Philip E. Vercoe
Harinder P.S. Makkar
Anthony C. Schlink
Editors

In Vitro Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies

Springer

IAEA
Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

In Vitro Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies

Philip E. Vercoe · Harinder P.S. Makkar ·
Anthony C. Schlink
Editors

In Vitro Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies

Springer

Editors
Dr. Philip E. Vercoe
School of Animal Biology
The University of Western Australia
35 Stirling Highway
Crawley WA 6009
Perth, Australia

Dr. Anthony C. Schlink
International Atomic Energy Agency
Animal Production & Health Section
Wagramer Str. 5
1400 Vienna
Austria
A.SCHLINK@iaea.org

Dr. Harinder P.S. Makkar
Universität Hohenheim
Institut für Tierproduktion in den
Tropen und Subtropen
70993 Stuttgart
Germany
makkar@uni-hohenheim.de

ISBN 978-90-481-3296-6 e-ISBN 978-90-481-3297-3
DOI 10.1007/978-90-481-3297-3
Springer Dordrecht Heidelberg London New York

Library of Congress Control Number: 2009939534

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Foreword

The Animal Production and Health Section of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture recognises that the trend towards intensification of livestock production in developing countries presents both opportunities and challenges. The potential opportunities are the flow-on benefits to the producers and local economy while the potential challenges are the flow-on costs to the environment, animal health and welfare. The intensification of livestock production can lead to higher levels of greenhouse gas emissions and a localisation or concentration of nutrients, which increases the risk of pollution of waterways, increased chemical and drug use to overcome disease transmission and put pressure on the livestock production systems as local communities strive to provide more and better quality feed for the animals. The growing global pressure from consumers for producers to engage in sustainable production systems, i.e. to produce high quality, wholesome and safe products in an efficient manner with minimal impact on the environment and human health, will also impact livestock production in developing countries. This will put producers in developing countries under similar pressures to those in developed countries to limit the input of, and find "natural" alternatives to chemical use by exploring alternative sources of feed resources.

The successful intensification of livestock production in developing countries will depend on the ability of local producers to design sustainable feeding systems based on locally available feed resources that are efficient, profitable and with minimum effect on the environment. To design these feeding systems, these producers need the technical capability to screen local plant resources for their nutritive value, anti-nutritional factors and/or toxicity. This would be followed by incorporation of the selected species in animal studies to measure the efficiency of nutrient utilisation, monitor reproductive efficiency and their effects on the health of the animals.

This publication stems from a meeting between the Joint FAO/IAEA Division and Writtle College, UK entitled "Alternative feed resources: a key to livestock intensification in developing countries" held in September, 2006 prior to the British Society of Animal Science meeting on ethnobotany/ethnoveterinary medicine entitled "Harvesting Knowledge, Pharming Opportunities". The participants included ten experts in nutrition, screening native plants for bioactive compounds for animal

production and health, rumen molecular microbiology, gut parasitology, and feeding behaviour from agricultural research organisations and universities in Germany (Dr. Evelyn Mathias and Dr. Harinder Makkar), India (Dr. Devki Kamra), Australia (Dr. Dean Revell, Dr. Chris McSweeney and Dr. Zoey Durmic), UK (Dr. Frank Jackson and Dr. John Wallace) and USA (Dr. Fred Provenza), as well as IAEA livestock production staff (Dr. Philip Vercoe, coordinating Technical Officer). The main objective of the meeting was to review the opportunities and challenges associated with in vitro screening of plants for bioactive properties and to use feeding behaviour and selection principles to develop systems that integrate novel plants and plant extracts into feeding systems.

The aim of this manual is to provide a comprehensive guide to the methods involved in collecting, preparing and screening plants for bioactive properties for use in manipulating key ruminal fermentation pathways and against gastrointestinal pathogens. The manual provides both isotopic and non-isotopic techniques for screening plant and plant products for extra-nutritional attributes to find "natural" alternatives to chemicals for manipulating ruminal fermentation and gut health. The isotopic techniques include the labelling of part or whole plants, protozoa and bacteria to improve the assaying of plant material for improved livestock production. Each chapter has been contributed by experts in the field and methods have been presented in a format that is easily reproducible in the laboratory. It is hoped that this manual will be of great value to students, researchers and those involved in developing efficient and environmentally friendly livestock production systems.

Contents

Contributors

Neeta Agarwal Centre of Advanced Studies in Animal Nutrition, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122, India

Klaus Becker Institute of Animal Production in the Tropics and Subtropics, University of Hohenheim, D-70593 Stuttgart, Germany

Mike Bennell Department of Water, Land and Biodiversity Conservation, Adelaide, SA 5001; Future Farm Industries – CRC, University of Western Australia, Crawley, WA 6009, Australia

Glen A. Broderick Agricultural Research Service, USDA, US Dairy Forage Research Center, Madison, WI, USA

Salvador Fernandez-Rivera International Livestock Research Institute, Addis Ababa, Ethiopia

Jean Hanson International Livestock Research Institute, Addis Ababa, Ethiopia

Trevor Hobbs Department of Water, Land and Biodiversity Conservation, Adelaide, SA 5001; Future Farm Industries – CRC, University of Western Australia, Crawley, WA 6009, Australia

Elen M. Hoffmann Institute of Animal Production in the Tropics and Subtropics, University of Hohenheim, D-70593 Stuttgart, Germany

Hervé Hoste UMR 1225 INRA DGER, INRA Ecole Nationale Veterinaire de Toulouse, 23 Chemin des Capelles, 31076 Toulouse, France

Steve Hughes South Australian Research and Development Institute, Plant Research Centre, Waite Campus, Adelaide, SA 5001; Future Farm Industries – CRC, University of Western Australia, Crawley, WA 6009, Australia

Peter G. Hutton School of Animal Biology, Faculty of Natural and Agricultural Sciences, University of Western Australia, Perth, WA, Australia; Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Palmerston North, NZ

Frank Jackson Moredun Research Institute, Pentland Science Park, Bush Loan, Edinburgh, EH26 0PZ, UK

Devki N. Kamra Centre of Advanced Studies in Animal Nutrition, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122, India

Greg W. Kemp CSIRO Livestock Industries, St. Lucia, Queensland 4067, Australia

Secundino López Department of Producción Animal, Universidad de León (ULE), E-24007 León, Spain

Harinder P.S. Makkar Institute of Animal Production in the Tropics and Subtropics, University of Hohenheim, D-70593 Stuttgart, Germany

Tim A. McAllister Agriculture and Agri-Food Canada, Lethbridge Research Centre, Lethbridge, Alberta, Canada

Chris S. McSweeney CSIRO Livestock Industries, St. Lucia, Queensland 4067, Australia

T.G. Nagaraja Department of Diagnostic Medicine/ Pathobiology, College of Veterinary Medicine, Manhattan, KS 66506-5606, USA

C. Jamie Newbold Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Llanbadarn, Aberystwyth, SY23 3AL, UK

Frederick D. Provenza Department of Wildland Resources, Utah State University, Logan, UT 84322-5230, USA

Dean K. Revell CSIRO Livestock Industries, Private Bag 5, Wembley, WA 6913; Future Farm Industries – CRC, University of Western Australia, Crawley, WA 6009, Australia

Natascha Selje-Assmann Institute of Animal Production in the Tropics and Subtropics, University of Hohenheim, D-70593 Stuttgart, Germany

Carla R. Soliva Institute of Animal Science, Animal Nutrition, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland

Philip E. Vercoe School of Animal Biology, The University of Western Australia, 35 Stirling Highway, Crawley WA 6009, Perth, Australia

Juan J. Villalba Department of Wildland Resources, Utah State University, Logan, UT 84322-5230, USA

R. John Wallace Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, Scotland

Colin L. White CSIRO Livestock Industries, Private Bag, PO Wembley, WA 6913, Australia

Introduction

The plant kingdom has been a source of medicinal, pharmaceutical and bioactive compounds for treating diseases and enhancing animal production, health and welfare as well as food processing for time immemorial. However, these gains are now seriously jeopardized by another recent development: the emergence and wide-spread incidence of chemical residues in human food and antimicrobial and anthelmintic resistance causing a surge of interest in the use of "natural" alternatives to chemicals in livestock production systems. In ruminant production, the main focus has been on identifying plants with extra-nutritional benefits that may be used to manipulate ruminal fermentation to improve the efficiency of nutrient utilization. Usually, the initial screening is conducted in vitro because of the large number of candidate plant species and the prohibitive cost of screening them in vivo. The number of species for in vivo testing is narrowed down over several stages of screening, and the top two or three are eventually evaluation in animal experiments. The focus of this book is on the in vitro techniques that are used to screen plants or plant products, with an emphasis on those that involve the use of nuclear and nuclear related technologies.

Researchers initiating a programme to screen plants for extra-nutritional benefits are confronted with a number of questions, for example, how to start the programme, how to choose the plants to screen, how to collect and store the plants, which parts of the plants to test, whether to test the whole plant or an extract from the plant and, of course, what technique to use to screen for particular characteristics. The chapters in the book have been chosen to help researchers embarking on this type of programme by addressing these questions and harmonising the screening techniques to be used. The first chapter provides an example of the type of processes that can be established to help make decisions about which plants to include in a screening programme. There is no "one size fits all". Some groups use botanical information that is available about families of plants and the likelihood of the presence of particular types of secondary compounds as a starting point, whereas others use a "random" approach and favour "novel" plants that have little known about them, or use geographical and climatic data to select plants that grow in a targeted region. However, the principles and approaches described in this chapter can be applied more generally to projects with different aims, budgets and manpower. The second chapter describes the collection, processing and storage of plants for nutritional analysis.

Chapters 3–10 are dedicated to various techniques used for screening a large number of plants and plant compounds for a wide range of properties, including; antimicrobial, anthelmintic, anti-proteolytic, anti-protozoal, and methane-reducing activities as well as their potential to modify ruminal fermentation, for example, improve fibre degradation or prevent acidosis. The final chapter discusses the challenges of extrapolating in vitro findings to in vivo evaluation of plant resources.

The chapters in this book are written by experts interested in exploring and making better use of plant biodiversity for improving livestock production and reducing its environmental footprint. This book will provide a guide to researchers in developing and developed countries to initiate and coordinate large-scale screening programmes of the local plant diversity and contribute to the global knowledge base on novel extra-nutritional benefits of plants and their extracts for use in animal agriculture. It will enable researchers worldwide to harmonise the techniques they use to screen for eight key bioactivities for manipulating ruminal fermentation and improved gut health. The information gathered could lead to the purification of specific compounds that could be used as feed supplements or for the development of new grazing systems involving multifunctional polycultures of plants to improve the long-term sustainability of ruminant production. There is little doubt that the more we explore the potential of our global plant biodiversity the greater the chances are of developing livestock production systems that are more clean, green and ethical.

Chapter 1
Selecting Potential Woody Forage Plants That Contain Beneficial Bioactives

Mike Bennell, Trevor Hobbs, Steve Hughes, and Dean K. Revell

Introduction

Current viewpoints on animal production systems are being challenged in many parts of the world by the importance of safeguarding their long-term environmental stability and improving productivity. Pressure for change is arising from a range of environmental problems including dryland salinity, degradation of rangeland grazing systems and desertification; the need to address growing resistance to chemical anthelmintic drugs [3] and pressure to reduce the use of antimicrobial drugs in livestock production [8]. Plants with anthelmintic properties are of special interest because of a growing problem of nematode resistance to the chemical anthelmintics. There is also concern that antibiotics used in stock feed will lead to development of resistant organisms that could harm human health. The European Union has applied a total ban on antibiotics in stock feed and producers in other countries will be under pressure to follow suit to gain entry into European markets. Global warming is also an important issue where we need to adapt to maintain productive capacity while contending with more variable rainfall patterns, while reducing greenhouse gas release into the atmosphere a particular issue with methane production from ruminant animals. These various pressures have led to an increase in the interest in exploring global plant diversity for solutions to these issues and "natural" alternatives to the chemicals used in livestock production. Financial and human resources determine the extent to which we can explore our plant diversity, which means we have to make a choice about which to include in a screening programme. In this Chapter, we have used our research programme as an example of an approach that can be taken to selecting plants for a large-scale screening programme. We acknowledge that ours is just one approach of many that can be taken and is shaped by the goals of our programme, but the principles behind our approach can be applied more broadly to any screening programme.

M. Bennell (✉)
Department of Water, Land and Biodiversity Conservation, Adelaide, SA 5001; Future Farm Industries – CRC, University of Western Australia, Crawley, WA 6009, Australia
e-mail: mike.bennell@anu.edu.au

P.E. Vercoe et al. (eds.), *In Vitro Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies*,
DOI 10.1007/978-90-481-3297-3_1,

In Australia, the focus on sustainability is stimulating research to develop new innovative farming systems that incorporate a much higher proportion of perennial species [6]. The potential of shrub based forage systems is gaining acceptance as a means of providing options that:

- Provide a feed base made up of a functional mixture of plant species including shrub options that are resilient to prolonged dry periods and provide feed in periods of seasonal shortfall;
- Integrate into a productive livestock enterprise based on current pasture options but are of a sufficient scale to have a positive impact on land management issues, and
- Provide the opportunity to include plants in a mixed assemblage that provide compounds of medicinal value, or compounds that have favourable effects on gut health through manipulating the micro flora and fauna of the digestive tract.

