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Functional foods: probiotics and prebiotics

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Introduction

The main role of diet is to provide nutrients to meet host physiological requirements. As research behind diet and health has evolved, so has the concept of 'functional foods' become popular. Foods which are touted as being 'functional' are thought to exert certain positive properties over and above their normal nutritional value. While not universally popular and sometimes plagued by inadequate research/claims, the concept is certainly commercially successful, e.g. The Institute of Grocery Distributors (http://www.igd.com) estimates that the functional food market in the UK in 2007 will have annual sales worth around £1800m. This shows an exponential rise from the 1996 figure of £134m. Examples of functional foods include organic and inorganic micronutrients, vitamins, anti-oxidants, dietary fibre, some proteins (e.g. lactoferrin), certain bioactive peptides and polyunsaturated fatty acids.

The concept has now moved markedly towards gastrointestinal function, in particular the impact of gut bacteria. Possibly this is driven by the ubiquity of gastrointestinal disorders but also the fact that diet is an important controlling factor with regard to indigenous microbiota activities. The gut microflora contains pathogenic, benign and beneficial microbial species. A predominance of the former can lead towards gut upset which can be both acute (e.g. gastroenteritis) and chronic (e.g. inflammatory bowel disease). Functional foods directed towards the gut microbiota would serve to influence the composition of activities towards a more positive metabolism.

Functional Foods and the Gut

Recent years have seen a major change in how activities of the human gastrointestinal tract are perceived. This has been driven by increased knowledge of the gut microflora composition and activities. This has been helped by a shift away from traditional microbiological culture methods (*Figure 1*) to the use of molecular markers of culture identity. The colon is the most heavily populated region of the gastrointestinal tract and, because of this resident microbiota, is one of the most metabolically active organs in the body. The concept of modulating activities directed towards improving gut microbial function has a long history, as diet can have a major effect on the gut microflora activities[1].

Whilst some indigenous bacteria can be pathogenic (e.g. proteolytic clostridia and

Figure 1. Studies on the complex microbial ecology of the intestinal tract have moved away from cultural procedures towards molecular biological methods. Courtesy of Dr Kieran Tuohy (University of Reading).

bacteroides), it is also the case that some genera/species may offer health promoting attributes. For example, bifidobacteria and lactobacilli are thought to exert powerful anti-pathogenic effects and are mainly responsible for 'colonisation resistance' in the gut. Moreover, the same genera have been attributed with other beneficial aspects: such as protection from bowel tumours and metabolism of cholesterol and other lipids in the gut[1]. Whilst many of the health promoting aspects have yet to be definitively proven in humans, it would appear that there is value in eliciting a change away from a gut flora dominated by potentially harmful bacteria towards a more benign, or beneficial, composition.

Probiotics

The most frequently used dietary method of influencing the gut flora composition is that of probiotics, whereby live microbial additions are made to appropriate food vehicles, usually fermented milks[2]. The concept was expounded in a scientific note by Metchnikoff[2]. He hypothesised that longevity in Bulgarian peasants was associated with their elevated intake of 'soured milks', i.e. dairy based drinks containing live bacteria. This was the basis of what is now recognised as the probiotic concept. A recent definition of probiotics was given as 'a live microbial feed supplement that is beneficial to health'[3].

Over the years, many species of micro-organisms have been used. They consist not only of lactic acid bacteria (e.g. lactobacilli, streptococci, enterococci, lactococci, bifidobacteria) but also *Bacillus* spp., *E. coli* and

fungi/yeasts such as *Saccharomyces* spp. and *Aspergillus* spp. The most common probiotics belong to the genera *Lactobacillus* (e.g. *L. casei, L. acidophilus, L. rhamnosus, L. johnsonii, L. reuteri*) and *Bifidobacterium* (e.g. *B. bifidum, B. longum, B. breve*). To be effective, probiotics must be capable of being prepared in a viable manner and on a large scale (e.g. for industrial purposes), whilst during use and under storage the probiotic should remain viable and stable, be able to survive in the intestinal ecosystem and the host should gain beneficially from harbouring the probiotic. The strains used should be generally regarded as safe.

Probiotics are marketed as functional foods, whereby they are ingested for their purported positive advantages in the digestive tract and/or systemic areas like the liver, vagina or blood-stream. Consumers should be provided with an independent assessment of physiological, microbial and safety aspects of these live microbial products – especially if they can improve health. Probiotic trials should use the best methodologies available. For probiotics to exert beneficial properties, they must have a high viability in the product and have robust survival properties in the gut, which is their first point of contact[4]. Moreover, they should not adversely affect immune up-regulation, produce toxins, disrupt colonocyte function or have the ability to transfer antibiotic resistance to the normal gut microflora. Food vehicles include live yoghurts, fermented dairy drinks, freeze-dried supplements (capsules, pills, liquid suspensions, sprays), cheese, fromage frais and fruit juices. Both single and multiple strain products are available.

