Chapter 8
Screening of Plants for Inhibitory Activity Against Pathogenic Microorganisms from the Gut of Livestock

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Introduction

One of the major hazards facing the livestock industries in food safety is commensal gut microorganisms (e.g. *Escherichia coli, Listeria monocytogenes*, *Yersinia enterocolotica*, *Campylobacter* spp. and *Salmonella* spp.), which contaminate animal products and cause illness in humans. Traditionally, pathogenic gut microorganisms have been controlled by antibiotic growth promoters [8]. Use of such supplements in livestock is being phased out because of the emergence of antibiotic-resistant human-pathogens [4]. The use of growth promoters as feed supplements in livestock was banned by the European Union at the start of 2006 and similar bans are under review in Australia [16]. A number of approaches, such as vaccination, probiotics, chemical inhibitors and dietary manipulation are being considered for reducing these organisms in livestock. One possible control strategy is the use of natural antimicrobial plant compounds as an alternative to antibiotic growth promoters. These plants could then be incorporated into animal feeding systems provided there were no adverse affects on the animal and the products from those animals [6, 3].

Often large numbers of natural products and extracts from plant or chemical libraries are available for screening. Therefore, the first step in identifying a bioactive compound in a plant is to develop a screening assay against the target organism. Success in finding a candidate bioactive compound is often dependent upon the ability to screen large numbers of these samples in a simple and rapid format.

Methods used in extraction of bioactive compounds from plants vary and are selected according to the overall objective of the research (e.g. water extractions will favour the polar compounds while extraction in chloroform will favour oils and terpanoids; if the research is aimed at finding volatile oils then chloroform extraction methods will be favoured). A variety of screening techniques are available to measure the in vitro susceptibility of microorganisms to antimicrobial agents and most assays are either broth or agar based [11, 12].

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Agar assays used in screening are divided into diffusion and replica plating assays. A diffusion assay is performed in a large petri dish containing agar-based media which is inoculated with the target organism. Extracts are then added to wells cut into agar or to a blotting paper disk, which is placed on the surface and the extract is allowed to diffuse into the agar. After incubation, a zone of clearing indicates inhibition but this depends on the ability of the bioactive compounds to diffuse into the agar [12]. As an initial screen, this approach provides an indication of the compound's inhibitory activity, but is influenced by its diffusion properties through the agar. The advantage of this assay is that a few compounds can be screened rapidly with simple and cheap equipment. However, the technique becomes laborious when large numbers of compounds need to be evaluated against several target organisms.

Replica plating techniques involve a series of test compounds being dissolved into individual agar plates and a set template of inoculums (several different microorganisms) being replicated on each individual agar plate. The technique enables the consistent reproduction of a pattern of bacterial colonies on each of the plates where growth and inhibition can be observed. Some limitations of the technique are that it requires a large amount of test compound and has limited use when many compounds are being tested [13].

A broth (liquid) culture assay differs from the agar-based screen in that the bioactive compound is dissolved into the medium, which has been inoculated with the target organism. Bacterial growth can be visually assessed or optical density measured at 620 nm (OD_{620}), after inoculation and incubation. The simplest form of assay is to measure growth at a set concentration of the bioactive target. Growth can be assessed as a change in OD_{620} over a set time or calculated as percentage inhibition. A more informative assay is to assess growth over a series of descending concentrations; this constitutes a minimum inhibitory concentration determination. Liquid assays can be performed in a variety of growth containers, but a useful format is the 96-well micro-titre plate. This enables preparation of the assay to be automated by the use of robotics allowing reproducibility and a relatively high through-put that can be scaled up to increase the number of compounds and bacterial strains under evaluation. Alternatively, multi-channel pipettes can be used to dispense media, bioactive compounds and target organisms into individual wells of the 96-well plate.