To address these multiple objectives it will be necessary to introduce a greater degree of perennial-based feed production together with an increased diversity of plants available to grazing animals. Combining this with a broader approach in plant selection that includes indigenous plant species offers exciting prospects for the future. For example, Australia's native flora is well adapted to the extreme conditions of the continent, can utilise water at depth in the soil profile, is responsive to "out of season" rainfall events, and has unrealized potential for domestication.

Australian plants have evolved to produce an array of secondary compounds as chemical defences against herbivores [2]. Extracts of Australian plants have been shown to inhibit the growth of one or more species of bacteria, with five extracts showing broad-spectrum antibacterial activity [5]. Extracts from the leaves of *Eremophila* species (Myoporaceae) were the most active.

A key goal of current research is the domestication of a larger number of productive native species with forage and health values. There is a significant pool of species identified from Australia's history of rangeland grazing industries that are palatable and have high nutritive value. Oldman Saltbush (*Atriplex nummularia)* is the only native species to date that has widespread usage as a cultivated forage species and is widely utilised in dryland salinity affected as well as agricultural areas. However even this species is at an early stage of development in regard to overcoming animal nutrition issues, improved agronomy or exploiting the potential for genetic improvement.

Overview of Process

We have developed a systematic approach to the identification of native species having forage potential that requires screening a large pool of native species. Our focus has been on woody perennial species for agricultural areas of the wheat/sheep belt of southern Australia. There are concurrent projects evaluating herbaceous species

[4, 7]. Southern Australia has in the order of 26,000 taxa for which there is limited information apart from taxonomic descriptions, recorded in ecological surveys or being noted as having potential value for a commercial purpose including grazing systems, and are often largely unknown to cultivation [1]. The general goal of this process is to identify a relatively small number of species (10–20) that have attributes making them suitable for domestication and inclusion into a plant improvement programme, and ultimately being incorporated into livestock production systems. The selection process can be simply described as a step-by-step process:

1. Define the plant attributes
2. Specify the regional characteristics (soil, climate, land-use) of the target areas
3. Identify the search area
4. Assemble a database of species occurring in the search area
5. Review family and genera and remove those that have characteristics not matching the specified plant attributes
6. Review literature and collect expert knowledge to identify species recorded as having forage value
7. Working list of potential species
8. Undertake a detailed collection of attribute information on working list species – Download and collate Global Positioning System (GPS) data on herbarium records
9. Develop indices and rank based on attributes and Geographic Information System (GIS) derived parameters
10. Undertake an expert review of listing of species
11. Germplasm collection of prioritised species and collection of samples for testing of nutrient value, impact on rumen function and anthelmintic effects.
12. Field evaluation of plant performance (productivity, adaptability, nutrient value, secondary compounds, toxicity, palatability)
13. Select target species for domestication

This allows information gained throughout the evaluation process to be entered into the database that informs an ongoing process of identification of superior species. For example, there is feedback of information from the field evaluation in Step 12 to Step 8 where data is fed back into the database and informs the final selection of species for domestication. Each of these parameters are defined in more detail below and divided into separate stages of the process.

Defining the Project Parameters (Steps 1–3)

The initial component of the screening process requires careful consideration of the goals and targeted regions of the project. This will include the general attributes of the plant species being sought and the geographic regions that have natural

populations of species likely to be adapted to the target region where the new crop plants are to be established for productive purposes. The key questions that need to be considered are:

Step 1

What are the target characteristics of the species you are seeking? Some of these characteristics will be particular to the location of the project but many will be common across different situations including productivity, feed value and secondary compounds.

Step 2

What are the characteristics of the region that is targeted for the introduction of the new species and systems? Identify the climatic and soil conditions, the nature of the existing land-uses and the characteristics of the production systems that the new species are to be part of. Geographic Information System mapping and spatial analysis can be a powerful tool in this process, allowing spatial mapping of major factors that will influence adaptability including climate, soil type and texture, salinity, potential for inundation and other features of the landscape.

Step 3

Define the geographic range that you will survey to locate likely species. It is most likely that species adapted to neighbouring areas of harsher climate/soil conditions will perform best in the better climatic conditions of the introduction zone. Species from wetter sites will frequently not be adaptable to drier conditions however be careful in making generalised assumptions.

In the Australian project on which this description is based, the aim of the process was to select woody native species with potential to be included in in-situ forage production systems providing feed or beneficial secondary compounds. Only perennial woody plants are being considered here with perennial herbaceous material being the objective of a parallel project [4]. There is expected to be a degree of overlap between the studies as there is a grey area where woodiness is a matter for definition. The degree of woodiness considered here is minimal but plants must have as at least a woody stump that the plant can be grazed back to and to be able to re-shoot from under favourable conditions. This will allow consideration of plants with a wide range of habit including ground cover species through to trees but with the majority being shrubs. Apart from being a woody perennial plant the guiding criteria for identification of a potential species included:

- Produce forage that is palatable and nutritious
- Is productive on a per hectare basis
- Contains secondary compounds that are beneficial for animal health
- Is resilient to environmental stress
- Be free of toxins
- Will re-grow following grazing
- Readily sets seed that is easily harvested
- Has resistance to insect and diseases
- Will propagate and establish readily
- Has a low potential of becoming an environmental weed

Database Collation (Steps 4 and 5)

Step 4

The development of a computer-based database is a critical step in the process providing the capacity to systematically capture the scattered information available and keep track of the originating source. Assemble a list of plant species occurring in the search region identified above together with taxonomic information including family and plant division information. This task can be complex due to the changing botanical names that arise as classification is reassessed by taxonomists. Uptake of new names can be different across national and state borders and close attention to synonyms is required during the development of the list. Taxonomic records will generally contain detailed plant descriptions and if in a digital form this can be drawn into the database at this stage. Information on habit, plant height and width and other morphological information will be useful in following steps.

In the Australian experience, a list of all plant species for the southern Australian states (Western Australia. South Australia, Victoria and New South Wales) was extracted from a range of state-based and national plant databases and compiled into an Access database. These databases principally contained information on plant taxonomic relationship that enabled the identification of the plant divisions, families, genera, species and subspecies level. The taxonomy of each database was standardised to create a common species list to cover the region. Some discrepancies in scientific names occurred due to the continual process of reclassification mentioned above. Some of these datasets contained information on plant life form, height, and crown width, and introduced and threatened species status under state and federal legislation that was incorporated into the database.

Step 5

Cull the list at the family and generic taxonomic level using the characteristics of target species defined above. This will be a multistage process commencing with identification of plant characteristics through taxonomic affiliations i.e. defining characters of the taxonomic levels of classification: division, family and genus.

For example, in our survey the first level of cull starts by considering only seed plants that includes the angiosperms and gymnosperms. Although some records of grazing of members of the gymnosperms exist, they were not considered further and only the angiosperms were retained. This division (Anthophyta) are the flowering plants, and are the largest and most diverse group. They are divided into two groups based on the number of cotyledons on the embryo, the dicots and the monocots. A characteristic of the monocots is the absence of secondary growth. Most seed plants increase their diameter through secondary growth, producing wood and bark but the monocots (and some dicots) have lost this ability (Some monocots produce a substitute however, as in the palms and agaves) but based on this general character the monocots were excluded from this study, as they will not meet our basic search criteria of woodiness.

The next levels of taxonomic classification – families and genera, can be reviewed at this point so that only those that include species fitting essential criteria of being woody perennials and not one of the specialised groups of plants such as arboreal parasites or only annuals are retained. Botanic texts that provide generic descriptions can be utilised at this point. In addition, plant species listed as endangered under conservation regulations were excluded from the primary selection list; and poorly described, hybrid or rare species variants were also excluded. In our study, this initial level of cull reduced the possible list of species from approximately 26,000 across southern Australia to about 7,000 angiosperms with a potentially woody habit.

Literature Search (Step 6)

More detailed species information will be required to support the next level of cull so that only plants with a history of forage utilisation are carried forward. It is expected that there will be a body of published information in the scientific, technical and popular literature that describes the history of plant utilisation in the region of investigation. In addition, there will be many individuals from the scientist to landowner with an interest in use of the flora by grazing animals who can be located and interviewed in order to share their knowledge on species suitability. This information can be entered into the database under headings such as; palatability (ranked), nutrient value, protein level, digestibility and metabolisable energy if available and/or a ranking of observed performance of stock using the feed source, presence of secondary compounds and evidence of toxicity. The output at this stage is the identification of species that have at least one reliable record of forage utilisation.

In the Australian study, there was a bias to semi-arid to arid species where there has been a longstanding reliance on rangeland plants to support a grazing industry and limited information on higher rainfall species occurring in regions were clearance and development of European style farming was the norm.

The rangeland livestock industry has declined in importance in recent times but has played an important role in the agricultural economy of Australia in the past.

This provided a substantial body of research and technical commentary on species with grazing value that provides much of the background information for the literature search. There were various other books, scientific papers, technical reports and fact sheets with information or commentary on forage value including palatability, nutritional value, toxicity and utilisation by stock that have been examined as well, although much of this is captured in the texts mentioned above. This material was collected and all observations of forage value for woody species entered into the database. Workshops and one to one discussions with botanists, rangeland experts and landowners were undertaken to gather local knowledge and experience to assist the survey staff in the species selection process. Observations on plant distributions; life histories; known physical, chemical and product values; and previous history of utilisation were collated and used to identify candidate species for further evaluation. All records were cross-referenced to original sources using Endnote® reference listing.

Working List (Step 7)

The base list can be reduced at this point to a working list of known potential species. An assumption is made that the species identified are indicative of genera that may contain species of potential, even if no other species in the genera have a reference to fodder value noted from the proceeding section. The existing records may suggest species in the same genera but occurring in other regions that could be worthy of examination in the future. All genera where there is no record are removed. Simultaneously the species that occur in retained genera but do not have an observation of forage value recorded against them are nominated within the working database as plants of potential but are not examined further at this stage. We have left at this stage with the working list of potential forage species with a referenced source to support the nomination.

Prioritisation (Step 8)

Once a core list of species is identified more detailed information can be obtained from herbarium databases. Herbarium records with GPS locations for plant collections can be downloaded to the database and utilised for basic GIS analysis. This potentially provides the opportunity to consider the natural geographic range of each species, the range of mean rainfall zones crossed and associations to major soil types. Now a smaller list of species has been created, a detailed literature search on each species can be undertaken. This includes detailed information on known animal utilisation, prior feed value testing, presence of secondary compounds and their medicinal value where known. In addition, information in the broader horticultural literature can be collected to add information the growth habit, growth rate, mature height and width, leaf density, ability to coppice and re-shoot after grazing, drought tolerance, seed bearing characteristics and ease of propagation. This

can be added to the database providing a basic level of information on the species of interest although this is likely to have many gaps.

In the Australian study, point location data for plant species was obtained from Australian government agencies. A Geographic Information System was used to identify the geographic and rainfall distribution for each plant record. Plant species records were plotted and matched to the rainfall and soil distributions. The number of point records for specimen collection for each species within the study region and within each rainfall and soil band was totalled. This provided an estimate of the frequency of occurrence of a species measured against the underlying environmental parameter and within the study area. Species that appeared to be vagrants or unsuited to the region were excluded. The availability of GIS herbarium location also provides the opportunity for application of bioclimatic modelling that uses climate parameters to predict the areas for which a species may be adapted. For the prioritisation process a preferred height based on the recorded mature height for the fodder species can be selected allowing a focus, for example, on shrubs between 0.5 and 2 m, or a sub-shrub or groundcover of less than 0.5 m.

Indices for Ranking (Step 9)

The data set developed so far can be used to produce a series of indices, for example, the number of rainfall increments the species occurs over, a possible indication of adaptability. A similar approach can be taken to soil types. Plant habit can be used to nominate a range for the ideal plant height or the recorded information on palatability or nutrient value used to create indices of forage value. The data set is most likely going to be incomplete and default values will need to be inserted in gaps. The indices used will depend on the objectives of the researcher and the amount of base information available. The approach taken in the Australian study is outlined below and can be used as a guide.

The important parameters used in our study are set out in Table 1.1. Indices were created based on some key selection criteria including:

- Rainfall range
- Plant volume/growth rate
- Palatability and nutrient value

To prioritise and rank species for further analysis and collection, a series of calculated indices were created:

- *Volume index* – Using maximum height and crown width the cylindrical volume (m^3) that each species occupies was calculated. The highly skewed distribution of volumes was normalised using a natural logarithmic transformation. The results were then rescaled into an index ranging from smallest volume to greatest volume. The index is a surrogate for the maximum potential yield at full maturity for each species;

Table 1.1 A summary of plant species attributes compiled for the southern Australian species selection process [1]

Information type (units or classification)
Genus, species and infra-specific variants (subspecies, varieties)
Family
Number of records in the study area
Mean annual rainfall (mm)
Minimum and maximum annual rainfall (mm)
Maximum height and crown width (m)
Life form (tree/mallee/shrub/subshrub/ground cover)
Growth rate (fast/moderate/slow)
Coppicing and suckering ability (yes/no)
Palatability to livestock (high/moderate/low/not palatable)
Presence of useful secondary compounds (presence/absence)
Fodder digestibility (% dry matter)
Crude protein (% dry matter)
Drought fodder persistence (high/moderate/low)
Calculated indices (indices between 0 = least desirable and 1 = most desirable)
Volume index – maximum potential space an individual plant occupies
Biomass priority index – a combination of volume, rainfall range and growth rate indices
Rainfall range index – rainfall range of a species as a proportion study region
Growth rate index – growth rate (fast, moderate, slow)
Fodder palatability index – palatability to livestock (high, moderate, low, not palatable)
Optimal fodder height index – height above optimal grazing height
Adaptability priority index – a combination of volume, rainfall range and growth rate indices
Fodder priority index – a combination of adaptability priority, fodder palatability and fodder height indices

- *Rainfall range index* – To indicate a species' adaptability to rainfall, and in part its spatial distribution, the overlap of each species' minimum and maximum rainfall records with the 200–700 mm annual rainfall zone has been expressed as a proportion and rescaled to lowest proportion of the range to across the entire range;
- *Growth rate index* – 3 categories of growth rate, based on expert observations or the literature, have been transformed into an index of growth rate (fast, moderate, slow). Species without reliable information on growth rate were assigned a moderate default value;
- *Fodder palatability index* – 4 categories of fodder palatability to livestock, based on expert observations or the literature, have been transformed into an index of fodder palatability (high, moderate, low, not palatable). Species without reliable information on palatability were assigned a moderate default value
- *Optimal fodder height index* – the maximum optimum grazing was nominated at 1.2 m (fodder height score of 1), to give a selection advantage to species that do not require any mechanical management in a grazing system. Fodder species taller than 1.2 m had their score reduced by their height above 1.2 m expressed as a proportion of the height of the tallest fodder species above 1.2 m. Fodder height scores were scaled from 0.25 (tallest fodder species) to 1 (below 1.2 m);

- *Adaptability index* – The average of volume, rainfall range and growth rate indices, with double weighting of Growth Rate Index; and
- *Fodder priority index* – The average of biomass priority, fodder palatability, useful secondary compound and fodder height indices.
- *Biomass priority index* – The average of volume, rainfall range and growth rate indices.

