Modern media and methods in food mycology

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Methods used in food mycology were largely developed from those used in food bacteriology. Enumeration by the dilution plate count, incubation at high temperatures, and reliance on a single general purpose medium, to provide a general 'yeast and mould' count, are all legacies of the influence of food bacteriology. However, food borne filamentous fungi have growth patterns quite different from those of bacteria, and grow under a much wider range of environmental conditions. They produce quite different kinds of toxins, and must be identified by quite different techniques. The challenge facing those studying food borne fungi has been to draw on the expertise of the bacteriologist, mycologist, plant pathologist and microbial physiologist to develop enumeration methods, media, isolation techniques and identification schemes specific for the fungi in foods. In the past decade, this challenge has been met with increasing effectiveness. This paper describes some of the ideas and techniques, the methods and the media, which are being developed specifically in the study of fungi in foods.

Improved enumeration techniques

Unlike most bacteria, fungi grow as filaments. This has three consequences of importance in the development of enumeration techniques: first, growth and the formation of particles (i.e. spores) which can readily be counted are two separate events, and not always directly related; second, fungal colonies spread and, if unchecked, rapidly become very difficult to enumerate; and third, fungi can penetrate solid foods. Techniques which enumerate fungi only from the surfaces of solid foods may be ineffective and misleading. The basic reproductive structures in fungi, the spores, are almost inert biologically, so enumeration of spores, especially from the surfaces of solid or particulate foods, may give little or no information about growth or toxin production within the food.

In developments in the enumeration of food borne fungi have come from many sources. The result of lacking of standardisation makes comparison of results from different laboratories very difficult, because of the plethora of techniques and methods used.

At a specialist workshop held in Boston in 1984 it was agreed that for powdered or liquid foods, certain of the techniques employed by bacteriologists were perfectly adequate. These included dilution techniques with appropriate diluents, spread plating, which is superior to pour plates for filamentous fungi, plates incubated upright, and incubation almost always at 25°C. However, for particulate or solid foods, for example nuts or grains, the workshop also recommended the technique known as direct plating. This is a quite radical departure from bacteriological techniques.

In direct plating, samples of particulate foods are surface dispersed in a choline solution, then plated on the surface of appropriate solidified media and incubated for a suitable time. Plates are then examined visually, usually with a stereomicroscope, and the types of fungal growth can be counted and recognised. Selected areas of fungal growth may then be picked off for examination with the compound microscope or inoculated onto suitable isolation or identification media (Figure 1).

For particulate foods such as nuts or grains, direct plating provides an estimate of the extent of infection of a commodity, and is usually expressed as a percentage. Surface sterilisation is an essential step in this process, it is an effective method for monitoring invasion of commodities by toxigenic fungi. For solid foods, where sampling involves cutting pieces of food for plating, direct plating is essentially a qualitative technique. Despite this, it is the only satisfactory isolation method for certain fastidious xerophiles discussed below.

Modern media

General purpose media

Fungal enumeration and isolation media should not only restrict fungal spreading but must prevent bacterial growth also. Decades ago, soil mycologists found that rose-bengal and ox galls reduced both spreading growth and bacteria. However, food microbiologists enumerating or isolating fungi still relied until much later on tetracycline agar, acidified potato dextrose agar being the most popular. Mossel et al. 10 showed that neutral pH media, with oxytetracycline added to control bacteria, were more effective for fungal enumeration than formulations which relied on acidity for bacterial suppression, because low pH often adversely affected mould cells or spores.

The problem of spreading colonies remains. Jarvis' devised rose-bengal chloramphenicol agar (RBC) which proved to be an effective general purpose enumeration medium. King et al. added dichloran 11 to produce dichloran-rose-bengal-chloramphenicol agar (DRBC), and greatly improved the suppression of spreading fungi such as Rhizopus and Mucor species. Put and Conway 12 reported that chloramphenicol was a superior antibiotic to chlorotetracycline: both RBC and DRBC are now commonly formulated with chloramphenicol. Both media have found wide acceptance among food mycologists, and both are in commercial production.