Prebiotics

An alternative, or additional, approach is the prebiotic concept. A prebiotic is 'a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that can improve the host health'[5]. Thus, the prebiotic approach advocates the administration of non-viable entities. Dietary carbohydrates, such as fibres, are candidate prebiotics, but most promise has been realised with non-digestible oligosaccharides, because of their selective metabolism. In particular, the ingestion of fructo-oligosaccharides (FOS) has been shown to stimulate bifidobacteria in the lower gut. As prebiotics exploit non-viable food ingredients, their applicability in diets is wide ranging. A further approach is synbiotics, where probiotics and prebiotics are combined[5].

The prebiotic activity of fructose-containing oligosaccharides has been confirmed in both laboratory and human trials[6-10]. This is because these carbohydrates have a specific colonic fermentation directed towards bifidobacteria[1,5]. Bifidobacteria are able to break down and utilise fructo-oligosaccharides due to their possession of a β-fructofuranosidase enzyme, providing a competitive advantage in a mixed culture environment like the human gut[11].

Galacto-oligosaccharides (GOS) are another class of prebiotics that are manufactured and marketed in Europe as well as Japan. These consist of a lactose core with one or more galactosyl residues linked via β1→3, β1→4 and β1→6 linkages[12]. They have found application in infant formula foods.

Recent documents have suggested that FOS and GOS are accepted prebiotics that fulfil current selection criteria[13, 14].

A prebiotic dose of 5 grams/day should be sufficient to elicit a positive effect upon the gut microbiota (in some exceptional cases this may be nearer to 8g/d). A possible side effect of prebiotic intake is intestinal discomfort from gas production. However, bifidobacteria and lactobacilli cannot produce gas as part of their metabolic process. Therefore, at a rational dose, of up to 20g/d, gas distension should not occur. If gas is being generated, then the carbohydrate is not acting as an authentic prebiotic. This is perhaps because dosage is too high and the prebiotic effect is being compromised i.e. bacteria other than the target organisms are becoming involved in the fermentation[5].

Possible Health Benefits

Several different avenues are being explored for pre/probiotics. These are largely mediated by affecting an increase in beneficial bacteria within
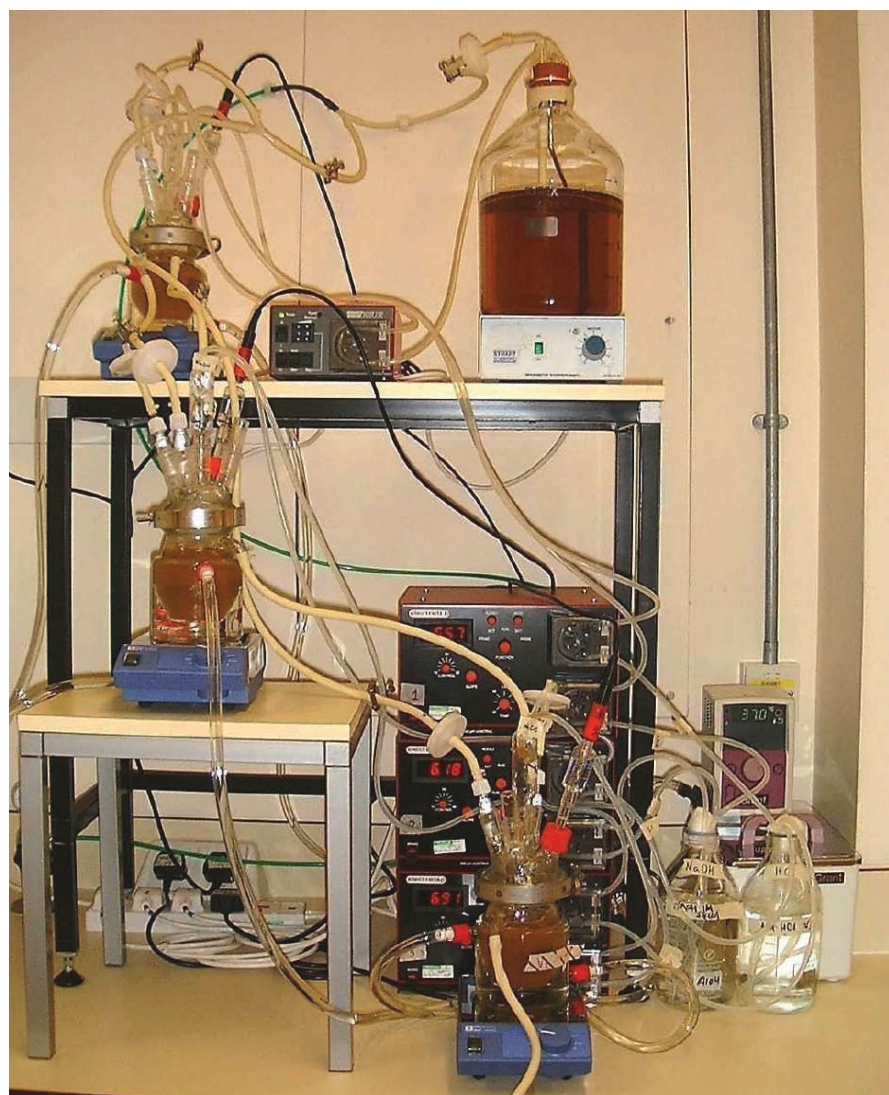
Figure 2. Model system of the large intestine used for *in vitro* studies on probiotic and prebiotic functionality. Courtesy of Dr Kieran Tuohy (University of Reading).
The system consists of 3 vessels of increasing size, aligned in series such that a sequential feeding of growth medium occurs. The vessels are pH regulated to reflect *in vivo* differences. Thus, vessel 1 has a high availability of substrate, bacteria grow quickly and is operated at an acidic pH, similar to events in the proximal colon. In contrast, the final vessel resembles the neutral pH, slow bacterial rate and low substrate availability which is characteristic of more distal regions. After inoculation with faeces, an equilibration period is allowed such that the bacterial profiles respond to their imposed conditions. Then, candidate pro/prebiotics are added and the fermentation profiles monitored.