The Adaptability index and Fodder priority index were then used to rank and prioritise potential fodder species.

External Expert Review (Step 10)

Once a prioritised list is created, evaluation by a panel of experienced individuals with practical experience in the study area and on the utilisation of native pastures by livestock will add depth and credibility to the preceding prioritisation process. The criteria for selection will need to be clearly established by the research team to provide a template for the panel.

A process of subjective evaluation has been employed by Hughes et al. [4] in a parallel study of exotic and native herbaceous species. In that case a process of information exchange and the compiled database was provided to a team of experts within the project team and international forage specialists at the N.I. Vavilov Research Institute (VIR), St. Petersburg, the International Centre for Agricultural Research in the Dry Areas (ICARDA), Syria, and the United States Department of Agriculture (USDA), and the University of Perugia, Italy. The representative team applied their expert knowledge, literature and experience to the species listed. Their expert knowledge base, together with an understanding of the problems (e.g. hydrological stability and commercial seed production) and objectives of the research team resulted in the addition of further species and identification of species of highest potential. Each new species was rated against the following criteria:

- Level of domestication and/or economical significance
- Tolerance to soil salinity
- Tolerance to saline water logging
- Tolerance to drought

The knowledge base for prospective Australian native woody species is much narrower, but within Australia, a small group of technical experts with a depth of knowledge in forage species and the management of rangeland pastures is available. An invaluable step in the species appraisal process was for these individuals to apply their own ranking to the list and to add any additional species or remove any they considered inappropriately included, together with comments as to their reasons. The reviewed lists were appraised and species inclusion or ranking adjusted to meet to consensus views of the panel when this occurred.

Germplasm and Sample Acquisition (Step 11)

The acquisition of seed or cutting material of the priority plants to establish nursery stock is the next key step. Plant able to be propagated will form the basis of field trials established in a few locations with soil and climate attributes representative of the broader study area. Concurrent with this collection, leaf samples can be collected to allow wet chemistry testing of the feed value and testing for the presence or absence of beneficial secondary compounds.

In Australia, germplasm for many species was poorly represented in existing institutional collections and needed to be assembled through the network of seed collectors and merchants that provide the majority of native seed in Australia. Many of the species were difficult to obtain as they occur in remote areas and are in low demand due to their obscurity. Direct collection of seed through in situ collections in the wild was also undertaken however drought conditions in recent years has impacted on much of the native range of many species and seed availability was poor. This acquisition phase needs to be undertaken over several years to compile a collection coming near to being a complete representation of the priority list. Sample material for testing was collected where possible and the results added to the database to contribute to selection and evaluation.

Field Evaluation (Step 12)

Undertake field evaluation of the selected species in a site(s) representative of the region targeted for introduction. Select a site of uniform topography and soil type so that the population is growing under conditions as even as possible to allow comparison of performance between species. The ease of seed collection and ability to germinate will be an early indication of the potential suitability of a species for eventual commercial adoption. The field evaluation trial will provide ongoing data on the productive potential of edible biomass from each species, adaptability, plant biology, response to simulated or actual grazing and will provide sample material for more detailed testing of a range of nutrition characteristics and secondary compounds with medicinal value or have a beneficial effect on rumen function. The data collected from this trial can be added to the database and assist in building a complete picture of the attributes of the candidate species.

The first step in southern Australia on the characterisation of the acquired germplasm was the establishment of spaced plant or row nurseries of up to 3 representative accessions of all species acquired, with the duel objective of obtaining sufficient seed for subsequent agronomic screening and of acquiring preliminary data on the agronomic value of the species. The nursery phase can be effectively utilised to advance selection if the desired traits or breeding objectives have been clearly defined and if the observed agronomic characteristics are maintained in the subsequent phases of plant selection. The objectives of the preliminary characterisation programme were:

- To reduce the number of species to more manageable numbers as efficiently as possible through collection of data on ease of propagation and establishment, productivity, shrub form, seed production, nutrient value and presence of secondary compounds. This process will allow selection of a smaller group of plant species for more extensive germplasm by environment trials and assessment of traits including palatability and ability to recover from grazing pressure.
- To make the best use of the restricted seed supply and ensure sufficient quantities of seed are available for further testing.

Species for Ongoing Development (Step 13)

As the evaluation trial data becomes available and is incorporated into the database the best performing species that match the original criteria determined in the initial stages of the project can be selected. These can then become the basis of a traditional plant improvement programme.

Oldman Saltbush has been elevated to this level in the Australian research programme with projects underway or being developed on germplasm collections at representative sites, evaluation of variability in the natural population of the species, planning a breeding programme, understanding of the animal responses to saltbush when used as a major component of feed rations and development of innovative management approaches to perennial pasture systems incorporating shrubs.

Conclusions

The approach described here is at an early stage of application in southern Australia. The process is emerging as being iterative and ongoing with the limited plant knowledge, acquisition of germplasm and overcoming seed dormancy mechanisms being particular barriers to progress. It is likely that new species will be introduced to trials over several years with feedback into the knowledge base leading to a steady trickle of potential species emerging over time.

References

1. Bennell, M., T. Hobbs, and M. Ellis. 2008. FloraSearch species and industry evaluation, p. 154. A report for the RIRDC/Land and Water Australia/FWPRDC/MDBC Joint Venture Agroforestry Program, Canberra.
2. Cork, S.J. and W.J. Foley. 1991. Digestive and metabolic strategies of arboreal mammalian folivores in relation to chemical defenses in temperate and tropical forests, pp. 133–136. In R.T. Palo and C.T. Robbins (eds.), Plant Defences Against Mammalian Herbivory. CRC Press, Boca Raton, FL, USA.
3. Hordegen, P., H. Hertzberg, J. Heilmann, W. Langhans, and V. Maurer. 2003. The anthelmintic efficacy of five plant products against gastrointestinal trichostrongylids in artificially infected lambs. Vet. Parasitol. **117**:51–60.

4. Hughes, S.J., R. Snowball, K.F.M. Reed, B. Cohen, K. Gajda, A.R. Williams, and S.L. Groeneweg. 2008. The systematic collection and characterisation of herbaceous forage species for recharge and discharge environments in southern Australia. Aust. J. Exp. Agric. **48**:397–408.
5. Palombo, E.A. and S.J. Semple. 2001. Antibacterial activity of traditional Australian medicinal plants. J. Ethnopharmacol. **77**:151–157.
6. Revell, D. and M. Bennell. 2006. Multipurpose grazing systems using perennial woody species. Report of Workshop held 8–9 December 2004. RIRDC Publication No 06/030, Canberra, ACT.
7. Rogers, M.E., A.C. Craig, R.E. Munns, T.D. Colmer, P.G.H. Nichols, C.V. Malcolm, E.G. Barrett-Lennard, A.J. Brown, W.S. Semple, P.M. Evans, K. Cowley, S.J. Hughes, R. Snowball, S.J. Bennett, D.G. Sweeney, B.S. Dear, and M.A. Ewing. 2005. The potential for developing fodder plants for the salt-affected areas of southern and eastern Australia: an overview. Aust. J. Exp. Agric. **45**:301–329.
8. Wegener, H.C. 2003. Antibiotics in animal feed and their role in resistance development. Curr. Opin. Microbiol. **6**:439–445.

Chapter 2
Collecting, Processing and Storage of Plant Materials for Nutritional Analysis

Jean Hanson and Salvador Fernandez-Rivera

Introduction

A solid sampling strategy for plant material is the first step in screening forages for nutritional analysis and extra-nutritional attributes to determine if potential forage species, with good adaptation and biomass production are suitable for use as livestock feeds. Since the morphological phenotype is rarely a good indicator of nutritional traits, nutritional analysis is essential when selecting plants as feeds. It is not possible to select forages based solely on biomass production without taking into account the nutritional and anti-nutritional attributes. Some species with leafy and high productivity may contain plant secondary metabolites that may be toxic and make them unsuitable for use as feeds. A good example of this is *Leucaena*, which is fast growing and yields up to 15 tons/ha of forage dry matter per year, but because of the mimosine in the leaves could initially only be fed in quantities up to 30% of the diet without causing toxicity symptoms. This was not apparent from looking at the plant and emphasizes the need to do a thorough nutritional evaluation before introducing new species as livestock feeds. However, identification of mimosine degrading rumen microbes now allows livestock to consume larger quantities [5] and makes this both a productive and nutritionally useful forage plant in many tropical livestock systems.

Sample Collection

Sampling strategies for assessment of nutritional attributes must consider plant diversity and replication. Not all plants are identical and considerable diversity occurs even within species in nutritional traits, giving the potential to select superior genotypes with both high yield and good nutritional attributes. In addition,

J. Hanson (✉)
International Livestock Research Institute, Addis Ababa, Ethiopia
e-mail: j.hanson@cgiar.org

P.E. Vercoe et al. (eds.), *In Vitro Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies*,
DOI 10.1007/978-90-481-3297-3_2,

some nutritional traits are also influenced by environment, plant age, sampling environment and time of sampling causing variation between samples from the same genotype or even within the plant. A good sampling strategy considers all these factors and aims for uniformity in sampling protocols so that environmental effects can be minimised and the true nutritional and extra-nutritional traits can be analysed. Several issues need to be taken into consideration when designing sampling strategies.

Diversity Within a Species

A large amount of diversity in nutritional traits and level and type of plant secondary metabolites has been observed within samples of genotypes from the same species whether grown at one location or collected from different geographical locations. These differences can be quite substantial and therefore it is important to account for the diversity and test samples from different genotypes within a species before drawing conclusions about its nutritional attributes. The tendency is to provide information at the species level, while in fact it would be more useful to provide this information at the variety or genotype level. A study on *Sesbania sesban* to determine influence of accession, environment and individual tree within an accession on nutritive value concluded that nitrogen, neutral detergent fibre, in vitro true digestibility, lignin content and polyphenolic compounds all differed significantly between accessions and sites [4].

Genotypic diversity is often seen within an accession of forage germplasm because sampling is either random or representative individuals showing phenotypic diversity are sampled from within the population at the time of plant collection to capture maximum diversity within the accession. Such accessions can include mixed genotypes. Some mixtures may show differences in agro-morphology while diversity in other traits may only show during laboratory analysis. The optimum way to ensure that all diversity within the accession is represented is to use large numbers of plants so that there is a high probability that genes in low frequency will be maintained [1]. However, using large numbers of plants will make sampling more time consuming and expensive and usually a balance has to be struck between capturing maximum diversity within the sample and practical issues involved in the screening programme. In order to capture diversity within the sample, it is recommended that leaf material be collected from a minimum of 10 plants and preferably 25 plants within each accession.

Physiological Age of Plants and Leaves

The chemical composition of leaves and pods of many forage types is transient owing to rapid biochemical changes occurring during the maturation process. Therefore, physiological age of the plant or plant part will often have a major effect on nutritional and extra-nutritional attributes. Nutritional quality deteriorates as the leaf to stem ratio reduces and the plant ages. Comparison of nutritional quality

among accessions should be undertaken at the same physiological age to provide meaningful data. Taking Napier grass as an example, trebling the time interval between cuts doubled yield, but halved the crude protein and leaf to stem ratio. The same is true for fodder trees where older leaves are less nutritionally useful. Genotypic differences can be clearly seen when age differences are controlled [7].

Juvenile stages tend to have higher levels of plant secondary metabolites. This ecological adaptation confers a competitive advantage when plants are young and more susceptible to grazing animals. It is well documented that polyphenolic compounds such as tannins are a common defence mechanism in plants [3]. Younger tissue on the same plant also shows differences in levels of these compounds. For example, the highest levels of alkaloids occur in young pods in lupins. Concentrations of 4-N-acetyl-2,4-diaminobutyric acid (ADAB), a toxic non-protein amino acid present in *Acacia angustissima* was tripled when ADAB was extracted from young leaves [10]. In order to make valid comparisons between plant material harvested from different plants or accessions, it is recommended to always harvest leaves of a similar physiological age from plants.