Media for xerophilic fungi

Xerophilic fungi are defined as being able to grow at the conditions of greatly reduced water activity. Such fungi are of great importance in the spoilage of dried and concentrated foods and bulk commodities. Many xerophilic fungi, such as Aspergillus and Penicillium species, have optimal aw for growth very close to 0.9, and can be effectively enumerated on general purpose media. However, other quite common occurring xerophilic species, including A. niger, Penicillium wakamatsu and many Eurotium species, have lower growth optima and hence germinate and grow poorly if at all on commonly used high aw media. For enumerating these fungi, dichloran-18 per cent glycerol agar (DG18) 13 was devised. Counts of fungi on dried foods such as peppers, chilies or dried fish can be up to 5 log cycles higher on DG18 than on DRBC. 11

Enumerating xerophilic species such as Xeromyces bisporus or the xerophilic Chrysosporium species is a special challenge. Xeromyces will not grow above about 0.95 aw, so will be missed if any normal isolation or enumeration medium is used, even if visual inspection of mouldy food indicates vigorous growth. C. xerophilum and C. imperfatum are little better. Also, experience has shown that these species are very difficult to isolate by dilution plating, even when low aw diluents are used. The recommended technique for enumeration or isolation of these fungi is direct plating, either of particulate foods, or by cutting pieces from solid foods such as dried fruit or licorice. When xeromyces or the chrysosporia are present in pure culture, and they often are, direct plating onto malt yeast 50 per cent glucose agar (MY50G) 14 is effective. If other xerophilic species such as Eurotium species are also present, recourse must be made to malt yeast 70 per cent glucose fructose agar (MY70G). 3 On this medium, 0.76 aw xeromyces will outgrow any other fungi.

Isolating and enumerating halophilic xerophiles from salty foods, particularly salted, dried seafoods, again requires special media. For...
Media for toxicigenic fungi
The first notable attempt to formulate a selective medium for toxicogenic food borne fungi was by Arthaud and Fennell. They produced a medium on which Aspergillus flavus and A. parasiticus, the aflatoxin producers, were strongly distinguished from other fungi by an orange colour reaction. However, the medium lacked selectivity. This was later improved by dichlor, refinements to the formula and altered incubation conditions, resulted in Aspergillus flavus and parasiticus, being differentiated from the large variety of innocuous yeasts which may contaminate raw materials and processing lines.

Detection of low numbers of yeasts
Perhaps the simplest way to detect small numbers of yeasts in liquid products is by membrane filtration for clear liquids, and enrichment techniques for products which cannot be filtered. A simple enrichment method is the product itself, diluted 1:1 with water. This has been used to detect yeasts in numbers as low as 1/g in cream products.

New methods
Various indirect methods of measuring fungal growth in foods have been proposed or are being investigated, to replace the traditional enumeration methods. Older methods include chemical estimation of ATP and ergosterol. Newer methods, such as impedimetry and estimation of fungal ATP, are also being evaluated but are not yet in general use. Perhaps the most promising new techniques for detecting and quantifying fungal growth in foods is the ELISA technique, using antigens specific for common food-borne fungi such as Aspergillus, Penicillium and Cladosporium species. The technique appears to be very specific, and possibly more sensitive than colony counting methods. However, ELISA techniques will need to be rigorously tested against the traditional methodologies before the results can be compared with our current concepts of acceptable and unacceptable levels of moulds in foods.

Conclusion
It has taken a relatively few years for food mycology to emerge from its origin in food bacteriology, with interaction from several other fields, as a discrete discipline. Much, however, remains to be done.

Better measures of fungal growth are urgently needed, both microbiological and chemical. Measures of growth, regardless of type, need to be linked mathematically to more fundamental properties, such as biomass production and respiration, and perhaps to more practical significance, such as sporulation, spoilage and toxigenesis.