the gut flora. At Reading, a model system of the human colon is in operation (*Figure 2*) whereby probiotic and prebiotic efficacy can be researched before moving onto human studies.

The health evidence is variable with the following being examples:

- *Improved tolerance to lactose:* it is thought that probiotics may help in this regard, through their β-galactosidase activity.
- *Protection from gastroenteritis:* the most compelling evidence for the success of probiotics and prebiotics probably lies in their ability to improve resistance to pathogens. Lactic acid excreting microrganisms are known for their inhibitory properties. There are a number of potential mechanisms for probiotic micro-organisms to reduce intestinal infections[1]. Firstly, metabolic end products such as acids excreted by these micro-organisms may lower the gut pH to levels below those at which pathogens are able to effectively compete. Also, many lactobacilli and bifidobacteria are able to excrete natural antibiotics which can have a broad spectrum of activity. Moreover, there is competition for nutrients and colonisation sites[15]. This inhibitory effect also has relevance for more chronic diseases thought to have an involvement of pathogens.
- *Reduced toxins:* stimulating a more beneficial community should reduce toxin levels, perhaps including carcinogens, some of which act systemically as well as locally. In humans, colorectal cancer is thought to have a bacterial origin, with around 10 different carcinogens described that have been attributed to microbial events[16]. Dietary strategies that lead to a reduced accumulation of such products may be possible. Dietary fibres and resistant starches may be fermented in the large gut to increase faecal bulk and reduce the residence time of such materials in the gut. Moreover, probiotics and prebiotics may modify the activities of enzymes that are involved in carcinogenesis.
- *Cholesterol reduction:* the lipid hypothesis purports that dietary saturated fatty acids lead to an increase in blood cholesterol levels. This may have the effect of depositing cholesterol in the arterial wall, leading to atherosclerosis and possibly coronary heart disease. Some studies have hypothesised a role for the lactic microflora in systemically reducing blood lipid values[17]. It has been suggested that some probiotics can degrade cholesterol in the gut as well as produce metabolites that interfere with its synthesis in

the liver. However, this has not been unequivocally proven, and there are contrasting data from human volunteer trials.