Position on the Plant

As well as age, micro-environmental differences may also result in chemical differences in leaf material depending on the leaf position on the plant. This is not very significant in small herbaceous legumes or grasses due to their size, but is relevant when considering fodder trees. This may be due to enhanced respiration or water balance in leaves in direct sunlight with elevated temperatures and light intensity compared to leaves growing in shade. Higher light intensity and temperatures are known to increase amounts of ascorbic acid in tomatoes, with the result that fruits harvested from different locations on the same vine have differing levels of ascorbic acid [8]. The same is true for other micronutrients and anti-nutritional factors, including plant secondary metabolites. Research has shown that there are significant differences in tannin content from leaves growing in shade and in direct sunshine in *Sesbania* (unpublished information). In order to ensure a representative sample, it is recommended that leaves be harvested at a similar stage of maturity from all around the plant.

Seasonality

Seasonal differences in nutritional compounds and plant secondary metabolites have been reported in several species. Many of these differences are compounded by physiological age effects, but there are also effects of environment involved in these changes. This is related to day length, temperature and amount of water available that will determine metabolism and growth rate within the plant. Studies have shown that samples of leaves of several fodder tree species with high moisture content collected during the rapid growing season showed different nutritional attributes to those collected in the dry season [9]. It is recommended that when plant sampling one should always record the sampling date and that the collection of samples

for comparative purposes should be carried out over a short time period within the same season to minimise seasonal effects when collecting leaf material for plant proximate analyses.

Methodology

Collection Method for Leaf Material for Proximate Analysis

Materials Required

- Secateurs or Cutters
- Scissors
- Strong paper bags of 80–100 g paper of size 200 × 400 mm
- Pencil, notebook and marker
- Balance (range 0–1600 g)

Procedure

1. Determine how many samples to take by observing the plants. If variable, sample more plants to obtain a representative sample of the population.

Note: A good representative sample is needed to ensure accurate results. If the plants look uniform, then randomly take samples from 10 plants per population. If the plants show variation, randomly take samples from 25 plants per population.

2. Determine how much to sample. Take approximately 6 times the weight you need for analysis/storage.

Note: Assume that plants will loose about 80–90% of their weight as water during drying. Use this as a guide to calculate the fresh weight you need to harvest to have the required amount of plant material after drying.

3. Cut leaf material of a uniform maturity stage from all sides of each plant. Cut material into small pieces with scissors or secateurs and mix well.
4. Place into a weighed and labelled paper bag. Weigh the fresh material plus bag and record the weight.

Collection Method to Freeze Dry Leaf Material for Analysis of Plant Secondary Metabolites/Bioactive Compounds

Materials Required

- Secateurs or cutters
- Scissors
- Balance (range 0–1600 g)
- Plastics bags (generally of size 150 × 300 mm)
- Marker

- Stapler
- Cooler box
- Ice flakes

Procedure

The procedure to determine number and quantity of sample, and the cutting and weighing are the same as for proximate analysis. Then:

1. Place the weighed fresh material into a labelled plastic bag and close.
2. Immediately place the bagged sample into ice in a cooler box. Transfer to a freezer (–20°C) as soon as possible for storage before freeze-drying.

Note: Work as quickly as possible to harvest the sample and place the bagged sample immediately on ice in the cold box to avoid changes in composition of extra-nutritional compounds during the sampling procedure.

Sample Processing

Results of nutritional analyses are usually reported as a percentage of dry matter. Plant samples commonly contain from 80 to 90% water and should be dried as soon as possible after sampling to reduce respiration and metabolism, and to prevent deterioration. Leaf material is most commonly air dried in a well-ventilated oven at 60°C to avoid deterioration during the drying process. Freeze-drying or lyophilization is a process where water from frozen materials is removed by converting frozen water directly to water vapour without passing the liquid phase. A vacuum is created in the drier to remove water vapour from the surface of the plant sample.

Selection of the drying method, temperature and time should be done with care to avoid substantial qualitative and quantitative changes in the nutritive and extra-nutritive attributes of samples. Many studies have been conducted to evaluate the effect of oven drying or freeze drying on the nutritional components of forages and have concluded that the drying method can have considerable effect on nutritional value. Freeze-drying usually preserves the quality of the sample and avoids heating, which can cause degradation of some nutritional attributes and inactivation of bioactive compounds. Studies with willow have shown that leaves that were put into a freeze-dryer without being prefrozen or subjected to room drying with desiccation had concentrations of most secondary compounds comparable to those found in fresh leaves [6]. Tannins may undergo oxidative polymerisation with heat, which reduces their solubility and leads to subsequently underestimation of tannin content during analysis.

Dzowela et al. [2] and Papachristou and Nastis [9] reported that oven drying at 40°C artificially increased the fibre and lignin concentration of leaves of a range of fodder trees when compared to air and freeze-drying. There was also a reduction in soluble tannins, total nitrogen and in vitro organic matter digestibility due to oven drying at 65°C in some common fodder trees [2, 11]. It is recommended

that drying temperatures should not exceed 60°C to reduce degradation changes during processing and that freeze-drying is the preferred method when assessing secondary plant compounds or for screening plants for bioactive compounds. In dry environments without access to oven drying, plant material may also be air dried when spread in a thin layer and a shady environment to avoid direct sunlight that can cause overheating and deterioration.

At this stage in the processing, when samples reach the laboratory for either oven or freeze-drying, they are usually assigned a sequential laboratory number. The details of accession number, trial entry number, replicate, collection site, plot number, plant part, maturity, date of harvest and any unique identifier provided by the collectors are usually entered into the register and/or computer file so that each sample can be linked back to its source through the laboratory number. Although some of these details appear unnecessary, it is always better to have all information that can be used to verify sample identification in any cases of errors in recording. When the collection is made from outside of the research station, it is important to have an exact record of the collection site to link the collection with environmental data. In these cases, a global positioning system (GPS) can be used to record the exact site (longitude, latitude and altitude) and the data recorded on the collection sheet and in the registry. Codes may be used for sites and full information kept in a separate code file (Table 2.1).

Table 2.1 An example of the recording system used in our laboratory

Lab no.	Unique identifier	Site code	Trial	Trial entry no.	Plot no.	Harvest date	Maturity	Plant part	Replicate

Oven Drying of Leaf Material for Proximate Analysis

Materials Required

- Pencil and notebook
- Strong paper bags of 80–100 g paper of size 200 × 400 mm
- Balance (range 0–1600 g)
- Large well ventilated or forced air oven capable of maintaining temperatures of 60°C

Procedure

1. Place the weighed and open paper bags with leaf material into a ventilated oven at 60°C for 3 days.

Note: Ensure bags are sufficiently well spaced for good air circulation to avoid uneven drying.

2. After 3 days, weigh the dry material plus bag and record the weight.
3. Calculate the dry weight of the leaf material. Percent dry matter is calculated by weight loss during oven drying:

$$\text{Percent dry matter by weight (\%w)} = (\text{weight of oven dried sample} \times 100)/(\text{weight of fresh sample})$$

Freeze Drying Plant Material for Analysis of Plant Secondary Metabolites/Bioactive Compounds

Materials Required

- Balance (range 0–1600 g)
- Sample bags
- Marker
- Stapler
- Freeze dryer

Procedure

1. A tray freeze dryer is most commonly used for drying plant samples.
2. In your notebook record the tray number where each sample will be dried.
3. Arrange the samples in thin layers for rapid drying in the numbered trays in the freeze dryer.
4. Follow the manufacturer's instructions for your freeze drier for creating the vacuum and setting the temperatures.
5. Freeze dry the material at –30 to –50°C for 60 h.
6. Turn off the freeze drier and allow the material to reach room temperature.
7. Empty each tray into a numbered sample bag, checking the sample and tray numbers carefully against the list and sample bag.

Grinding Plant Samples

After drying, most plant samples are ground to small particles to ensure homogenous samples for the analysis. Oven dried, freeze-dried and air-dried samples are all ground in the same way. A range of grinder types can be used for grinding plant samples including hammer mills; Wiley mills and cross-beater mills are all suitable machines, providing they have a range of sieves to ensure uniform particle size. Thomas-Wiley, Laboratory Mill, Model 4 mills are often used in our laboratory. The particle size of the ground material is important to ensure reproducible results in the nutritional analyses. Different analyses require samples ground to different particle size. In some cases where several analyses are carried out on the same sample, it is important to grind sub-samples to a specified size, as required for that analysis. Samples that pass through a 1 mm mesh sieve are suitable for proximate analysis while samples should not be ground through a screen smaller than 2 mm for

nylon bag degradability studies. For quantification of plant secondary metabolites, a screen size of 0.5 mm should be used.

Materials Required

- Grinder with 2, 1 and 0.5 mm sieves
- Stiff brush for cleaning the grinder
- Notepad and pencil
- Marker pen
- Sample cups or plastic bags

Procedure

1. Arrange all bags with dried samples in the order of the list and check that all samples are present.
2. Open the first bag and mix the sample well in the bag.
3. Pass the sample through a clean grinder with the required size of screen for the analysis selected.

Note: Ensure a uniform particle size and avoid fine grinding to reduce differences in analysis from coarse and fine ground samples. Where very fine particles of a 0.5 mm screen is required, it is possible to first grind the entire sample through a larger screen size of 1 mm or 2 mm. After careful mixing, a sub-sample can then be taken and ground to the smaller screen size.

4. Collect the ground sample in a labelled plastic bag or sample cup and seal to prevent absorption of moisture.
5. Clean the grinder thoroughly and carefully after each sample.

Storage of Dried Plant Samples

Dried plant samples will not deteriorate during storage for several years if stored in good storage conditions. It is important to store samples until all analysis and experiments are completed and you have verified that there is no need to repeat any laboratory work. It is common to store samples for at least 2 years and possibly longer if there is a likelihood of continuing research that requires returning to earlier samples for additional analysis. Ground leaf materials should be stored in cool, dry and dark environments in sealed containers to maintain quality during storage.

Note: Remember to make a list and arrange containers in order of the list for easy access to samples later.

Materials Required

- Balance (range 0–1600 g)
- Labels and permanent pen
- Plastic containers with airtight lids

Procedure

1. Prepare labels for inside and outside each container.
2. Pack weighed ground samples in airtight sealed and well-labelled containers.
3. Arrange in numeric order in cartons or on shelves and prepare a list of samples and storage containers so that you can easily locate samples later.
4. Store in a cool place out of direct sunlight.

References

1. Bray, R.A. 1983. Strategies for gene maintenance, pp. 157–168. In J.G. McIvor, and R.A. Bray (eds.), Genetic Resources of Forage Plants. CSIRO, Melbourne.
2. Dzowela, B.H., L. Hove, and P.L. Mafongoya. 1995. Effect of drying method on chemical composition and in vitro digestibility of multipurpose tree and shrub fodders. Trop. Grassl. **29**:263–269.
3. Harborne, J.B., and R.J. Grayer. 1994. Flavonoids and insects, pp. 589–618. In J.B. Harborne (ed.), The Flavonoids: Advances in Research Since 1986. Chapman and Hall, London.
4. Heering H., J.D. Reed, and J. Hanson. 1996. Differences in *Sesbania sesban* accessions in relation to their phenolic concentration and HPLC fingerprints. J. Sci. Food Agric. **71**(1): 92–98.
5. Jones, R.J. 1985. Leucaena toxicity and the ruminal degradation of mimosine, pp. 111–119. In A.A. Seawright, M.P. Hegarty, L.F. James, and R.F. Keeler (eds.), Plant Toxicology – Proceedings of the Australia–USA Poisonous Plants Symposium. Queensland Department of Primary Industry, Brisbane.
6. Julkunen-Tiitto, R., and S. Sorsa. 2001. Testing the effects of drying methods on willow flavonoids, tannins, and salicylates. J. Chem. Ecol. **27** (4):779–789.
7. Lyons, R.K., R. Machen, and T.D.A. Forbes. 1996. Why range forage quality changes. Texas Agricultural Extension Service Bulletin B–6036.
8. OECD. 1993. Safety evaluation of foods derived by modern biotechnology – concepts and principles, p. 74. Organization for Economic Co-operation and Development, France.
9. Papachristou, T.G., and A.S. Nastis. 1994. Changes in chemical composition and in vitro digestibility of oesophageal fistula and hand plucked forage samples due to drying method and stage of maturity, Anim. Feed Sci. Technol. **46**:87–95.
10. Reed, J.D., G. Gebremariam, C. Robinson, J. Hanson, A. Odenyo, and P.M. Treichel. 2001. Acetyl diamino butanoic acid (ADAB), a potential lathyrogenic amino acid in leaves of *Acacia angustissima*. J. Sci. Food Agric. **81**:1481–1486.
11. Stewart, J.L., F. Mould, and I. Muller-Harvey. 2000. The effect of drying treatment on the fodder quality and tannin content of two provenances of *Calliandra calothyrsus* Meissner. J. Sci. Food Agric. **80**:1461–1468.

Chapter 3
In Vitro Methods for the Primary Screening of Plant Products for Direct Activity against Ruminant Gastrointestinal Nematodes

Frank Jackson and Hervé Hoste

Introduction

Although the search for novel phytotherapeutics is an area of current research focus, man has always sought plant products in an effort to alleviate illness and infection in both humans and animals. During the latter part of the twentieth century, the emergence of the modern pharmaceutical industry and the development of a range of effective medical and veterinary treatments tended to focus attention away from these traditional resources. However the subsequent emergence of resistance amongst veterinary microbial, protozoal and metazoan pathogens, the high cost of veterinary products to resource poor farmers, and consumer interest in reducing chemical treatments in food producing animals have all served to re-awaken interest in bioactive plant products.