Inhibitory activity of extracts is estimated as minimum inhibitory concentration, which is defined as the lowest concentration of a given extract where growth is completely inhibited [11]. This is performed with the use of a series of descending concentrations of a pure compound or crude extract from a plant. The medium is inoculated with the test organism and the minimum inhibitory concentration calculated as the concentration where growth is inhibited. This has several advantages over other screens. The extent of information about the compound is increased and with forethought about concentration used in the minimum inhibitory concentration assay, percentage inhibition can be calculated within the one assay. The disadvantage is that each compound now takes at least four wells with corresponding increases in time and resources.

Initial screening assays against gut pathogens are performed under aerobic conditions since many of these microorganisms are facultative anaerobes. A screening assay, which involves testing an extract against a monoculture of a microorganism, is a simplistic approach when the objective is to inhibit the target organism in the anaerobic environment of a complex gut microbial ecosystem. The bacteria in the rumen or digestive tract of the animal could modify natural compounds within the test plants. A better model might involve a mixed culture anaerobic fermentation of gut inoculum in the presence of ground plant material and periodic sampling for enumeration of the microorganisms on a medium, which is selective for the target organism. Such an experiment is complex and time consuming, but is an excellent secondary screen.

It is difficult to provide a generic technique for screening bioactive compounds against pathogenic organisms since each application will vary in the nature of the bioactive compound, the target organism and assay conditions. It has been shown that conditions, such as media composition, pH and temperature within the same assay can vary the outcome. Therefore, methodology must be carefully controlled to ensure consistency in results [14].

In our laboratory, we have developed a set of general methods to screen a collection of plant extracts for inhibitory activity. The present paper addresses key elements of screening against pathogenic *E. coli* as follows:

1) the extraction of dried plant material;
2) culture selection, inoculation and growth conditions;
3) agar diffusion assays;
4) broth assays using 96-well plates to calculate percentage inhibition and minimum inhibitory concentration; and
5) in vitro assays to simulate gut conditions of ruminant animals.

The methods described can be adapted to provide a guide for researchers who wish to employ similar strategies for identifying bioactive molecules in nature, which could be used to control pathogenic organisms in livestock.

Experimental Approach

This section describes some of the methods such as the preparation of extracts, choice of bacterial strains and preparation of inoculum involved in these assays which are common to most screening tests. These methods will need to be adapted depending on the compounds/extracts/substances under evaluation and the target organism.

Plant Extractions

Freeze-dried, ground plant material (0.25 g) is extracted with 5 mL of ethanol/water (0.7:0.3, v/v) in polypropylene centrifuge tubes (10 mL; Starstedt, Nümbrecht,

Germany). The mixture is gently agitated on a rotary mixer for 60 min with vortexing every 30 min. After centrifugation (2000*g*, 5 min) the supernatant is removed, 5 mL of ethanol/water (0.7:0.3, v/v) is added and the agitation and vortexing repeated (90 min). The tubes are again centrifuged and the supernatants combined. The resulting ethanol extract is evaporated under nitrogen at 25°C in a pre-weighed glass vial for 36 h and then freeze-dried for 48 h. Vials are weighed and the extraction efficiency calculated.

Dissolving Plant Extracts for Testing

The greatest difficulty with testing extracts is to dissolve them into a solvent and their subsequent solubility within the testing media [14]. Freeze-dried extracts are dissolved in *N,N*-dimethylformamide (DMF; Auspep, Parkville, Australia) resulting in a 40 mg/mL solution. The solvent DMF is preferred in our laboratory as it is regarded as a universal agent, which dissolves both polar and non-polar compounds. To dissolve particulate matter, the mixture is gently shaken at 37°C for 1 h. A pipette tip is then used to crush any solid material and the solution is returned to the shaker (1 h). The solutions can also be dissolved at 160 mg/mL for testing at higher concentrations, although this may make the solubilization more difficult.