Better selective media are urgently needed, especially for toxicogenic fungi. Ideally a medium should select for one or more related species, and also develop some distinct colour or other reaction to allow differentiation from extraneous organisms, so that non-specialists can enumerate or isolate the desired species. Such methods need to be as rapid as possible, so that they can be of value in quality control. Greater standardisation of existing methodologies is also an urgent requirement.

References

Figure 3: Isolation of Xeromyces bisporus from pelleted animal feed vitamin supplement by direct plating of the sample onto a low water activity (a_w) medium, MY50G, the left hand Petri dish contains an uncontaminated sample (left) and a moudly sample (right). When plated onto MY50G, the moudly sample yielded an almost pure growth of the xerophilic fungus X. bisporus. This species will not grow on high a_w media such as DRBC.
Lyme disease: a perspective

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Lyme disease is a recently recognised disease affecting both man and animals and is caused by the spirochaete Borrelia burgdorferi. The disease is transmitted by the bite of ixodid ticks, *Ixodes ricinus* being the principal European vector (Figure 1) and *Ixodes dammini* the main vector in North America. Early symptoms include the formation of a characteristic skin rash at the site of the tick bite, flu-like symptoms and lymphadenopathy. Later manifestations of Lyme disease affect the central nervous system, heart and may be associated with chronic skin disease and arthritis. Although Lyme disease responds well to antibiotic therapy, prevention gives rise to problems. Clinical and laboratory criteria for diagnosis are poorly defined with physical presentations sometimes complicating the picture.

**History**

Signs now known to be synonymous with Lyme disease have been described since 1909, when Abbeius described a skin rash, erythema migrans, which was associated with the bite of a tick. In 1922, Garin-Bujadoux recorded the neurological complication of meningopolyneuritis which was associated with tick bites. By 1951, pencilin was shown to aid the resolution of erythema migrans and associated meningitis. Human to human transmission of erythema migrans was demonstrated in 1960, further supporting an infective cause. Sixteen years later, in NE America a cluster of cases of suspected juvenile rheumatoid arthritis were observed in Old Lyme, Connecticut. This resulted in 'Lyme disease' being reported to the Yale University by two concerned mothers. The subsequent investigation by Allan Steere showed that 39 children and 12 adults from a population of 12,000, had what became known as Lyme arthritis. Twenty-five per cent of cases also described a skin rash weeks before the onset of arthritis. Epidemiological analysis of cases in Connecticut in 1979 and 1980 showed an anthropod vector which was later found to be *I. dammini*. A later prospective analysis of patients revealed that arthritis was only one of the sequelae of this disease. Patients also developed a variety of neurological and cardiac complications; as a consequence of this the condition was renamed Lyme disease. The parallel was drawn between the skin rash of Lyme disease and erythema migrans previously seen in Europe. It was not until the early 1980's when Dr Willy Burgdorfer at the National Rocky Mountain Fever Laboratory in Montana, while examining ticks from Shelter Island for ricettia, observed spirochaetes in the mid gut of an adult female *I. ricinus*. These spirochaetes (later termed *Borrelia burgdorferi*) reacted with sera from patients with Lyme disease when tested by an indirect immunofluorescence technique and produced skin lesions resembling erythema migrans after intradermal inoculation in rabbits. DNA homology studies confirmed that these spirochaetes were a genetically distinct *Borrelia* species.

**Epidemiology**

The true incidence and exact prevalence of Lyme disease in the United Kingdom, and indeed the rest of the world, is difficult to determine. The medical and veterinary professions are only just becoming aware of the diverse spectrum of presenting features of Lyme disease; diagnostic tests, although improving, are not sufficiently sensitive and specific to detect all cases, and there is accumulating evidence of asymptomatic cases of Lyme disease. All of these factors have so far prevented the exact incidence of the disease from being known. Between 1986 and 1988, our laboratory has serologically investigated over 4000 people in the UK and, as mentioned above, this underestimates the true incidence of the disease. During the summer, the sexes appear equal in a peak incidence in the 30 to 50 age group. Other studies have found a predominance of cases in girls, possibly as a result of increased exposure to ticks through work or recreation.