Because of the threat helminths pose to the health and welfare of ruminants throughout the world, anthelmintics have for more than 40 years been the chief means of controlling these debilitating diseases. However, resistance has been reported against the three current broad-spectrum anthelmintic families and in some countries multiple anthelmintic resistances is now a common phenomenon.

The search for cheap, effective and safe plant based alternatives for the control of ruminant nematodes is being conducted in many countries. Plants and their products can not only have direct effects against parasite populations resident in the gastrointestinal tract but also by improving host nutrition can also serve to enhance immunity against these parasites. The search for local forages that optimise host immuno-regulatory capacity and/or have direct antiparasitic effects is particularly relevant for resource poor farming communities who would clearly stand to benefit from the availability of "nutraceutical" plants, i.e. plants that are used first for their beneficial effects on health rather than for their nutritive value. The techniques used to study effects upon host immunity of plant products are somewhat specialised and

F. Jackson (✉)
Moredun Research Institute, Pentland Science Park, Bush Loan, Edinburgh, EH26 0PZ, UK
e-mail: Frank.Jackson@moredun.ac.uk

P.E. Vercoe et al. (eds.), *In Vitro Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies*,
DOI 10.1007/978-90-481-3297-3_3,

are not within the realms of this article which is focused on in vitro methods for screening plant products for direct antiparasitic effects.

Since it is reasonable to assume that local forages that ruminants currently consume have, at best, only modest direct effects on the hosts' parasite burden, the search for novel phytotherapeutics has naturally tended to focus on other plant species that are not currently consumed in large quantities. Given the bewildering array of plants available for testing, the first imperative is to find some way of reducing the numbers entering the screening process to a feasible level. The initial screening process to exclude known toxic plants and those which may be unsuitable on agronomic grounds or to select plants using some knowledge of their biochemistry, use in ethno-veterinary medicine or selective animal feeding behaviour is not the focus of this article, suffice to say that the best results will be achieved through collaborative efforts involving a range of specialists. If only a small number of plants are to be screened then there is little doubt that the best approach is to screen them is in vivo; feeding or administering the plant products to infected ruminants. The reasons for this are very simple; the extent of presentation of complex bioactive compounds to the intended target parasite will be influenced largely by the physical and biochemical conditions prevailing at the site of infection. Since these conditions change throughout the gut and it is invariably impossible to duplicate them under in vitro conditions, testing in the host is the best way to determine efficacy. However where large numbers of plants are being examined it is clearly not feasible to test them all in animals and researchers will need to resort to the use of in vitro techniques to provide primary screening. The two key processes involved in primary screening are:

1. Preparation of parasite material, the isolation of different pre-parasitic stages from faecal material.
2. In vitro screening, using a range of different bioassays all of which measure efficacy in comparative terms, examining the disruptive effects of a plant product on some vital biological process.

The various methods used to prepare the plant products and extracts are described in Chapter 2 and within other chapters of this book. Some pre-screening experimentation is almost inevitable to decide on an appropriate concentration ranges. There are two main reasons for this. Firstly, the way in which the whole plant or some extract from it will be used is important. If for example forage is the sole food source, for a period then it would be appropriate to test it, or products from it, at a higher concentration range than if it were only being used to provide a fraction of the daily dry matter intake. Secondly, differences in the parasite species being subject to testing will also influence the choice of concentration used in the screening process simply because of between species differences in susceptibility to the bioactive products.

Wherever possible it is useful to incorporate a series of controls. Negative control data provides the base line against which the effects of the plant product are measured. Positive control data obtained by using either chemical or known

bioactive plant products is useful for not only confirming that the bioassay is working but can also help to indicate the type of bioactive substance(s) implicated in activity. Finally, it must be remembered that primary screening using in vitro bioassays will inevitably throw up a number of positives that will, due to the very different physicochemical conditions in the gut, have little or no effect in vivo.

Preparation of Parasite Material

The preparation of clean parasite material is important since dirty preparations are difficult to count and the presence of faecal debris can interfere with the action of some plant secondary metabolites. Eggs, first and third stage larvae and adult worms recovered from post mortem material can be used in in vitro bioassays.

Mass Extraction of Nematode Eggs

Description

Nematode eggs are isolated from fresh faecal material for use in egg hatch assays, or for further culture to first stage (L_1) larvae. Faecal material is thoroughly dispersed in tap water before being passed through a series of sieves and parasite eggs are further cleaned by floatation in saturated salt solution.

Materials

- Fresh ruminant faeces
- Top pan balance
- 130 × 230 mm polythene bags
- 1 mm, 500, 212, 75 and 38 μm sieves
- Beckmann polyallomer centrifuge tubes (Cat. No. 337986)
- 15 mL polystyrene or glass centrifuge tubes (Sterilin or similar)
- Cover slips, glass slides (26 × 76 mm)
- Saturated sodium chloride solution
- Centrifuge
- Artery forceps
- Micro pipette and disposable tips (20–400 μL)
- Stereo or compound microscope fitted with a mechanical stage

Procedure

1. Collect fresh faeces directly from the donor animal's rectum into polythene bags no more than 1 h prior to extraction.
2. Add tap water and disperse the faecal material to give a smooth liquid suspension, water may be added as required.
3. Wash suspension over sieves in order; 1 mm, 500, 212 and 75 μm, collecting filtrate in a bucket or large beaker.

4. Pass the above filtrate over 38 μm sieve and collect retentate (material off the sieve). Transfer the retentate into centrifuge tubes and wash it with tap water, followed by centrifugation, as described below in steps 5–9 (polyallomer tube method) or 5a–9a (polystyrene/glass tube method)

Polyallomer Tube Method

5. Polyallomertubes are deformable semi rigid tubes that can be clamped externally to isolate the upper reaches of the suspension to isolate nematode eggs. Fill the tubes with the retentate collected at step 4 and centrifuge at 203*g* for 2 min.
6. Remove supernatant with vacuum line leaving approximately 1 mL pellet and water or alternatively tip off supernatant leaving a faecal pellet.
7. Re-suspend the pellet with saturated sodium chloride solution (specific gravity 1.2), seal the top of the tube and invert gently several times to loosen the faecal pellet.
8. Re-centrifuge at 203*g* for 2 min. Clamp tubes just below meniscus using forceps (eggs will be on top of the meniscus), pour off top layer into 250 mL beaker, wash onto 38 μm sieve and rinse thoroughly with tap water.
9. Collect retentate and resuspend with tap water and centrifuge at 203*g* for 2 min, remove supernatant with vacuum line. Steps 6–9 may be repeated to remove further debris.
10. Make volume up to 10 mL with tap water and count eggs in 100 μl of suspension by streaking this volume along the glass slide and examining using the stereo or compound microscope.

Polystyrene/Glass Tube Method

5a. Fill the tube with the retentate collected at step 4 and centrifuge at 203*g* for 2 min.
6a. Remove supernatant with a vacuum line leaving approximately 1 mL pellet and water or alternatively tip off supernatant leaving a faecal pellet.
7a. Resuspend the pellet with saturated sodium chloride solution (specific gravity 1.2), shake gently to mix and then fill the tube completely to form a positive meniscus (see Fig. 3.1). Carefully place a cover slip on top of the tube and place in the centrifuge.
8a. Centrifuge at 203*g* for 2 min, carefully remove the tube with its cover slip.
9a. Lift off the cover slip (the eggs will be held in the surface film attached to it) and wash into a beaker with tap water. Pass the contents over 38 μm sieve and rinse with tap water. Collect the retentate into a beaker.
10. Sediment contents of the beaker, and reduce the volume to 10 mL by removing upper liquid portion with a vacuum line and count the numbers of eggs in 100 μL by streaking this volume along the glass slide and examining using the stereo or compound microscope.

Clean eggs collected in this way can be used in ovicidal assays or may be used to provide first stage larvae.

Fig. 3.1 Re-suspended faecal material in a tube filled to a positive meniscus

Culture of First Stage (L_1) Larvae from Nematode Eggs

Description

Nematode eggs obtained using Method 1 are cultured at room temperature until hatched to first stage larvae, and then filtered using a Baermann apparatus to remove debris and unhatched eggs.

Materials

- Nematode egg suspension
- 10 cm plastic Petri dishes (Sterilin Ltd., or similar)
- Baermann apparatus and filter collar (made from plastic tubing or similar material plus plastic collar, with elastic band to hold filter material in place) (see Fig. 3.4 for general structure of Baermann apparatus)
- Suitable high wet strength filter paper such as Cottom Bottoms nappy liners
- (Boots Ltd., UK) or 20 μm nylon mesh (Nytal, Sefar Ltd or similar)
- 250 mL Beaker (Nalgene) or similar to support filter collar
- Glass slides (26 × 76 mm)
- Micro pipette and disposable tips (20–400 μL)
- Stereo or compound microscope fitted with a mechanical stage

Procedure

1. Place freshly extracted egg suspension into a suitable culture vessel. This should ideally have a large liquid surface area to allow sufficient gas exchange for the eggs to hatch.
2. Incubate at room temperature or in an incubator should the ambient room temperature is likely to fall below 10°C for 24 h.

3. Examine the culture microscopically to ensure hatching has occurred.
4. Prepare the Baermann filter; the filter consists of a 22 mm diameter plastic tube about 5 cm long 20-μm nytal mesh has been glued over one end of the tube.
5. Fill the beaker with tepid water (22°C).
6. Pour the eggs and larvae onto the mesh of the Baermannn apparatus, ensuring that the sample is distributed evenly over the mesh.
7. Immerse the Baermann apparatus in the warm tap water in the beaker.
8. Allow 1 h for the larvae to migrate through the fine mesh, and then carefully remove the filter collar. Allow the larval suspension in the beaker to settle and then reduce the volume using a vacuum line or centrifugally.
9. Count the larvae present by examining microscopically a small sub-sample taken with a pipette (100 μL) by streaking this volume along a glass slide and counting on a stereo or compound microscope fitted with a mechanical stage.

First stage larvae obtained in this way can be used in larvicidal assays or those that measure the disturbance of normal behavioural activity such as the larval feeding inhibition assay.

Culture of Third Stage (L3) Nematode Eggs from Sheep Faeces

Description

Faeces from infected sheep are incubated to allow nematode eggs to hatch and develop into third stage larvae [12]. Faeces are flooded with water until larvae migrate out of pellets, and then the resulting suspension is cleaned using a Baermann filter.

Materials

- Faeces from monospecifically infected donor animal
- Culture trays
- Polythene bags
- 22°C incubator
- Sieve – approximately 1 mm pore size
- Glass slides (26 × 76 mm)
- Baermann apparatus (20 cm diameter 5 cm long plastic tube with two layers of high wet strength paper held over one end by a rubber band)
- Micro pipette and disposable tips (20–400 μL)
- Stereo or compound microscope fitted with a mechanical stage.

Procedure

1. Place faeces collected from infected donor animal in a culture tray to a maximum depth of 30 mm and seal tray inside polythene bag. If the faeces are very loose (diarrhoeic), then it may be necessary to add vermiculite or washed peat or

charcoal to provide better culture conditions. Sufficient material needs to be added to enable the mixture to be formed into balls. Puncture bag to allow airflow.
2. Incubate tray at 22°C for 7 days.
3. Flood tray with tepid tap water (22°C). Allow to soak for 1 h.
4. Sieve fluid through a 1.0 mm sieve and collect filtrate.
5. Sediment filtrate for 2 h at 4°C. Reduce volume using a vacuum line and filter using Baermann apparatus as described for L1 culture. Make the larval suspension up to a suitable volume and count the numbers of larvae present in 100-μL sub sample taken with a micropipette and streaking the sub sample along a glass slide and counting on a stereo or compound microscope fitted with a mechanical stage. Calculate the total numbers of larvae present.
6. Larvae may be stored at 4–10°C until required.

Infective larvae may be used in larvicidal assays or behavioural assays such as the larval migration inhibition assay, which measures the effect of a test substance on locomotion.

Larval Identification

Where field (mixed species) infections are used to provide material for the tests, it can be useful to determine the prevalence of the different species simply because there can be large interspecies differences in the sensitivity of nematodes to bioactive substances.

The following references are the most appropriate sources of information for identification (speciation) of ruminant nematodes based on the morphology of third stage larvae [12, 17].

Isolation of Adult Nematodes

Description

Adult worms are isolated from gastrointestinal contents by combining the contents with agar and soaking mixture in water. Worms will migrate from the agar slab and can be collected from the water. Where only small numbers of adult worms are required they can be recovered directly by using a stereo microscope to examine the washings/contents collected from the abomasa, small intestines or large intestines.