- *Vitamin synthesis:* bifidobacteria can synthesise various vitamins, largely of the B group. The physiological value of this in the lower bowel is questionable, however.
- *Irritable bowel syndrome:* IBS is a major drain on general practitioners' time, and some evidence has implicated a 'dysfunctional' gut flora. This may be addressed through pro/prebiotics. Evidence is very contradictory, however.
- *Improved digestion and gut function:* an active gut flora helps to adequately digest the food that enters the adult colon each day.
- *Food allergy:* it has been suggested that gut flora modulation may down-regulate gut inflammation and hypersensitivity that would otherwise lead to atopic eczema.
- *Immune regulation:* a stimulation of the non-specific immune response through non-pathogenic means may help improve resistance to infection.
- *Mineral bioavailability:* a reduced pH in the bowel because of a lactic fermentation is thought to better sequester calcium and perhaps magnesium.

Conclusions

The incidence of acute and chronic gut disorders continues to rise, with many diseases being untreatable. The functional food industry's perception of the importance of gut microbiology in human health and nutrition has led to a major increase in probiotic and prebiotic-based products. Not all products will be reliable in terms of their efficacy, however, and it is important that these are not allowed to skew an important area of human health and the functional food concept generally. Moreover, claims on particular products cannot be extrapolated to others, e.g. if one probiotic strain elicits a particular positive effect, it cannot be assumed that this is applicable to others (even of the same species). A further issue is public acceptance, with dietary response to change being weak – it is estimated that only about 8% of UK citizens consume at least 5 pieces of fruit/vegetables per day, and this is a well understood health message.

Next, legislation is loose and open to abuse from manufacturers launching untested products. This will be tightened up in time and is needed. However, if food law and claim hurdles are set too high, a degree of reluctance among manufacturers with good products may ensue. For the full value to be realised, it is imperative that developments are based upon sound

scientific principles and research that provide reliable information on efficacy – effect as well as mechanisms involved.

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Chromogenic media: bacteriology in colour

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Introduction

Laboratory managers are often faced with difficult decisions in terms of allocation of resources, not only with regard to staffing and prioritisation of work tasks, but also procurement of the most effective tools for the job within tight budgetary constraints. These decisions can have a significant effect on the efficiency of sample processing and turnaround times to the reporting of results. By its very nature, microbiological bench work is often very labour intensive, time consuming and requires skill and experience. Whilst new, rapid methods of sample analysis, such as real-time PCR and other automated molecular techniques, inevitably generate an air of excitement amongst scientists and represent important advances in scientific technology, the importance and relevance of advances in culture methods in routine microbiology must not be forgotten or ignored. The introduction of new culture media is a crucial factor that can have a significant impact on cost-effective, accurate and timely results in food, water, clinical and industrial laboratories worldwide.

Culture media

For over a century, since Robert Koch's early work with "the mixture of nutrient liquid and gelatin", solid culture media have been used for the cultivation of an ever-expanding array of micro-organisms. It was the work of Walther Hesse (1846-1911) and his wife Fanny (née Eilshemius) (1846-1934) that established the use of agar as a superior gelling agent for solid media[1]. The main problem with such general purpose nutrient media was the inability to distinguish between pathogenic and non-pathogenic organisms by morphological characteristics alone. At a time when many new strains were being discovered, microbial identification and taxonomy were in their relative infancy and the need to establish biochemical profiles of bacterial species (and thus provide a means of differentiation) became apparent. However, this involved extensive further testing of individual colonies of bacterial growth and was highly labour intensive. Furthermore, the work was highly skilled and experience was in the hands of a few.

Gradually, as more became known about the biochemical distinctions between different genera and species (i.e. their ability to degrade certain substances or produce specific biochemical substances as end-points of metabolism), so culture media evolved to incorporate additional components, such as specific carbohydrate sources and a suitable pH indicator, to aid differential identification. Inhibitory agents (e.g. bile salts, certain dyes and other compounds) also became commonly used to reduce or eliminate growth of unwanted organisms. Thus, media could be designed to be selective as well as differential. A classic example of this is MacConkey Agar (*Figure 1*), which contains lactose and neutral red for the differentiation of lactose fermenting organisms, along with bile salts for the inhibition of bile-sensitive species. These properties make it useful in the identification of intestinal pathogens such as salmonellas (of importance in clinical, food and water microbiology), which are generally non-lactose fermenting (NLF), as well as commensals such as *Escherichia coli* that are able to ferment lactose (a key indicator of faecal contamination, also of importance in food, water and industrial microbiology).

In many cases, the specificity of conventional selective media has been improved by the addition of antibiotic supplements to inhibit unwanted organisms. One example of this is mannitol salt agar with oxacillin for the detection and isolation of meticillin-resistant *Staphylococcus aureus* (MRSA) (*Figure 2*). Many variants of *S. aureus* are halophilic and able to ferment mannitol. Only those resistant to oxacillin (the first widely-used surrogate marker for meticillin resistance) are able to survive the presence of this antibiotic. Such organisms appear as yellow colonies with yellow haloes on this medium. However, owing to other, less clinically significant staphylococci (e.g. *S. haemolyticus*) that also possess these qualities, a significant number of false positives are encountered. This has a significant effect on increasing the volume of confirmatory testing required. Also, there are emerging variants of MRSA that are unable to ferment mannitol, yielding false negative results.