**Geographical distribution**

Almost all European countries have reported indigenous Lyme disease cases, including Great Britain, France, Germany, Austria, Belgium, Switzerland, Sweden, Italy and European Russia. The southern European limits is above the 32°C isotherm. Cases have also been reported from as far afield as Canada, Japan, China and Australia. In America, Lyme disease has been reported in at least 32 states in the USA and has been concentrated in the Northeast (Connecticut, Massachusetts and New York), the West (California, Oregon and Utah), and the Northern Midwest (Wisconsin and Minnesota).

**The vector**

Currently five species of ixodid ticks have been incriminated as vectors of Lyme disease. These are *I. ricinus* in Europe (Figure 1), *I. dammini* in the NW United States, *I. pacificus* on the West coast of the United States and *I. scapularis* in the south. *I. pacificus* is postulated as the vector in Japan. *B. burgdorferi* has been detected in all three stages of Ixodes ticks. However, the nymphal and adult stages harbour *B. burgdorferi* to a much greater extent than the early larval form. The life cycle requires three hosts and can be completed within two years (Figure 2). Although transovarial passage of *B. burgdorferi* has been experimentally demonstrated, it occurs at a low frequency (1.9%). Larval ticks tend not to transmit *B. burgdorferi*, but acquire the infection by feeding on infected reservoir hosts such as mice. Mice may harbour spirochaetes in their circulation for up to three months following infection and therefore serve as a good source of infective material. Once the tick is infested the spirochaete persists in the mid gut of the tick throughout its life stages. The seasonal variations in infection reflect the tick ecology, with high relative humidity and temperatures above 13°C required for the tick to quest, explaining the spring and autumnal peaks of the disease. Although other ticks, and indeed other haematophagous arthropods may harbour *B. burgdorferi*, their ability to act as vectors for Lyme disease appears to be minimal.

**Animal reservoirs**

A large number of animals and birds have been suggested as reservoirs for *B. burgdorferi*. Immature *I. ricinus* ticks commonly parasitise rodents such as mice, squirrels, rabbits, voles and birds. Adult ticks show a predilection for larger mammals, especially sheep and deer. Mice are believed to be the major reservoir for *B. burgdorferi*, with infected mice harbouring the spirochaete for up to three months thereby permitting ample opportunity to infect immature ticks. Infected ticks are then able to transmit the infection to larger mammals. Birds may have an important role for dissemination of Lyme disease and following migrations may lead to the establishment of new disease foci. Deer have a different role in the epidemiology of Lyme disease, providing a host able to support large numbers of adult ticks so permitting completion of the tick's life cycle (Figure 2). A wide variety of other animal species, including domestic dogs and cats, have now been identified as having circulating antibody against *B. burgdorferi*. *B. burgdorferi* has also been cultered from these animals, which rarely show symptomatic infection.

**Control measures**

Several methods of control have been investigated including the destruction of vegetation in endemic areas by

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<td>Nymphs</td>
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<td>Larval</td>
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| Figure 1: Nymph stage of *Ixodes tick*. |

**Figure 2: Life cycle of ixodid ticks.**

| Figure 3: Erythema migrans: lesion showing expanding red border and central clearing. |

| able host, at the tick's expense. The individual exposed to ticks should take precautions to prevent tick bites, for example tucking trousers into socks or wearing boots. Examination for any attached ticks following exposure is advisable and prompt removal of any ticks reduces the probability of infection.

**Symptoms**

There has been great temptation to divide the symptoms of Lyme disease into three stages by analogy with syphilis. Stage one comprises of the non-specific systemic changes and the characteristic skin rash erythema migrans (formerly termed erythema chronicum migrans), the second stage is characterised by neurological and cardiac sequelae whilst stage three includes the chronic skin, neurological and arthritic symptoms. These stages can overlap and may even be absent, so that it is preferable to classify disease symptoms into those representing either early or late disease.