Materials

- Abomasum or small or large intestine from freshly killed animal
- Physiological saline (0.85% w/v NaCl solution)
- Technical grade agar (Sigma Aldrich Ltd, catalogue number -A7002)
- Baermann mesh apparatus and lid (see Figs. 3.2 and 3.4)

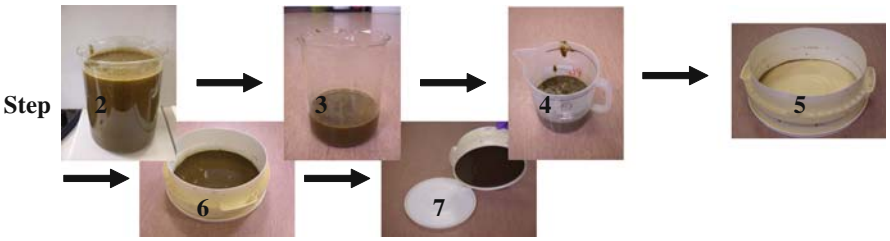

Fig. 3.2 Steps 2–7 of the isolation of adult nematodes

- 10 L Funnel fitted with tap at base
- Micro pipette (200–1000 μL) and tips
- Stereo microscope

Procedure

1. Heat two batches of saline to 56°C and maintain at that temperature.
2. Collect and cut open gut sample, empty contents into 10 L bucket containing 5 L saline kept at 39°C, wash gut surface in further 1 L saline, pool the contents.
3. Sediment and remove the liquid portion to adjust volume.
4. Dissolve 1.8 g agar per 100 mL saline (56°C), allowing a total volume of 500 mL per sample. Add the 500–200 mL of digesta stirring continuously throughout the mixing process. Cool it to 45°C.
5. The Baermann mesh apparatus consists of the upper portion of a bucket that has a snap on lid. A 1 mm mesh support is fitted within the apparatus next to where the lid is attached. Carefully fit the lid and place lid down onto a cool surface (approximately 10°C).
6. Pour the agar-digesta mix over mesh and allow to set for 15 min. The lid of the apparatus ensures that the mix does not leak out of the apparatus during the period when the agar/digesta mix solidifies.
7. Remove lid from Baermann apparatus and place the apparatus in a Baermann funnel filled with warm 0.85% saline and maintained at 37–39°C. The Baermann funnel should have a wide bore (4 mm internal diameter) tap fitted to enable the collection of worms migrating out of the agar bed.
8. Add saline to ensure contact between saline and agar slab.
9. Incubate for 4 h at 37°C (or 39°C), remove any worms from top surface using forceps, draw off the remaining worms using the tap fitted at the bottom of the funnel, concentrate the adult worms and store in 0.85% saline at 37°C (or 39°C), until required. Adjust to a suitable volume (between 100 mL for small numbers of worms and 1000 mL for large numbers of worms. Take a 1 mL sub sample (remember to clip the tip off the pipette tip to give an internal diameter of about 4 mm to allow the adult worms to be sucked into the tip) and count the numbers of worms in 1 mL using the stereo microscope.

Adult worms can be used in adulticide trials or those measuring disruption in locomotory behaviour because of exposure to plant material.

In Vitro Screening

A number of different bioassays using either the pre-parasitic stages of nematodes or adult nematodes can be used to screen plant extracts. A number of plant products and plant secondary metabolites have been shown to have direct anthelmintic effects in these assays. These include condensed tannins, saponins, flavonols, lectins and various proteases. However, since most of these assays operate best within a narrow pH range around pH 7 it cannot be assumed that activity observed in vitro will be seen under the very different conditions that exist in vivo.

The principal assays and their target stages are shown in Table 3.1 below. Wherever possible it is useful to incorporate positive controls using an appropriate anthelmintic (a compound or forage) to confirm that the assay is working correctly.

Although all of these assays can be used for primary screening, the simple and robust assays such as the EHT, LMIT and LFIT have tended to be used in the first instance. The more time consuming or expensive assays such as the LEA, LDT and AMT are generally used with products that are known to have some efficacy. Inevitably, laboratories may need to adapt the methodologies described here to suit to their facilities and budgets.

Table 3.1 Bioassays used to test plant extracts

Bioassay	Target stage(s)	Process disrupted	References
Egg hatch (EHT)	Eggs	Hatching to L1	[6, 10, 11]
Larval migration inhibition (LMIT)	L_3	Locomotion of L3	[4, 8, 15]
Larval feeding inhibition (LFIT)	L_1	Feeding of L1	[1, 7]
Larval exsheathment (LEA)	L_3	Exsheathment of L3 stage	[3]
Larval development (LDT)	Eggs $\rightarrow$ L_3	Development to L3	[2, 5, 9, 16, 18]
Adult motility (AMT)	L_5/adults	Motility (viability) of worms	[13, 14]

Preparation of Stock Solutions of Plant Material

Description

Since plant material for use in the bioassays can come in a variety of different forms including whole plants, freeze dried, finely ground (milled) material, semi or fully purified (following solvent extraction) it is not possible to provide details of the full range of stock solutions that might be required.

For simplicity, the stock solution concentrations mentioned with the following assays are those based around a possible 10% inclusion rate of whole plant material and represent a concentration range that might be expected to occur in

the gut under those circumstances. Again for simplicity the stock solutions are also aqueous solutions, not based on the use of organic solvents and represent a range of concentrations that are effective against the drug sensitive populations of *Teladorsagia, Haemonchus* and *Trichostrongylus* maintained at Moredun Research Institute (Pentland Science Park, Edinburgh, Scotland, EH26 0PZ), or in UMR 1225 INRA DGER (Ecole Nationale Veterinaire de Toulouse 23 Chemin des Capelles, 31076 Toulouse France).

The use of a phosphate buffer saline (PBS; 0.1 M phosphate containing 0.05 M NaCl, pH 7.2) to prepare the stock solution might also be recommended to avoid any non specific effects due to acidic or basic pH sometimes related to dilution of plant extracts.

Using ruminal fluids collected from animals being fed bioactive forages has also been proposed as a means of mimicking in vivo conditions. However, due to the difficulties of maintaining, defining and standardizing this medium, this approach has not found favour in many laboratories.

Materials

- Top pan balance
- 10 mL volumetric flask
- 0.2 μm syringe filters
- Micro pipettes and tips
- Bench top micro centrifuge
- 2 mL eppendorf tubes
- Plastic universal tubes

Preparation of a 10 mg/mL Aqueous Plant Material Stock Solution

1. Weigh 0.3 g of freeze dried finely milled plant product into a 10 mL volumetric flask.
2. Add distilled water to the required level and mix with a vortex mixer for 2 min.
3. Pipette the suspension into labelled 2 mL eppendorf tubes.
4. Microfuge the sample for 3 min at 18,000*g*. Transfer the supernatant to a syringe and pass through a 0.20 μm syringe filter into a plastic universal bottle.
5. Serially dilute the stock solution in PBS or distilled water as required to produce working solutions of 10, 5, 2.5, 1.25 mg/mL, etc.
6. Maintain these stock solutions at 4°C and use within 24 h.

Egg Hatch Test

Description

Freshly extracted eggs are incubated in varying concentrations of thiabendazole for 48 hr, after which they are fixed and stained and the numbers of hatched larvae and

unhatched eggs are counted [6, 10, 11]. The concentration of extracts that is required to inhibit 50% of the eggs from hatching (ED_{50}) is calculated to determine the ED_{50}. Ideally, the assay should incorporate a solvent control (solvent used for preparing plant extract) to confirm that the solvent has no effect on the bioassay and a positive control (thiabendazole) to confirm the bioassay is working. The solvent control well should contain the same amount of solvent that is present in the highest plant product concentration. All of the test and control wells should be run in duplicate.

Materials

- Fresh nematode egg suspension
- 15 mg/mL plant extract stock solution in PBS or distilled water
- 1000 μg/mL thiabendazole (TBZ) stock solution
- 1000 μg TBZ/mL of di-methyl sulphoxide [DMSO]
- 10 mL volumetric flasks
- 24 well culture plate
- Helminthological iodine (50 g iodine and 250 g potassium iodide in 500 mL distilled water)
- Micro pipettes (200–1000, 40–200, 1–10 μL) and disposable tips

Procedure

1. Prepare the 15 mg/mL stock solution of plant material as described in the previous section. Prepare working solutions of 15, 10, 5, 1 and 0.1 mg/mL in distilled water. When used in the assay these will give final concentrations of 14.25, 9.5, 4.75, and 0.95 mg/mL.
2. Add 10 μl of the TBZ stock solution (1000 μg/mL) to each positive control well (in a total volume of 2 mL this will give a final concentration of 5.0 μg/mL of TBZ, sufficient to prevent hatching of nematode eggs).
3. Count eggs present in suspension and adjust volume to provide a concentration of 1000 eggs/mL.
4. Add 100 μl of the egg suspension to each well, ensuring that the suspension is thoroughly mixed prior to every aliquot being taken as the eggs will settle very rapidly.
5. Add 1900 μl of working plant stock solution to the wells to give a total volume of 2000 μl. Mix by pipetting in and out several times. For the control wells simply use 1900 μl of distilled water. Both the test and control wells should be run in duplicate.
6. Place the culture plate in a sealed polythene container on some wet paper towel to ensure a high relative humidity and incubate at 25°C for 48 h.
7. Following incubation, add 50 μl of helminthological iodine to each well. This both fixes and stains the nematode egg and larvae.
8. Count the number of eggs and first stage larvae using an inverted or stereo microscope (Table 3.2).

Table 3.2 Raw data and ED_{50} estimates generated using Genstat on the egg hatch test data

Plant concentration (mg/mL)	Replicate 1			Replicate 2			
	Eggs	Larvae	% Hatch	Eggs	Larvae	% Hatch	Average hatch
0	2	95	98	1	99	99	98
0.95	3	92	97	6	95	94	95
4.75	21	75	78	25	83	77	77
9.5	75	28	27	72	34	32	30
14.25	98	1	1	93	2	2	2

$ED_{50} = 7.386 \pm 0.2072$ (estimate $\pm$ s.e.m)

9. Calculate mean numbers of eggs and larvae at each concentration and the percentage hatch using the formula:

   $$\text{Percentage hatch} = [(\text{numbers of larvae})/(\text{number of eggs} + \text{numbers of larvae})] \times 100$$

10. Plot the percentage hatch at each concentration on a graph (Fig. 3.3), and using a suitable statistical technique such as probit analysis calculate the ED_{50} estimate – the concentration at which 50% of the eggs fail to hatch.

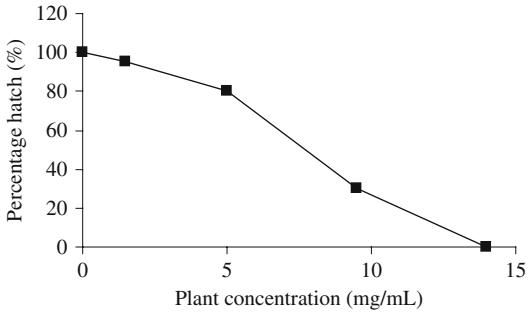

Fig. 3.3 Graphical representation of egg hatch test results

Larval Migration Inhibition Test

Description

The larval migration inhibition assay measures effects upon locomotion [4, 8, 15]. Ensheathed or exsheathed third stage larvae can be used. They are incubated in a range of concentrations of the test substance and then their capacity to migrate through 20-μm nylon mesh is determined. The anthelmintic levamisole can be used to provide positive control data. Although the technique as described uses multiwell plates and requires an inverted stereo microscope, it is also possible to use centrifuge tubes to collect migrating larvae and to subsequently determine larval numbers using a standard stereo microscope.

Materials

- 15 mL centrifuge tube
- Sodium hypochlorite solution (2% w/v)
- Filter collars to fit wells of 24 well cluster plates
- 20 μm nylon mesh (Nytal, Sefar Ltd or similar)
- 24 well culture plates
- Levamisole stock solution (1000 μg/mL distilled water)
- Micro pipette and disposable tips (20–200 μL)
- Helminthological iodine (50 g iodine and 250 g potassium iodide in 500 mL distilled water)
- Inverted stereo microscope

Procedures

Rapid Exsheathment of infective nematode larvae

1. Dispense 10 mL larval suspension into centrifuge tube and add 1 mL 2% sodium hypochlorite solution.
2. Remove 100 μL aliquot and place onto a microscope slide. Examine slide under microscope and watch while larvae shed their protective sheath.
3. When all the larvae have exsheathed, centrifuge at 203*g* for 2 min and remove supernatant. Wash the larvae by resuspending them in tap water and centrifuging. Repeat washings and centrifuging two more times to remove sodium hypochlorite solution.

Larval migration inhibition assay

1. Fit mesh to collars as per Fig. 3.4.
2. For the negative control wells, use distilled water and for the positive control wells use levamisole at a final concentration of 40 μg/mL of solution in the well.
3. Count the exsheathed larvae and adjust the concentration to approximately 1000 larvae/mL.
4. Dispense 100 μl of the larval suspension (approximately 100 larvae) into eppendorf tubes and add 1 mL of each of the working plant stock solutions (40, 20, 10 and 5 mg/mL). Incubate for 2 h at 37°C.
5. Centrifuge at 3000*g* for 2 min and remove the supernatant, reducing the volume to approximately 200 μl.
6. Add 1800 μl of each dilution of the plant extract to the appropriate wells on the culture plate and place a filter into the well ensuring that the mesh is fully submerged and that there are no air bubbles trapped beneath the mesh.
7. Mix each larval suspension thoroughly and add 200 μl to the matching filter.
8. Place a cover over the plates and incubate for 2 h at 37°C.