Many plant extracts contain pigments, which can mask OD_{620} when growth is measured (Fig. 8.1). Another problem is that the extract, when added to media at high concentrations may precipitate and settle to the bottom of the well, blocking the optical path of the OD_{620} measurement. These complications should be taken into account by visual inspection of the assay before OD_{620} analysis is performed and data interpreted accordingly. When pigments and precipitates interfere with the optical density analysis, it is possible to assess metabolic activity within the assay as an indicator of growth (see "Detection of metabolic activity using p-iodonitro tetrazolium violet," described later in the chapter).

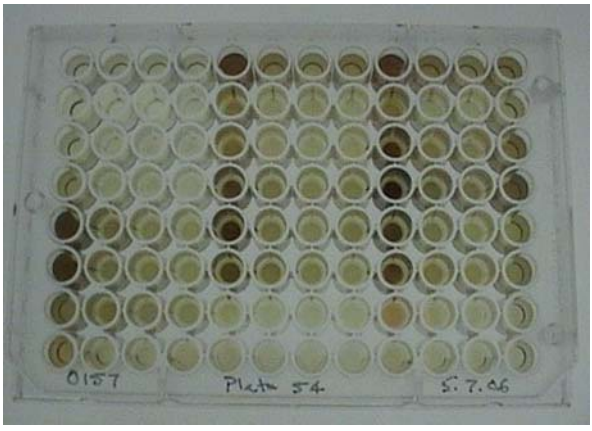

Fig. 8.1 A typical 96-well micro-titre test plate showing the masking effect (i.e. darker wells) of the test compounds

Microorganism Selection, Inoculation and Culture Conditions

Microbial Panel Selection

The panel of microorganisms used in screening assays should be selected with respect to the task being undertaken. A typical panel consists of a single species or series of target organisms and adjunct strains (i.e. strains being tested but not direct targets of the assay). For example, a Gram-positive and Gram-negative organism and a negative control such as yeast.

When the target organisms are pathogens, it is a common practice to include several strains of the pathogen in the test panel. In our laboratory, we use the pathogenic *E. coli* serotypes O23, O111 and O157 as targets representing the enterohaemorrhagic *E. coli* group. However, the pathogens must not pose a significant risk for the laboratory workers. Therefore, safe handling and good laboratory practice should be considered when selecting the panel of strains. In some cases, it may be advisable to use non-pathogenic strains of the target organism in initial screens.

Adjunct strains should be selected based on continuity with other experiments. For example, our laboratory has routinely used *Staphylococcus aureus* ATCC 25923, *E. coli* ATCC 35218 and *Candida albicans* ATCC 24433 in antibacterial assays for several years. Thus comparisons can be made between assays. These three strains are used as standards in the National Committee for Clinical Laboratory Standards (NCCLS) for determining antibiotic susceptibility [11, 12].

Media Selection

The culture medium used in the assays will vary depending on the organisms being targeted. Our laboratory routinely uses Muller-Hinton broth (Oxoid, Basingstoke, UK) to grow the cultures for inoculation and as the medium in the screening assays. This broth is also used as the basis of solid agar (Difco Laboratories, Detroit, MI, USA, 1.2% medium) in diffusion assays. Specialised media may be used for more fastidious strains of test bacteria. Media is usually made and used under aerobic conditions for screening against gut pathogens, but screening can be performed using anaerobic culturing techniques provided the laboratory facilities are available. The facilities and equipment used routinely in an anaerobic microbiology laboratory are described by McSweeney et al. [9].

Microbial Inoculation Culture

Starter cultures of all bacterial and yeast strains used in both the plate diffusion and the 96-well dilution assays, begin with a sub-culture from a fresh plate, inoculated into broth (6 mL) and shaken at 37°C for 5–6 h. These cultures are then diluted according to the assay in which they are used.