**Early symptoms**

Onset may follow 1–2 weeks following the introduction of the spirochaete by the bite of a tick, non-specific symptoms of fatigue, malaise, headache, fever, chills, arthralgia, myalgia, regional
lymphadenopathy and stiff neck may be experienced. A skin rash, erythema migrans may develop at the site of the bite (Figure 3). This characteristic rash is the hallmark of Lyme disease and occurs in approximately 70% of cases two to three weeks following a tick bite. The rash slowly expands with central clearing to form an annular lesion with a bright red border. Late complications of Lyme disease A small percentage of people will progress to later complications of Lyme disease. Approximately 15% of patients develop neurological manifestations, especially cranial nerve palsies, meningoradiculitis and encephalitis.13 Lyme meningitis is typified by a lymphocytic pleocytosis with approximately 100 cells per mm³, the protein slightly raised and glucose normal or slightly reduced. A range of neurological symptoms which have been associated with Lyme disease are listed in the accompanying table. Chronic skin eostrophia facialis lesions, lymphadenosis benigna cutis, acneformitis chronica atrophicans (ACA) may develop within six months but may be delayed for many years after initial infection. ACA starts with an inflammatory phase followed by cutaneous atrophy rather like morphae and occurs in up to 6% of Swedish and central European cases.13 These complications although found in the UK seem to be less common. Cardiac complications occur weeks to months after infection with an incidence of 8% in American patients, however, this is seen less often in European countries. Symptoms of Lyme carditis include pericarditis and interstitial myocarditis but have shown the true incidence of rheumatological complications to be nearer 10%. Patients experience migratory episodes of joint pain and a minority may subsequently develop episodes of arthritis weeks to years after the onset of Lyme disease. Arthritic attacks may recur over several years and may lead to chronic synovitis with erosion of the joint. Treatment Early disease responds well to treatment which both shortens the duration of symptoms and reduces the incidence of later complications. Late disease responds less well, especially for neurological complications. Tetracyclines are generally considered the most appropriate treatment for uncomplicated Lyme disease. Penicillin or erythromycin may be used as an alternative, however, treatment failures have been reported. Late Lyme disease is more refractory to treatment. Parenteral therapy with third generation bactericidal cephalosporins such as ceftriaxone or cefotaxime are more effective.15 Jarisch-Herxheimer reactions may occur at the commencement of treatment but are rarely as life threatening as seen in the relapsing fever borreliosis.

Animal symptoms Veterinary case reports have described symptoms of arthritis in dogs,14 erythema migrans in a horse15 and a wasting disease and arthritis in a cow.16 There have also been reports of kidney, liver and testicular failure by death and symptoms of heart block involving the atrioventricular node in dogs.17

Laboratory diagnosis Microscopy Microscopic examination of material for B. burgdorferi can be achieved using a variety of different methods including dark field microscopy, silver stains and fluorescent staining with either monoclonal or polyclonal antibodies. None of these methods are sensitive and all require lengthy examination of material for spirochaetes. Dark field examination requires the presence of a large number of spirochaetes and is thus probably best restricted to the examination of cultured borrelias rather than direct examination of clinical material. False positive results may be observed with clinical material, for example, collagen fibres resembling spirochaetes. This has been reported when examining material for leptospira. A variety of stains can be used to visualise spirochaetes most of which are variations on silver staining methods. None of these are particularly sensitive or specific. The Warthin-Starry and Deltele staining techniques are the two most popular methods. Spirochaetes can also be stained with acidine orange, however, an immunofluorescence method is more sensitive, particularly for extrasympathetic (Figure 4). Immunofluorescence with either polyclonal or monoclonal antisera specific for B. burgdorferi will simultaneously visualise and identify spirochaetes present. Electron microscopy is not a practical method for detection of spirochaetes in clinical material but has a role in confirming that the spirochaetal forms seen on dark field are genuine. Electron microscopy also distinguishes borrelia from treponema by the number of periplasmic flagella present (Figure 5).