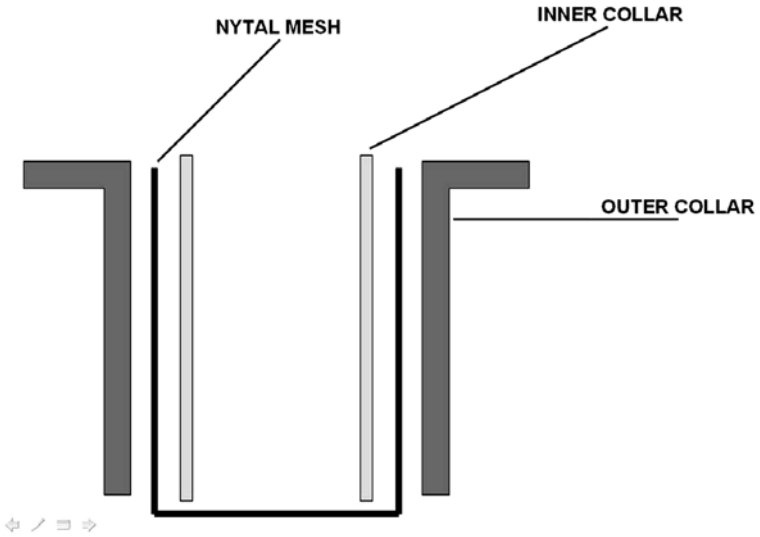

Fig. 3.4 Illustration of the fitting of the mesh and collar to the well

9. Carefully remove the filters and wash any remaining larvae into individual labelled petri dishes and stain with a few drops of helminthological iodine.
10. Add 50 μL of helminthological iodine to each well on the culture plate.
11. Using an inverted microscope at × 100 magnification, count the number of larvae in each well and in each Petri dish.
12. Calculate the percentage migration for each concentration using the standard formula:

$$\text{Percentage migration} = (\text{Nm}) \times 100/(\text{Nm} + \text{Nr})$$

Where: Nm = number of larvae migrating through mesh (i.e. found in the well) Nr = number of larvae retained by the mesh (i.e. washed off from mesh).

13. Plot a graph of drug concentration against percentage migration and using the data in probit analysis calculate the LM50 value (concentration at which 50% of larvae fail to migrate).

Larval Feeding Inhibition Assay (LFIA)

This assay determines the effect of plant products on the feeding behaviour of first stage larvae [1, 7]. First stage larvae that have been exposed to different concentrations of the test (plant) product are subsequently offered lyophilised *Escherichia coli* that are labelled with fluorescein isothiocyanate (FITC). Larvae that have fed can be readily identified using an inverted fluorescence microscope by the presence of the labelled *E. coli* in their gut.

Materials

- Lyophilised *E. coli*
- Fluorescein isothiocyanate FITC
- Bicarbonate buffer (pH 9.6)
- Phosphate Buffered Saline (PBS)
- Inverted fluorescence microscope (Blue Filter 475–490 nm)

Procedures

Fluorescein isothiocyanate (FITC) labelling of lyophilised *Escherichia coli* for use in larval feeding inhibition assay (LFIA)

1. Incubate 1 mL of concentrated *E. coli* (2250 μg *E. coli* in 1 mL bicarbonate buffer containing 1 mg of FITC) in a 2 mL microcentrifuge tube at 20°C for 2 h.
2. Centrifuge *E. coli* suspension at 18,000*g* for 2 min.
3. Remove supernatant using a vacuum line. Re-suspend *E. coli* pellet in 1 mL of PBS.
4. Repeat steps 2 and 3 twice.
5. Re-suspend *E. coli* in 1 mL of PBS.
6. Aliquot in 500 μL portions and store at –20°C for subsequent use.

Larval Feeding Inhibition Assay (LFIA)

1. Add 100 first stage larvae in 100 μL of distilled water to 500 μL of the plant extract (at 10, 5, 2.5, 1.25 mg/mL in distilled water) and mix thoroughly with a further with 900 μL of distilled water. For negative control add 100 μL of the larval suspension to 1400 μL of distilled water and for the positive ivermectin controls add 100 μL of the larval suspension to 1390 μL of distilled water and 10 μL of 1000 μg/mL ivermectin solution.
2. Incubate tubes horizontally at 25°C for 2 h.
3. Add 10 μL of FITC labelled E. coli and incubate tubes horizontally for a further 18 h at 25°C.
4. Microcentrifuge tubes at 3000*g* for 20 s to "pellet" worms, remove 750 μL of supernatant.
5. Examine larvae from the pellet under inverted fluorescence microscope fitted with blue filter.
6. Determine the number of feeding/fed and non-feeding/unfed larvae at each test concentration (Fig. 3.5).
7. Calculate mean numbers of eggs and larvae at each concentration and the percentage hatched using the formula:

$$\text{Percentage feeding} = \text{(Number of fed larvae)}100/\text{(Number of fed and unfed larvae)}$$

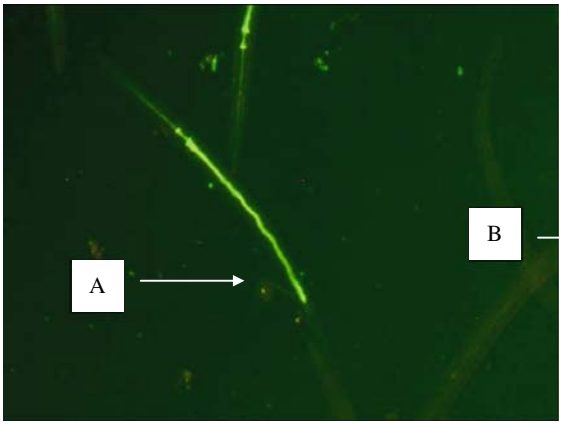

Fig. 3.5 First stage larvae that have fed on FITC labelled *E. coli* (**a**) and those that have been unable to feed (**b**) as seen under a fluorescence microscope

Plot the percentage feeding at each concentration on a graph and calculate the LF_{50} value (concentration of compound at which 50% of the larvae fail to feed).

Larval Exsheathment Assay (LEA)

This test uses infective third stage larvae in a two-stage process aimed at examining the effect of the test plant product on larval exsheathment induced by a diluted sodium hypochlorite solution. This is an adaptation of the rapid exsheathment process described previously [3]. The aim is to obtain a progressive exsheathment of the larvae, making microscopic observation of the process feasible. Examples of ensheathed and exsheathed larvae at × 100 magnification are provided in Figs. 3.6 and 3.7 respectively. For control, a 100% exsheathment after 60–70 min is sought.

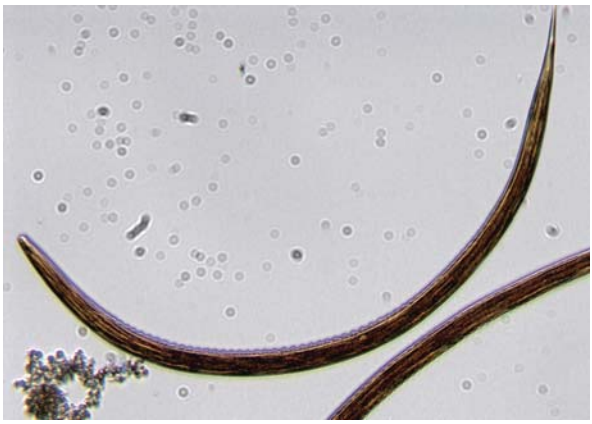

Fig. 3.6 Ensheathed third stage larva (× 100 magnification)

Fig. 3.7 Exsheathed third stage larva (× 100)

Negative controls of PBS and extracts of rye grass (or some other forage that is known to be non-bioactive) are incorporated into the assay.

Because the rate of exsheathment varies with the parasite species and/or strains, it is important in a preliminary step, to undertake a preliminary test on control larvae, to determine the best dilution to apply for the exsheathment fluid in order to obtain a gradual exsheathment process within a 60-min time interval. For this, the step 2 of the procedure described herein is applied with a range of concentrations of exsheathment fluid, usually from 1 in 100 to 1 in 500.

Materials

- Infective larvae are harvested from monospecific cultures in the manner described previously
- PBS (0.1 M phosphate containing 0.05 M NaCl, pH 7.2)
- 2% w/v sodium hypochlorite solution
- 16.5% w/v sodium chloride solution
- 24-well multiwell plates

Procedure

1. Pre-incubate 1500 ensheathed L3 larvae in PBS at pH 7.2 with the test product (600 μg/mL) or rye grass extract (600 μg/mL) or PBS (pH 7.2) at 22°C for 3 h.
2. Examine the larvae and if no exsheathment has occurred then move to the second stage of the procedure.
3. Wash the larvae centrifugally 3 times using PBS (pH 7.2) and reduce volume so that 100 μL contains 100 ensheathed L3 larvae. Dispense the larvae (100 μL) into the wells using 6 replicates per plant product. Also, use larvae that have been exposed to PBS alone or rye grass extract (600 μg/mL) to provide negative controls

4. Dilute the 2% w/v hypochlorite solution and the 16.5% w/v sodium chloride solutions 1 in 300 with PBS (pH 7.2) add 1900 μL of this mixture to each well that contains 100 ensheathed larvae in 100 μL.
5. Observe the rate of exsheathment by examining material from the wells at × 200 magnification at 10, 20, 30, 40, 50 and 60 min after adding the exsheathment fluid.
6. Calculate the mean exsheathment rate for the test substances and analyse differences using an appropriate statistical technique such as GLM.

Larval Development Assay (LDA)

This test uses fresh eggs like the egg hatch assay, but is carried out over a longer period. It measures the ability of the parasite to hatch and develop to the infective third stage larva in the presence of the test substance [2, 5, 9, 16, 18]. In this assay, it is possible to test for activity of plant products against eggs and developing larva. Dilute 1000 μg/mL stock anthelmintic solution with distilled water to produce test concentrations. Pure anthelmintics such as thiabendazole (TBZ), ivermectin and levamisole may be used in this assay to provide positive controls. Final concentrations in the control wells should be 2.5 μg/mL for TBZ and 20 μg/mL for Levamisole and Ivermectin. Since the technique involves a 7-day incubation period, which increases the risk of bacterial or fungal "overgrowths", it is necessary to incorporate antibiotics and antifungals in the assay. Although the technique has clear advantages, the long incubation period has meant that fewer laboratories have used the assay as the primary screen for activity in vitro.

Materials

- Fresh nematode egg suspension
- Phosphate buffered saline (PBS) solution
- 5 mg/mL Amphotericin B in PBS
- 30,000 U/mL Nystatin in distilled water
- Streptopen solution (10,000 μg streptomycin 10,000 units penicillin per mL PBS)
- Earls balanced salt solution (EBSS)
- 1 g/90 mL yeast extract in 0.85% (w/v) saline solution
- Helminthological iodine (250 g Potassium iodide and 50 g Iodine in 500 mL water)
- Sealed plastic humidity chamber

Procedure

1. Harvest eggs from faeces as described above. Incubate eggs overnight in the antifungal and antibiotic solution made up with PBS and 2% Nystatin, Amphotericin and Streptopen solutions. Following the overnight incubation, centrifuge in a

microcentrifuge at 18,000*g* for 2 min, then remove supernatant and resuspend the eggs in sterile distilled water. Repeat the washing step using sterile distilled water and centrifugation three times.
2. Count ten aliquots to determine the concentration of eggs. Adjust volume with sterile distilled water to give a concentration of 100-eggs/60 μL.
3. Add 20 μL of yeast extract suspension, 20 μL of lyophilised *E. coli* and 60 μL of egg suspension to each well of microtitre plate. Add 20 μL streptopen, 20 μL amphotericine B and 10 μL nystatin to each well. Fill each of the outer wells of the microtitre plate with 200 μL distilled water and seal using the microtitre plate lid.
4. Incubate at 22°C for 24 h and then add plant product or anthelmintic to duplicate wells. The control wells should incorporate DMSO at the same concentration that it is present in the positive control (anthelmintic) test wells.
5. Re-cover the microtitre plate with a lid and seal its edges with PVC tape. Incubate in a 100% relative humidity chamber at 22°C for 7 days.
6. Add 10 μL of helminthological iodine to each well and count the numbers of L_3 larvae at × 40 using an inverted microscope. The mean larval development for each drug concentration is calculated using the standard formula:
7. Larval development = (Number of live L3/total number added to wells with anthelmintic)]/[(Number of live L3/total number added to control tubes)
8. Plot the percentage larval development at each concentration on a graph and calculate the LC_{50} value (concentration at which 50% of the parasites fail to develop) using an appropriate statistical method such as probit analysis.

Adult Motility Assay (AMA)

This test uses freshly collected adult worms that are maintained in a culture medium or in the medium containing the test plant product for several days [13, 14]. Activity of the worms is scored over the period by counting the numbers of active worms at regular times throughout the assay. Paralysing anthelmintics such as ivermectin or levamisole can be used to provide positive control material for the assay. Negative controls (i.e. worms in PBS) are also included.

Materials

- Freshly collected adult worms
- Plant product dilutions (suggested range 75–1200 μg/mL in distilled water). Phosphate buffered saline (PBS)-antibiotic solution (with 4% w/v penicillin and 4% streptomycin)
- 2% w/v Levamisole solution for positive control
- 24 (for *Haemonchus*) or 48 (for *Teladorsagia* and *Trichostrongylus*) multiwell
- Plates
- Inverted microscope or stereomicroscope.

Procedure

1. Add 1 mL of the plant product in the PBS antibiotic solution to each of three wells.
2. For the 3 negative control wells, use 1 mL of the PBS/antibiotic solution.
3. For the 3 positive control wells, add 1 mL of a 1% w/v levamisole solution made in the PBS-antibiotic solution.
4. Count adult worms into each of the wells for large worms such as *Haemonchus* and then use about 2–3 worms per well and increase the numbers for smaller worms (e.g. about 4–6 for adult *Teladorsagia* and adult *Trichostrongylus*). If too many worms are used in the assay, it becomes difficult to make accurate observations.
5. Observe and score the activity of the adult worms after 6, 24, 48 or 72 h of incubation at 37°C, 100% relative humidity in a 5% CO_2/air mixture. In general, the survival of control *Haemonchus* is less (48 h) than for *Trichostrongylus* or *Teladorsagia* (72 h).
6. Change the incubation medium every 24 h. Medium with the plant concentration can be prepared at the start of the assay and stored at 4°C but the solution have to be placed at 37°C before the changes.
7. For each time point, calculate a motility index based on the numbers of immobile worms divided by the total number of worms in the well.
8. Analyse the survival curve, taking into account the different concentrations, using a suitable non-parametric test such as the stratified Cox regression test run using suitable software.