In the plate diffusion assay, *E. coli*, *S. aureus* and *C. albicans* cultures are diluted 250, 100 and 10 times respectively into sterile normal saline (0.9% w/v, NaCl; BDH Chemicals, Kilsyth, Australia). These specific dilutions are modifications of the

NCCLS methods for antibacterial resistance [11, 12] and were determined as the lowest dilution to result in confluent growth on an agar plate.

For 96-well plate assays, the 5–6 h culture is diluted 2500, 1000 and 10 times for *E. coli*, *S. aureus* and *C. albicans* respectively. For *E. coli* and *S. aureus,* the dilutions are carried out in two consecutive steps. All dilutions have been determined so that growth is observable after 18 h of incubation at 37°C in a 96-well plate using 10 μl of inoculum in a well containing 190 μl of media.

Detection of Metabolic Activity Using p-iodonitro tetrazolium Violet

It is sometimes difficult to determine bacterial growth using OD_{620}. This is due to the plant extracts precipitating and/or being dark in colour. To overcome this, *p*-iodonitro tetrazolium Violet (INTV; 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride; Sigma, St. Louis, MO, USA) can be added (10 μl of 2 mg/mL) to the wells in question and incubated for 1–3 h. *p*-Iodonitro tetrazolium Violet is an indicator of metabolic activity, which turns the media purple in the presence of respiring bacteria. When the purple colour develops, the dilution series can be examined visually and the minimum inhibitory concentration determined readily [5]. However, the use of INTV is target species dependent, since it works well for the *E. coli* but not for *S. aureus* or *C. albicans*.

Control Inhibitor Selection

A microbial inhibitor of known activity (positive control) should be included in all screens to demonstrate the efficacy of the assay. Typically, a broad-spectrum antibacterial substance which inhibits both Gram-positive and Gram-negative bacteria (e.g. Tetracycline; MP Biomedicals, Irvine, CA) could be used as a control inhibitor. A standard inhibitor for yeast or fungi (e.g. amphotericin B; Sigma) is also required if these microorganisms are part of the testing panel. Additionally, a negative control should be included, which normally is the solvent used to dissolve the plant extract for the assay.

Agar Plate Diffusion Assay

From the diluted inoculation culture, described above, a sterile cotton swab is dipped into the mixture and rotated several times. Excess inoculum is removed and the swab is streaked over the entire surface of a Mueller-Hinton medium agar plate. The swabbing is repeated twice, with the plate being rotated 120° between streaks to ensure confluent growth on the plate.

An alternative inoculation technique is to add 200 μL of diluted starter culture (see *Microbial inoculation culture*) to 20 mL of molten agar, then overlay this mixture on the agar plate. This is a particularly useful method for testing species that swarm or produce mucoid colonies on agar plates.

A sterile blank anti-microbial disk (Oxoid) is placed onto the surface of the agar in a predetermined pattern. Disks must be no less than 24 mm from centre to centre. A sample 5.0 μl of the inhibitor (20 mg/mL) is then aliquoted onto a disk in a specific location thus giving 100 μg of sample per disk. Positive and negative controls are also added to other disks on the same plate (See control inhibitor selection). Extracts are allowed to soak into the agar from the disc, which takes about 10 min. The plates are then inverted and incubated at 37°C for 16–18 h.

Plates are read by measuring the zone of clearing (not including the disk radius). The zone of clearing is an indication of the antimicrobial activity of the compound or extracts activity, but this is influenced by the diffusion characteristics of the inhibitor in the agar [12].

Broth Microbial Growth Assay

Extracts and compounds can be screened for inhibitory microbial activity in a liquid 96-well plate assay. Master plates (96-well v-bottom poly-vinyl; Nunc, Roskilde, Denmark) are created by adding stock extract solutions either as single extracts or as a series of dilutions of the extract. The master plate is used to create daughter test plates for optical analysis (96-well flat bottom polycarbonate; Nunc), containing media and a small aliquot of the extract at the test concentration.