Although conventional selective, differential media have effected a reduction in the volume and extent of confirmatory testing required compared to the original Nutrient Agar, their overall specificity remains comparatively limited. Despite their limitations, many of these types of media remain useful microbiological tools and are still extensively used today.

Given the widespread use of fluorogenic and chromogenic substrates in biochemistry, it is somewhat surprising that their application to microbiology did not really take off until the 1980s. The catalyst for research in this field was the desire of water microbiologists to develop a

Figure 1. MacConkey agar inoculated with *Escherichia coli* (ATCC 25922) and *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC 13076). Lactose fermenters (including *E. coli* and other coliforms) appear as pink to red colonies, while non-lactose fermenters (NLFs) (including Salmonellas and Shigellas) produce colourless or straw colonies with orange to yellow halos.
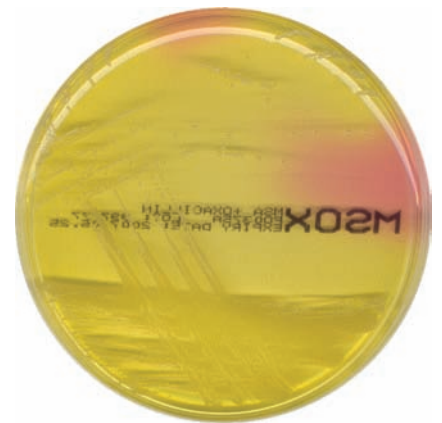
Figure 2. Mannitol salt agar with oxacillin after 48 hours incubation at 37°C following inoculation with meticillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300).

rapid screening method for the faecal indicator *E. coli*[2]. Since the work of Feng and Hartman[3], who pioneered the use of 4-methylumbelliferone-β, D-glucuronide for the detection of *E. coli* in water and food samples, an explosion of research and development in the field of chromogenic culture media has ensued.

Chromogenic substrates

A chromogenic substrate may be defined as "a compound or substance that contains a colour-forming group"[4]. Commercially synthesised chromogenic substrates (or chromogens, for short) are available for the detection of many hydrolase enzymes, including glycosidases, peptidases, phosphatases and esterases (*Table 1*). This group of enzymes includes many gene products specific to certain genera (or in some cases species) of bacteria and their detection can often be an invaluable aid to differentiation and identification. This can often significantly reduce the amount of work required to confirm the identity and significance of the suspect colony.

Glycosidases exhibit specificity not only for the sugar type, but also for its steric conformation (D- or L-) and the conformation of the glycosidic bond (α- or β-)[2]. For example, β-D-glucoside chromogens are specific for detecting β-D-glucosidase activity and their usefulness in bacterial differentiation is well documented[5-7].
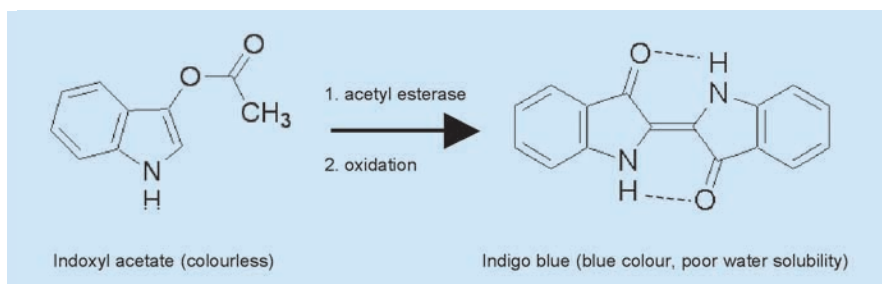
Indoxyl chromophores

Detection of specific hydrolase activity can be achieved by attachment of a chromophore (the "colour-forming group") to the target substrate, such that hydrolysis of the substrate yields a specific colour, dependant on the type of chromophore used. Various types are available, but the most commonly used in solid culture media are derivatives of indoxyl. In its simplest form, indoxyl is a colourless, water soluble compound that rapidly oxidises in air to form indigo blue, a coloured, insoluble, dimeric compound (*Figure 3*).

