs, the cylindrical feed container continually replaces the feed as it is consumed and as a result microbial numbers are kept low.

Feed in the layer trial was collected from pen 3 and again mould numbers were generally lower in the feed than in the litters. However, feed numbers in this shed were higher than in the broiler shed. In the layer house, feed is supplied by a chain system of pan feeders. Thus old feed in the pans is regularly mixed with new feed from the reservoir. Such mixing occurs once or twice a day depending on the amount of feed consumed by the active birds. Samples were collected early in the evenings before the second mixing took place. Therefore, although mould numbers maintained relatively low levels, there was sufficient time during the day for mould numbers to rise, possibly because of exposure to dust with a high mould content.

Moisture contents (m.c.) of weekly samples of litter in the broiler trial increased from approximately 6% to approximately 12%. In the layer trial the range was from 6% to 19%. Kunkle et al. (1981) reported aged litter to have a m.c. of 23.8%, whereas in the study conducted by Dennis and Gee (1973), used litters had higher m.c. values, i.e. 25-38%, with fresh litters having m.c. values close to 9%. Litter becomes moist mainly due to the excreta and respiration of the chickens. In the layer trial the number of birds per pen was larger than in the broiler trial and the layers were much more active. This, as well as the fact that the layer trial was much longer, explains why m.c. values were higher in the layer trial.

In the feed, the m.c. ranged fom 5-7%, in the two trials. This would be due to the regular addition of dry, new feed to the feed troughs or pans. Since the birds tend not to get into the feed con-

tainers, the feed does not get contaminated by urinary moisture. The slight increase in m.c. which was observed in the feed could be due to the increase in atmospheric moisture.

M.c. may play a role in determining the total fungal numbers. A substrate that is moist can be expected to carry more microbes than one that is dry, as most microorganisms require moisture for growth and proliferation. In this study, incresing m.c. values were accompanied by increasing mould numbers. Dennis and Gee (1973) suggested that initially, fresh wood shavings have a low m.c. and have certain fungi, eg. the A. glaucus group that can grow at low moisture levels, and that when they release moisture by their metabolic activities, the litter becomes more favourable to other fungi, such as Penicillium and A. flavus. In this investigation Penicillium was found throughout the trials, and not necessarily only later on. The only genus which was found only at the beginning on fresh litter, was Paecilomyces on day 1. A. flavus and most of the other moulds isolated, were found to increase during the course of the trials, as the litter/feed got older.

In the preliminary experiments to test the strip-bait in the present investigation, it was found that a substrate m.c. of 20% was the optimal for fungal isolation. As the m.c. values increased in the trials, fungal numbers increased. Thus m.c seems to play an important part in the numbers of fungi present.

In addition to the m.c., weekly samples were measured for their pH values. In both feed and litter samples, pH values were initially on the acidic side and then increased, becoming more alkaline with time. pH values of litter ranged from approximately 5 to 8 in the broiler trial and approximately 5 to 11 in the layer trial. For feed, pH values ranged from approximately 5 to 6.5. A rise in pH is observed due to the release of ammonia from the decomposition of uric acid by bacteria (Schefferele, 1965b). There are conflicting opinions on the effect of such pH rises on the total mould population. Reports of Schefferele (1965a) and Halbrook, Winter and Sutton (1951) agreed that moulds and yeasts decreased with time due to the increasing alkalinity of poultry litter since the effect could be accentuated by the addition of lime. However, Lovett (1971) found that pH had very little effect on the levels of individual species within the microbial population. In the present study, a relationship has been described between pH of samples

and total fungal counts (Results 2.3). In both trials, as the pH reached the alkaline side, total fungal counts increased. However, the increasing fungal counts have also been shown to be associated with other variables eg. m.c., thus it is difficult to establish a clear relationship between counts and any one environmental factor.

The presence of high frequencies of A. flavus and A. fumigatus in the poultry environment is significant. In view of the large number of A. flavus isolations it was appropriate to test these strains for the production of toxin, and also to analyse samples of feed and litter for the presence of any other mycotoxins.

Over 300 A. flavus isolates obtained from litter and feed throughout the layer trial were subcultured onto coconut agar to test for the presence of aflatoxin (AT). This screening test yielded a number of AT-positive isolates.

Many of the positive isolates exhibited strong fluorescence at 3 days. Some were negative at 3 days but positive at 7 and 12 days, and others were positive only at 12 days, showing a weak fluorescence. It is interesting to note that on most sampling days, 20-30% of isolates tested were AT-positive. Of the litter isolates tested 21% were AT-positive, and of feed isolates tested 28% were positive.

Some of the A. flavus cultures were then further studied on semisynthetic liquid medium (SMKY). Those strongly positive on coconut agar were found to be positive for aflatoxin production in SMKY medium, as seen by fluorescent spots on TLC plates which matched those of the standard aflatoxin. The weakly fluorescent cultures produced no fluorescent spots after growth in SMKY medium and neither did the negative cultures. Either the weakly fluorescent cultures did not contain toxin in high enough levels to be detected on the chromatograms, or the original coconut agar test was falsely positive.

Interesting ecological information can be obtained by this approach of isolating fungi from samples and testing strains individually for their potential toxigenicity. The presence of AT-positive cultures does not, however, necessarily mean that the toxin has been produced in the substrate. Even if toxigenic strains are isolated, these may have been derived from dormant spores, conditions within the substrate not having

been suitable for active fungal growth. But the ability of cultures to produce toxin may be significant when large numbers of the mould are isolated.

The multimycotoxin method was carried out on selected samples of feed and litter to test for the presence of aflatoxin, ochratoxin, T-2 toxin and zearalenone. The method involved chloroform extraction of the samples, silica gel column chromatography for the separation and isolation of the different mycotoxins and TLC analysis of the column eluates. None of the samples, however, was found to contain any of these mycotoxins. All samples spotted onto TLC plates, developed and observed under UV light of the appropriate wavelength, were negative for fluorescence.

The production of mycotoxins in litter has not been widely reported, but Lovett (1972) stressed the importance of this possibility, both to the health of the birds themselves and in view of the proposed incorporation of poultry house litter as a nitrogen source in livestock feed. In feed, however, various reports have shown that mycotoxins may be present, and these have been reviewed by Smith (1982). The increasingly high peaks of A. flavus found in this study are significant as toxins may be formed in the litter by the moulds which are toxigenic. Also, spores which contain toxin can be found in the air, and this could be dangerous if inhaled by the birds (Whitlow and Shotwell, 1983).

In this study, the abundance of fungi in poultry houses has been demonstrated and an attempt has been made to elucidate patterns of fungal succession formed in litter, feed and the air. Levels of most fungal genera were shown to increase during the time of housing of the birds. High levels were also found of those fungi which pose a threat to the health of the chickens, and some A. flavus cultures were positive for the production of aflatoxin. Thus it has been shown that fungi are a significant part of the microbial component in the poultry environment. It is important, therefore, that their abundance and the consequent possible dangers to flock health be recognized so that problems of fungal diseases in poultry can be avoided.

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