Each well in the master plate should contain approximately 50 μL of extract to be tested (40 mg/mL), negative controls such as a DMF and positive controls such as tetracycline and/or amphotericin B (2 mg/mL) should be included in the plate. Positive controls will depend on the panel of microorganisms to be tested.

When diluting extracts on the master plate, 100 μL of each extract should be placed in the first well and 50 μL of DMF added to consecutive adjacent wells. The compound is then diluted in 2-fold increments. This type of procedure can be done by hand with a multi-channel pipetter or programmed into a liquid handling robot such as the Beckman Biomek 2000 (Beckman, Fullerton, CA, USA).

When the master plate is complete with compounds or dilution series, a daughter plate is produced for each strain of microorganisms to be tested. Each well on the daughter plate will contain 2× Muller-Hinton Broth (100.0 μL, Muller-Hinton contains Beef Dehydrated Infusion 200 g/L, casein hydrolysate 17.5 g/L and starch 1.5 g/L at pH 7.3), sterile water (84.9 μL) and an aliquot (5.1 μl) from a corresponding position on the master plate. Again, the use of multi-channel pipettes or robotics is invaluable in producing these plates (Fig. 8.2).

The final concentrations for inhibitors in the wells on the daughter plate are given in Table 8.1 and are based on a dilution of 5.12 in 200 in each well of the daughter plate. For dilution series, each consecutive well will contain a natural halving of the concentration.

All daughter plates are then inoculated with 10.0 μL of the diluted inoculation culture. The OD_{620} is read immediately after inoculation for each plate; this measurement is used as a blank within the assay. The plates are observed after

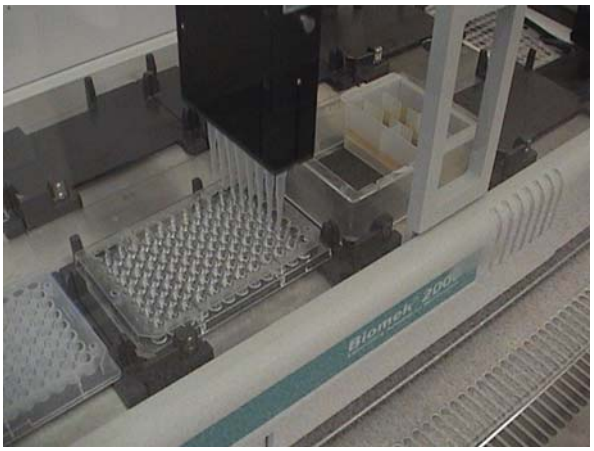

Fig. 8.2 Plates being loaded with media on the Beckman Biomek 2000^{TM} robot

Table 8.1 Concentration of extracts, compounds and internal standards in the dilution series contained on the daughter plates

Extract, compound or internal standard	Concentration (μg/mL)			
	1st Column	2nd Column	3rd Column	4th Column
Extract or compound at 40 mg/mL	1024.0	512.0	256.0	128.0
Tetracycline and amphotericin B at 2 mg/mL	51.2	25.6	12.8	6.4

16 and 40 h of incubation at 37°C and an appraisal is made of the growth followed by calculation of percentage inhibition or minimum inhibitory concentration or both.

Percentage Inhibition

Percentage inhibition is calculated on wells that have a test concentration of 1024 μg/mL. The OD_{620} measurements for unincubated wells are subtracted from those for the incubated wells. By comparing the resulting value with OD_{620} of both DMF (0% inhibition) and tetracycline or amphotericin B, (100% inhibition) for bacteria and yeast respectively, a percentage inhibition is calculated. The relative OD_{620} of each test compound is reported as the percentage with reference to these results (Fig. 8.3).