In practice, indoxyl generally gives a weak colour when used in culture media, but the indoxyl ring may be modified by the addition of one or more halogens at certain locations on the ring. This results in changes in absorbance in the visible spectrum and consequently yields different coloured end-products. Examples of commonly used indoxyl-based chromophores and their respective colours are shown in *Figure 4*. Furthermore, subtle changes to these colours and their intensities can be achieved by the inclusion of other components in the medium (e.g. cations, peptones, inducers, etc.).

Table 1. Examples of commercially available hydrolase substrates (modified from Bovill and Druggan, 2005).

Enzyme	Chromogenic substrate
Aminopeptidase	A wide variety of substrates are available, containing single amino acids through to longer peptide lengths.
Esterase (carboxilic)	A range of substrates containing various fatty acid chain lengths: C2, C4, C6, C9, C10, C12, C14, C15, C16 and C18.
Esterase (inorganic)	Phosphate, phosphodiester, venom phosphodiester, sulphate.
Glycosidase	α-L-arabinoside, β-D-cellobioside, α- and β-L-fucoside, β-D-fucoside, α- and β-galactosaminide, α- and β-D-galactoside, α- and β-D-glucosaminide, α- and β-D-glucoside, β-D-gluconoride, β-lactoside, α- and β-D-maltoside, α- and β-mannoside, α-L-rhamnoside, β-xyloside.
Others	Substrates for lysozyme and phosphoinositol phospholipase C.

Figure 3. Formation of indigo blue by enzymic hydrolysis of indoxyl acetate in the presence of oxygen.
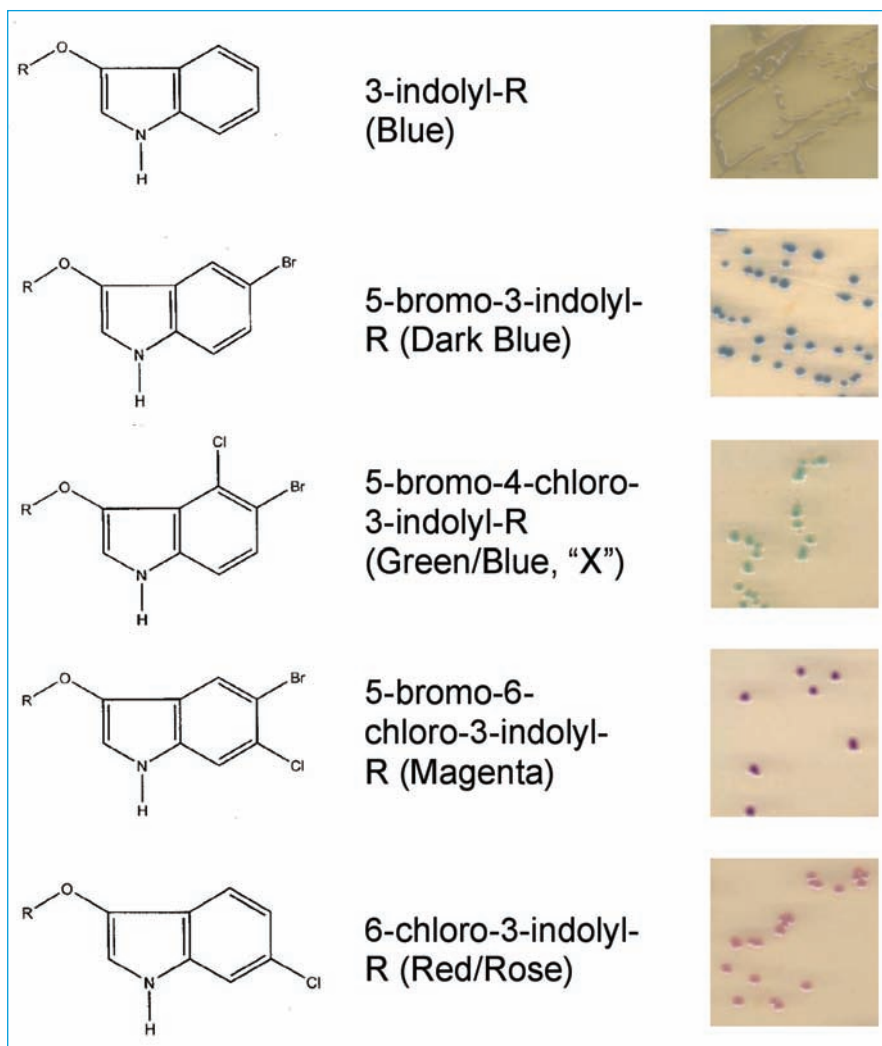
Figure 4. Examples of indoxyl chromophores, showing chemical structures and resultant colour formation. The addition of halogens at specific positions on the indoxyl ring affects the resultant colour formation in colonies of bacterial growth possessing the hydrolase enzyme necessary to release the chromophore from the specific chromogenic substrate.
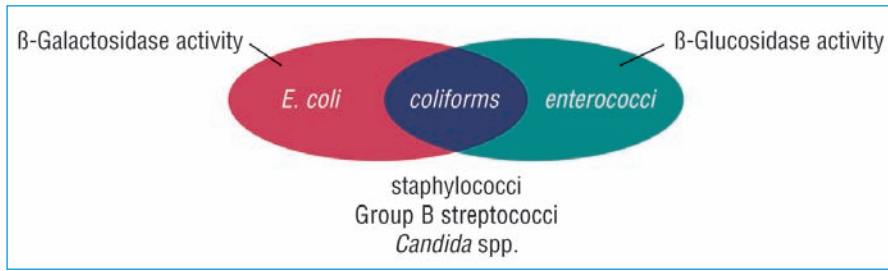
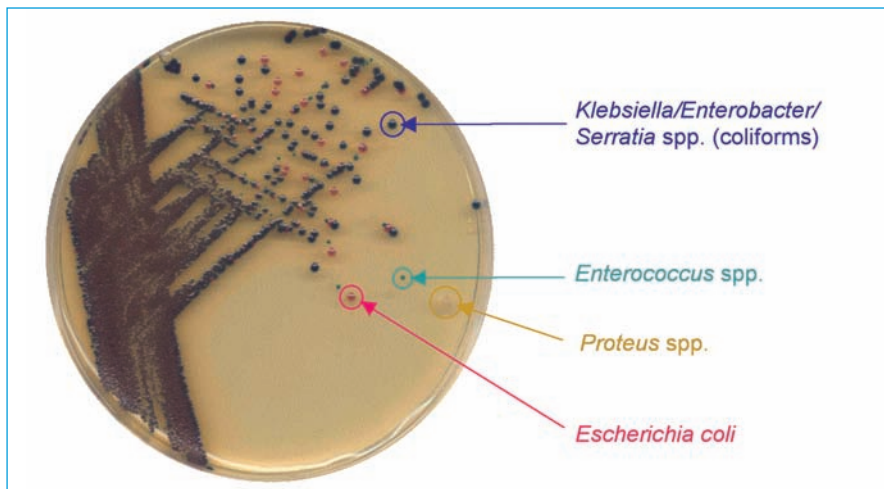**Figure 5.** Venn diagram of colour reactions.