Culture Borreliae have been cultured from a variety of specimens including ticks, skin biopsies, CSF, blood and synovial fluid. Culture is a low yield procedure with microscopy positive material not infrequently failing to yield growth of borreliae. The medium used is modified Barbour-Stoenner-Kelly medium (BSK).17 It is a highly nutritious complex medium but suffers from batch to batch variation and has a short shelf life. The medium can be made selective by the addition of antibiotics such as kanamycin, streptomycin, rifampicin, and colistin sulphate.

Serology As a result of the problems with microscopy and culture, serology remains the only practical method for the diagnosis of Lyme disease.20 The two principal methods used are indirect immunofluorescence (Figure 4) and enzyme linked immunosorbent assay methods (ELISA). Immunoblotting can be used to confirm serology results. Serology can also be used to analyse other body fluids such as CSF and synovial fluid to assist the identification of areas of disease activity.

During early disease serology is less useful with patients with erythema migrans not producing significantly elevated levels of antibodies. The IgM response is often not usually detectable during the first two weeks and peaks between the third and sixth weeks. The IgG response is not usually detectable until the fourth week following infection and may remain elevated years after clinical remission. Prompt antibiotic treatment may prevent significantly elevated antibody levels. Although serodiagnosis is now readily available, it is not without shortcomings. Sensitivity of the tests is poor during early disease. Serological tests for Lyme disease not very specific, patients with other spirochaetal diseases and active collagen disorders may give false positive results. Subjectivity is an additional problem encountered with IFA and immunoblotting methods. The problems of serodiagnosis of Lyme disease have recently been reviewed.22

Concluding remarks As a result of the large number of animal species which serve as hosts, it is unlikely that a control programme could be implemented in the near future. Avoidance and prompt removal of ticks and treatment of clinical symptoms as they occur will help to reduce the risk of late complications of Lyme disease. Although serological tests are now available for the diagnosis of Lyme disease, they should not be used in isolation from the clinical symptoms.

Acknowledgements I thank Dr DJM Wright, Medical Microbiology, Charing Cross Hospital, for his help.

References

Table 1: Neurological complications of Lyme disease.

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<td>Radiculitis</td>
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<td>Spastic paraparesis</td>
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Late complications of Lyme disease Small percentage of people will progress to later complications of Lyme disease. Approximately 15% of patients develop neurological manifestations, especially cranial nerve palsies, meningoradiculitis and encephalitis. Lyme meningitis is typified by a lymphocytic pleocytosis with approximately 100 cells per mm³, the protein slightly raised and glucose normal or slightly reduced. A range of neurological symptoms which have been associated with Lyme disease are listed in the accompanying table. Chronic skin eostrophia facialis lesions, lymphadenosis benigna cutis, acneformitis chronica atrophicans (ACA) may develop within six months but may be delayed for many years after initial infection. ACA starts with an inflammatory phase followed by cutaneous atrophy rather like morphae and occurs in up to 6% of Swedish and central European cases. These complications although found in the UK seem to be less common. Cardiac complications occur weeks to months after infection with an incidence of 8% in American patients, however, this is seen less often in European countries. Symptoms of Lyme carditis include pericarditis and interstitial myocarditis but have shown the true incidence of rheumatological complications to be nearer 10%. Patients experience migratory episodes of joint pain and a minority may subsequently develop episodes of arthritis weeks to years after the onset of Lyme disease. Arthritic attacks may recur over several years and may lead to chronic synovitis with erosion of the joint.

![Figure 4: Immunofluorescence stain of spirochaetes x 50 (UK strain). Note tendency of spirochaetes to clump.](image-url)

![Figure 5: Electron microscopy showing cross section of B.burgdorferi (UK strain).](image-url)

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