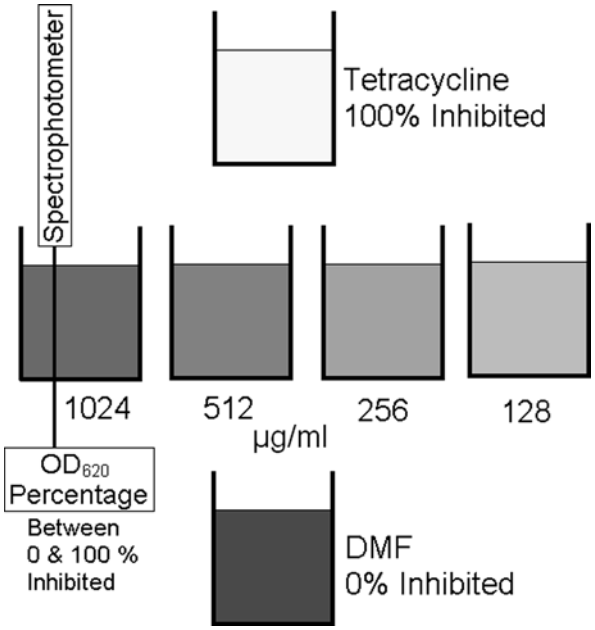

Fig. 8.3 Calculation of percentage inhibition from OD_{620} values compared between the 0 and 100% inhibition standards

Minimum Inhibitory Concentrations

We consider growth to be inhibited when the OD_{620} is less than 0.07. The minimum inhibitory concentration is reported as less than the lowest concentration where the OD_{620} is below this level (see Fig. 8.4).

In Vitro Testing with Rumen Fluid Inoculum

It is possible that compounds contained within the plant may undergo metabolic transformation in the gut of animals which could induce or reduce the activity of the compound against the target organism. To address this issue we mixed plant extracts and rumen fluid together in an anaerobic medium to determine whether compounds identified as inhibitory in the primary screening process demonstrated similar activity in a mixed culture fermentation from the rumen.

Anaerobic Culture Techniques and Media Preparation

The anaerobic techniques of Hungate [7] as modified by Bryant [1] are used for the growth of organisms and preparation of media. A more detailed description of these methods and other types of media [10] that could be employed for rumen-simulated media is given in McSweeney et al. [9]. In our experiments on *E. coli*

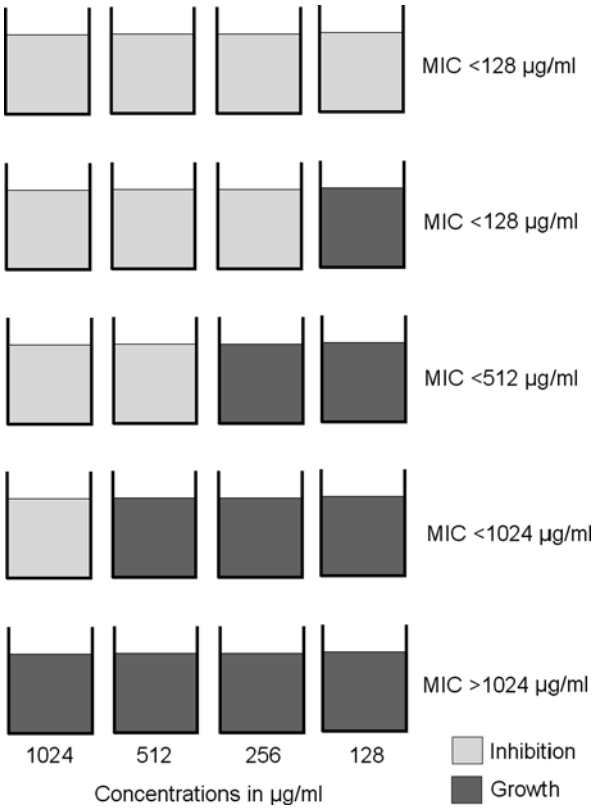

Fig. 8.4 Identification of the concentration where growth is inhibited for a particular target organism and calculation of minimum inhibitory concentration