Figure 6. Differentiation of organisms commonly isolated from urine samples on a chromogenic medium by their ability to produce either, both or neither β-galactosidase or β-glucosidase.
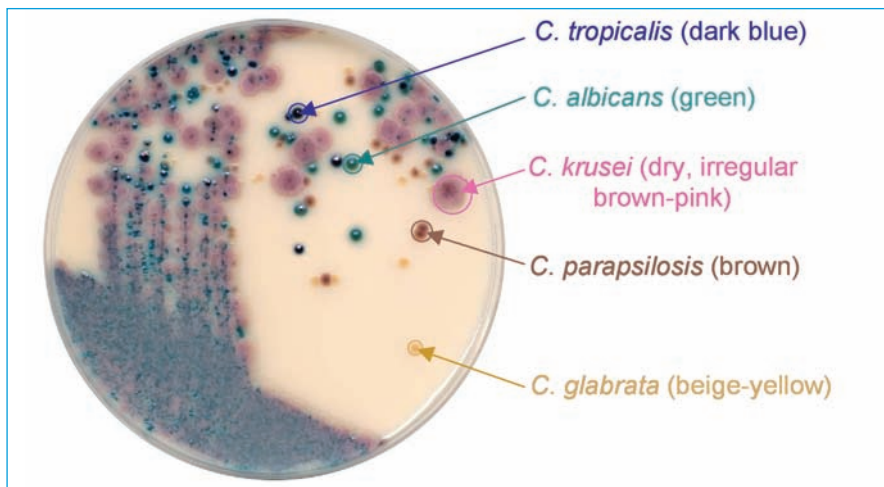

Figure 7. Enzyme-dependant colour production and consequent differentiation of clinically significant *Candida* spp. on a chromogenic medium for the detection of hexosaminidase and alkaline phosphatase activity.