inhibition, a basal medium (12 mL, see Appendix) is dispensed into Balch tubes containing pasture grass, (60 mg) and test plant (15 mg) both of which are finely milled, oven and freeze-dried. The media and plant material is immediately stoppered and inoculated with an overnight *E. coli* O157 culture (1.5 mL; approximately 5×10^8 CFU) and rumen fluid (1.5 mL). The rumen fluid for inoculation is prepared using a freshly collected digesta sample from an animal fed a conventional diet. The collected rumen digesta is strained through muslin cloth into an insulated vacuum flask and kept at 39°C for use within 1–2 h. The inoculated tubes are incubated at 37°C for 60 h and samples taken at 12 h intervals for analysis of *E. coli* growth.

Enumeration of Target Bacteria

Culturing techniques and selective media for enumeration will vary depending on the target organism. In the case of detection of *E. coli*, the Petrifilm "Coliform count

plate" (3 M Microbiology, St Paul, MN, USA) was used. Firstly, the mixed culture sample is serially diluted in 10 fold increments into aerobic peptone water (Oxoid, Basingstoke, UK). Normally the dilutions tested are 10^{-7}, 10^{-6} and 10^{-5}. From the selected dilutions, 1 mL is inoculated onto a Petrifilm plate and incubated overnight at 37°C. Individual coliforms appear as dark red colonies on a pink background and are easily counted. As the experiment progresses and counts are observed, the dilution steps for forthcoming samples are adjusted to ensure that the counts remain on scale.

Conclusions

For discovery of compounds possessing antibacterial activity, there is a lack of uniform and harmonised testing methods [15]. This is understandable since the target organisms; the source and nature of compounds under investigation, and the environment in which the antimicrobials will be employed usually differ markedly for each application. In our experiments, we have tried to maintain continuity between past and present procedures by using similar culture methods and bacterial panels that overlap between different antimicrobial testing studies. Our studies are primarily designed to identify plants that could be used as dietary supplements for ruminant animals. However, the initial steps in identification of plant compounds for antibacterials, drug discovery or dietary supplements remains the same. The difference is what is done after identification of the crude bioactive sample. In drug discovery, lead compounds need to be isolated from the plant material characterised and modified for specific uses.

The process of bioactive discovery is a compromise between what will provide the most useful information with given resources and the practicality of the approach. It has been shown that conditions of testing can be critical for the final outcome [15]. Conditions can be varied at many levels (e.g. pH, type of media, culture used etc), but it is difficult to justify the increased resources to test plants/compounds at more than a limited set of conditions in the initial screen. Our methods utilise a defined set of standard conditions and the results are used to determine the next stage of testing. This strategy is also suited for screening large libraries of bioactive materials.

Testing plant and compound libraries can create extremely large sets of raw and calculated data. It is essential that such data sets be handled in a manner that allows visualization of results. For example, Microsoft Excel® using the background programming language or visual basic for applications, can sort and categorise with colour coding, subsets of the data contained within the spreadsheets. This allows rapid identification of compounds that can be selected for further phases within the screening process. The importance of this aspect of the screening process cannot be underestimated when dealing with large libraries or an extensive bioactive discovery project.

Appendix – Basal Media Composition

Table 8.2 Basal media components as modified from Caldwell and Bryant [2]

Basal media components	
Mineral solution 1 (Table 8.3)	38 mL
Mineral solution 2 (Table 8.3)	38 mL
Pfenning trace solution (Table 8.4)	1 mL
$Na_2HPO_4.12H_2O$	18 g
VFA Mixture (Table 8.5)	100 mL
Clarified rumen fluid	200 mL
Sodium bicarbonate ($NaHCO_3$)	6 g
Resazurin solution[a]	1 mL
Hemin solution[b]	1 mL
Yeast Extract (Oxoid)	0.5 g
Water	625 mL
$Na_2S.9H_2O$	0.25 g
L-cysteine HCl (Added last)	0.25 g

[a]Resazurin solution is made up to a concentration of 0.1% in 20 mM, NaOH
[b]Hemin solution is 0.05% in 50 mM, NaOH

Table 8.3 Mineral solutions used as components of the basal media

	Minerals (g/L)	
Mineral solutions	1	2
$CaCl_2$	0.2	
$MgSO_4$	0.2	
K_2HPO_4	1.0	6.0
$NaHCO_3$	10.0	
NaCl	2.0	

Table 8.4 Pfennings trace elements solution used in the basal media

Trace elements diluted in distilled H_2O	mg/L
H_3BO_3	300
$ZnSO_4.7H_2O$	100
$MnCl_2.4H_2O$	30
$CoCl_2.6H_2O$	20
$NaMoO_4.2H_2O$	30
Na_2SeO_3	10
$NiCl_2$	20
$CuCl_2.2H_2O$	10
$FeCl_2.4H_2O$	150

Table 8.5 Volatile fatty acid mixture used as a component of the basal mixture

Volatile fatty acids	mL/L
Acetic acid	17
Propionic acid	6
n-butyric acid	4
n-valeric acid	1
iso-valeric acid	1
iso-butyric acid	1
2-methylbutyric acid	1

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References

1. Bryant, M.P., 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Am. J. Clin. Nutr. **25**:1324–1328.
2. Caldwell, D.R., and M.P. Bryant. 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. Appl. Microbiol. **14**:780–794.
3. Callaway, T. R., R.O. Elder, J.E. Keen, R.C. Anderson, and D.J. Nisbet. 2003. Forage feeding to reduce preharvest *Escheriscia coli* populations in cattle, a review. J. Dairy Sci. **86**:852–860.
4. Dibner, J.J. and J.D. Richards. 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poult. Sci. **84**:635–643.
5. Eloff, J.N. 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta. Med. **64**:711–713.
6. Hovde, C.J., P.R. Austin, K.A. Cloud, C.J. Williams, and C.W. Hunt. 1999. Effect of cattle diet on *Escherichia coli* O157:H7 acid resistance. Appl. Environ. Microbiol. **65**:3233–3235.
7. Hungate, R.E. 1969. A roll tube method for cultivation of strict anaerobes, pp. 117–132. In I.R. Norris, and E.W. Ribbons, (eds.), Methods in Microbiology. Academic Press, New York.
8. Jenson, H.M. 2006. Health management with reduced antibiotic use – experience of a Danish pig vet. Anim. Biotech. **17**:189–194.
9. McSweeney, C.S., S.E. Denman, and R.I. Mackie. 2005. Rumen bacteria, pp. 23-37. In H.P.S. Makkar, and C.S. McSweeney (eds.), Methods in Gut Microbial Ecology for Ruminants. Springer, The Netherlands.
10. Menke, K.H., L. Raab, A. Salewski, H. Steingass, D. Fritz, and W. Schneider. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedstuffs from the gas production when they are incubated with rumen liquor. J. Agric. Sci. **93**:217–222.
11. National Committee for Clinical Laboratory Standards. 1997. M7-A4. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Wayne, PA, USA.
12. National Committee for Clinical Laboratory Standards. 1997. M2-A6. Performance standards for antimicrobial disk susceptibility tests. Wayne, PA.
13. Österblad, M., T. Leistevuo, and P. Huovinen. 1995. Screening for antimicrobial resistance in fecal samples by the replica plating method. J. Clin. Microbiol. **33**:3146–3149.

14. Recio, M.C., J.L. Rios, and A. Villar. 1989. A review of some antimicrobial compounds isolated from medicinal plants reported in the literature 1978-1988. Phytother. Res. **3**:125–177.
15. Rios, J.L., and M.C. Recio. 2005. Medicinal plants and antimicrobial activity. J. Ethnopharma. **100**:80–84.
16. Wegener, H.C. 2003. Ending the use of antimicrobial growth promoters is making a difference. ASM. News **69**:443.