Chromogenic Culture Media

Incorporation of more than one chromogen in a medium can improve both its specificity and differential properties. A medium containing both 5-bromo-4-chloro-3-indoxyl β-D-glucopyranoside (X-Gluc) and 6-chloro-3-indoxyl β-D-galactopyranoside (red-Gal) is useful for the differentiation of potential urinary tract pathogens, as illustrated in *Figures 5* and *6*.

Organisms that express β-galactosidase cleave the red-Gal substrate to produce pink/red colonies (e.g. *E. coli*), while expression of β-glucosidase results in cleavage of the X-Gluc

to form green colonies (e.g. *Enterococcus* spp.). Expression of both enzymes results in dark, blue-purple colonies and is indicative of *Klebsiella, Enterobacter* or *Serratia* spp. (KES group). Staphylococci (with the main exception of *S. saprophyticus*) and streptococci do not produce either enzyme and grow as white or colourless colonies. Differentiation of these genera is rapidly established by a catalase test (staphylococci are positive for this enzyme, streptococci negative). *Proteus* spp. may be differentiated by inclusion of tryptophan, forming tan coloured colonies due to the tryptophan deaminase (TDA) reaction.

This technology has also been applied to the differentiation of *Candida* spp. by the use of indoxyl chromogens to detect the presence of hexosaminidase and alkaline phosphatase. This is illustrated in *Figure 7*.

The solubility of the initial substrates and the insolubility of the end-products are characteristics that make the indoxyl group of chromophores particularly suitable for use in solid culture media. This is because colouration is restricted to the cellular mass, enabling colonies of a species possessing the relevant hydrolase to be easily recognised in a mixed culture. A drawback of the indoxyl chromogens is their reliance on oxidation, making them unsuitable for detection of anaerobic bacteria. Alternative chromogens have been described that overcome this problem, notably the metal chelators (e.g. esculin, 8-hydroxyquinoline, dihydroxyflavones and alizarin)[2,8].

Other chromophores

Other chromophores, such as nitrophenol and nitroaniline, are available in a variety of substrate forms. Indeed, ortho-nitrophenol-β-D-galactoside (ONPG) is still widely used in biochemical differentiation of bacteria. However, nitrophenol and nitroaniline substrates suffer two drawbacks: they have a low extinction coefficient, often resulting in poor sensitivity due to insufficient colour production; and the end-product is highly soluble, rendering them better suited to liquid (broth) assays than to solid media. Nitroaniline (and other amine-containing) substrates are particularly suitable for the detection of amino-peptidases when linked to a peptide. However, these require the addition of a developer (usually dimethylaminocinnamaldehyde) to illicit the observed colour reaction (formation of the Schiff base) and this makes them impractical for use in solid culture media.

Benefits of chromogenic media

A wide range of chromogenic media are commercially available for the detection of many organisms of significance in food, water, clinical and industrial microbiology (e.g. *Listeria, Salmonella, Bacillus cereus,* clostridia, *Candida,* enterococci, staphylococci, *E. coli* and coliforms). The main benefits of these over conventional media are their improved sensitivity and specificity. In some cases improved sensitivity may lead to a reduction in incubation time (e.g. chromogenic agars for MRSA detection), allowing a faster turnaround time to reporting of results. These properties also make them ideally suited as high-volume screening media owing to the resultant reduction in

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confirmatory testing required. From a laboratory manager's perspective, the key benefits of chromogenic media can be summarised, as follows:

- *Ease of use and interpretation:*
  – minimal training required;
  – allow for more appropriate use of experienced staff;
- *Improved performance:*
  – greater confidence in results compared to conventional media;
  – faster results;
  – reduced volume of follow-up work;
- *Cost effectiveness:*
  – reduced confirmatory testing outweighs extra cost of media;
  – significantly cheaper than PCR and other automated molecular methods.

Although molecular techniques are rapidly gaining recognition and credibility for certain applications, owing to their expense they are generally only cost-effective for a very large throughput of samples or where detection is not achievable by conventional means. These techniques do not allow subsequent culturing of the organism. This is not necessarily a problem in certain circumstances, but can be a major disadvantage, especially in many scenarios, where further phenotypical characterisation is required (e.g. antimicrobial sensitivity patterns). The main advantage of molecular systems is a faster time to result. The impact of this in real terms can be measured only by the efficiency of the reporting system itself and in certain cases there may be no time benefit at all.

Overall, chromogenic media represent a cost effective way of achieving improved sensitivity and specificity of results without the expense of automated molecular techniques. Further applications of chromogenic technology are being found all the time and are limited only by the quest to find more differentially useful substrates.

Acknowledgements

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