References

1. Alvarez-Sanchez, M.A., J.P. Garcia, D. Bartley, F. Jackson, and F.A. Rojo-Vazquez. 2005. The larval feeding inhibition assay for the diagnosis of nematode anthelmintic resistance. Exp. Parasitol. **110**:56–61.
2. Amarante, A.F.T., W.E. Pomroy, W.A.G Charleston, D.M. Leathwick, and M.T.T. Tornero. 1997. Evaluation of a larval development assay for the detection of anthelmintic resistance in *Ostertagia circumcincta*. Int. J. Parasitol. **27**:305–311.
3. Bahuaud, D., C.M.O. De Montellano, , S. Chauveau, F. Prevot, F. Torres-Acosta, , I. Fouraste, and H. Hoste. 2006. Effects of four tanniferous plant extracts on the in vitro exsheathment of third-stage larvae of parasitic nematodes. Parasitology. **132**:545–554.
4. Barrau, E., N. Fabre, I. Fouraste, and H. Hoste. 2005. Effect of bioactive compounds from Sainfoin (*Onobrychis viciifolia* Scop.) on the in vitro larval migration of *Haemonchus contortus*: role of tannins and flavonol glycosides. Parasitology. **131**:531–538.
5. Coles, G.C. and K.G. Simpkin. 1977. Resistance of nematode eggs to the ovicidal activity of benzimidazoles. Res. Vet. Sci. **22**:386–387.
6. Coles, G.C., C. Bauer, F.H.M. Borgsteede, S. Geerts, T.R. Klei, M.A. Taylor and P.J. Waller. 1992. World-Association-For-The-Advancement-Of-Veterinary-Parasitology (WAAVP): methods for the detection of anthelmintic resistance In nematodes of veterinary importance. Vet. Parasitol. **44**:35–44.
7. Geary, T.G., S.M. Sims, E.M. Thomas, L. Vanover, J.P. Davis, C.A. Winterrowd, R.D. Klein, N.F.H. Ho, and D.P. Thompson. 1993. *Haemonchus-contortus* – ivermectin-induced paralysis of the pharynx. Exp. Parasitol. **77**:88–96.

8. Gill, J.H., J.M. Redwin, J.A. van Wyk, and E. Lacey. 1991. Detection of resistance to ivermectin in *Haemonchus-contortus*. Int. J. Parasitol. **21**:771–776.
9. Hubert, J. and D. Kerboeuf. 1992. A microlarval development assay for the detection of anthelmintic resistance in sheep nematodes. Vet. Rec. **130**:442–446.
10. Hunt, K.R. and M.A. Taylor. 1989. Use of the egg hatch assay on sheep faecal samples for the detection of benzimidazole resistant nematodes. Vet. Rec. **125**:153–154.
11. Le Jambre, L.F., W.H. Southcott, and K.M. Dash. 1976. Resistance of selected lines of *Haemonchus contortus* to thiabendazole, morantel tartrate and levamisole. Int. J. Parasitol. **6**:217–222.
12. Ministry of Agriculture, Fisheries and Food. 1986. Manual of Veterinary Parasitological Laboratory Techniques, Her Majesty's Stationery Office, London. Edition 3.
13. O'Grady, J. and A.C. Kotze. 2004. *Haemonchus contortus*: in vitro drug screening assays with the adult life stage. Exp. Parasitol. **106** (3–4):164–172.
14. Paolini, V., I. Fouraste, and H. Hoste. 2004. In vitro effects of three woody plant and sainfoin on third-stage larvae and adult worms of three gastrointestinal nematodes. Parasitology. **129**:69–77.
15. Rabel, B., R. McGregor, and P.G.C. Douch. 1994. Improved bioassay for estimation of inhibitory effects of ovine gastrointestinal mucus and anthelmintics on nematode larval migration. Int. J. Parasitol. **24**:671–676
16. Taylor, M.A. 1990. A larval development test for the detection of anthelmintic resistance in nematodes of sheep. Res. Vet. Sci. **49**:198–202.
17. Van Wyk, J.A., J. Cabaret, and L.M. Michael. 2004. Morphological identification of nematode larvae of small ruminants and cattle simplified. Vet. Parasitol. **119**:277–306.
18. Varady, M. and J. Corba. 1999. Comparison of six in vitro tests in determining benzimidazole and levamisole resistance in *Haemonchus contortus* and *Ostertagia circumcincta* of sheep. Vet. Parasitol. **80**:239–249.

Chapter 4
Assessing Antiprotozoal Agents

C. Jamie Newbold

Introduction

Nitrogen metabolism in the rumen affects both the efficiency of ruminant production and the environmental impact of excreta from ruminant livestock production. Inefficient N retention by rumen microorganisms is compensated in production terms by feeding excessive amounts of dietary protein to the animal to meet required output levels. This leads directly to the excretion of N-rich wastes. Microbial protein synthesis in the rumen is the major source of amino acids entering the small intestine and available for absorption in ruminants [1, 4]. However, microbial protein turnover in the rumen may result in the net microbial protein outflow being less than half the total protein synthesised [9]. In vitro studies suggest that engulfment and digestion of bacteria by protozoa is by far the most important cause of microbial protein turnover in the rumen, with autolysis, other lytic factors and endogenous proteolysis being of minor importance [11]. Thus, it is apparent that removing ciliate protozoa from the rumen (defaunating) should avoid the recycling of nitrogen between bacteria and protozoa and thereby increase the efficiency of nitrogen metabolism in the rumen and stimulate the flow of microbial protein from the rumen [12]. A variety of techniques to remove protozoa from the rumen has been tested experimentally, but none is used routinely, because of toxicity problems, either to the rest of the rumen microbial population or to the host animal [12]. Recently, there has been an increased interest in plant secondary metabolites for use as possible defaunating agents. Here we describe three methods for screening plant material for antiprotozoal activity.

C. J. Newbold (✉)
Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Llanbadarn, Aberystwyth, SY23 3AL, UK
e-mail: cjn@aber.ac.uk

P.E. Vercoe et al. (eds.), *In Vitro Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies*,
DOI 10.1007/978-90-481-3297-3_4,

Screening of Antiprotozoal Agents Based on Their Ability to Inhibit the Breakdown of ^{14}C-Leucine-Labelled *Selenomonas ruminantium*

As noted above engulfment and digestion of bacteria by protozoa is responsible for the majority of bacterial protein breakdown in the rumen. Thus it has been suggested that it should be possible to screen antiprotozoal agents based on their ability to inhibit the breakdown of ^{14}C-leucine-labelled *Selenomonas ruminantium* as described by Wallace and McPherson [11], Newbold et al. [8] and Teferedegne et al. [10].

Basis of the Method

The cellular proteins of the rumen bacteria *S. ruminantium* are labelled by growth in a ^{14}C-leucine enriched media. The labelled bacteria are incubated in mixed rumen fluid in the presence of an excess of ^{12}C-leucine and the release of ^{14}C TCA soluble material during the incubation reflects degradation of the labelled bacteria and hence protozoal activity.

Preparation of Labelled Bacteria

1. *S. ruminantium* can be obtained from the American Type Culture Collection (ATCC) (www.lgcpromochem-atcc.com).
2. Cultures are easily maintained in modified Hobson, medium no. 2 (M8, Table 4.1) [5] under anaerobic conditions in Hungate tubes (details of anaerobic technique can be found in: Hungate [6] and Bryant [2], Hungate tubes can be obtained from Bellco Glass; www.bellcoglass.com)
3. On the night prior to experiments a sub culture (7 mL) is labelled by growing overnight at 39°C in Wallace and McPherson media [11] containing ^{14}C-leucine as the sole N source (Table 4.1).
4. On the morning of the experiment bacteria are harvested by centrifugation (3000*g*, 15 min) and resuspended in 7 mL of anaerobic 50 mM potassium phosphate buffer (pH 6.8) containing 5-mmol ^{12}C-L-leucine to prevent re-incorporation of released ^{14}C-leucine.

Bacteriolytic Activity of Protozoa

1. Rumen fluid is collected via a rumen cannula or by stomach tube or at slaughter and strained through a double layer of muslin and stored under CO_2 at 39°C with ^{12}C-L-leucine added to a final concentration of 5 mmol/L.
2. Strained rumen fluid (4.5 mL) is added to a Hungate tube containing the antiprotozoal agent (concentration will need to be determined empirically but our studies have typically used 1 and 10 g/L of ground plant material).

Table 4.1 Composition of media (/100 mL) used to culture and label *Selenomonas ruminantium*

Ingredients	M8	Wallace and McPherson modified medium
Bacto-casitone (g)	1	–
Yeast extract (g)	0.25	–
Glucose (g)	0.2	0.2
Maltose (g)	0.2	0.2
Cellobiose (g)	0.2	–
$NaHCO_3$	0.4	0.4
Rumen fluid (mL)	20	20
Mineral solution I (mL)	15	15
Mineral solution II (mL)	15	15
Resazurin (mL) (0.1% w/v)	0.1	0.1
Vitamins solution –N4a	–	10
Dist. H_2O (mL)	49	40
Cysteine HCl (g)[a]	0.1	0.1
[^{14}C]leucine		1.26 μCi/7 mL

Mineral solution I: KH_2PO_4, 3 g, Dist. H_2O, 1 L
Mineral solution II: KH_2PO_4, 3 g, $(NH_4)SO_4$, 6 g, NaCl, 6 g, $MgSO_4$, 0.6 g, $CaCl_2$, 0.4 g, Dist. H_2O, 1 L
Vitamins solution –N4a
Pyridoxine HCl, 0.2 g, Riboflavine, 0.2 g, Thiamine HCl, 0.2 g, Nicotinamide, 0.2 g, Ca-D-pantothenate, 0.2 g, P-aminobenzoic acid, 0.01 g, Folic acid, 0.005 g, Biotin, 0.005 g, Vitamin B_{12}, 0.0005 g, Dist. H_2O, 100 mL
[a]The ingredients were mixed and the solutions were boiled once and bubbled with O_2 free CO_2. Cysteine HCl was added after boiling.

3. This mixture is pre-incubated for 1 h at 39°C before adding 0.5 mL of ^{14}C-leucine labelled *S. ruminantium* (for convenience this can be done by injection using a sterile 1 mL plastic syringe with a 23 gauge needle). The incubation is continued under CO_2 in shaking water bath (Grant Instrument Ltd, Cambridge, 80 strokes/min) at 39°C.
4. Samples (0.5 mL) are removed (again a 1 mL plastic syringe with a 23 gauge needle can be used) at 0 h and at 1 h intervals up to 3 h into Eppendorf tubes containing 0.125 mL trichloroacetic acid (25%, w/v) The Eppendorf tubes are then centrifuged (11,000 g, 5 min).
5. Samples of the supernatant fluid are counted by liquid-scintillation spectrometry (Packard 1900 CA, Berkshire, UK). Generally, we add 200 μL of the supernatant to 2 mL of scintillation fluid.
6. A sample of the ^{14}C-leucine labelled *S. ruminantium* bacteria should also be counted by liquid-scintillation spectrometry (Packard 1900 CA, Berkshire, UK). Generally, we add 50 μL of the bacterial culture to 2 mL of scintillation fluid.
7. The degradation of ^{14}C-leucine labelled *S. ruminantium* at each incubation time is calculated from the acid soluble radioactive label and expressed as a percentage of the total dpm (disintegration per minute) present in labelled bacterial suspension.

8. The rate of degradation per hour is calculated as the difference from the linear portion of the degradation curve (normally 0–3 h).
9. When testing large numbers of plants over several days it is normal to see considerable variation in absolute values from day to day due to variation in the protozoal population collected, thus it is useful to express results relative to a control (no addition) run on each day.

Calculations

As noted above it is necessary to determine the radioactive label (as dpm) in the sample of ^{14}C-leucine labelled *S. ruminantium* as added in stage 3 above and typically we count 50 μL of this sample.

We also count 200 μL of the supernatant after centrifugation in stage 4.

Assuming that:

- the count obtained from 50 μL of the ^{14}C-leucine labelled *S. ruminantium* added in stage 3 is 10,000 dpm
- the counts recovered in 200 μL of supernatant from stage 4 at 0, 1, 2 and 3 h are 250, 469, 654 and 830 dpm for the control sample and 268, 377, 460 and 560 dpm in the sample preincubated with the potential antiprotozoal agents respectively.
- The released counts in 0 h sample from the control is: dpm ×100 (to change to a percentage) ×5 (to correct to a total sample volume of 5 mL) ×5 (to correct from 200 μL to 1 mL) ×1.25 (to correct for dilution of the acid)/(10,000 × 10) (to correct from 50 μL to 0.5 mL in the added bacteria) = 7.8%

Thus by the sample calculation the released activity is 7.8, 14.7, 20.4 and 25.9% at 0, 1, 2 and 3 h incubation in the control and 8.4, 11.8, 14.4 and 17.5% in the sample preincubated with the potential antiprotozoal agents.

When plotted as a graph (Fig. 4.1) the rate of degradation of *S. ruminantium* is 6%/h in the control and 3%/h in the sample preincubated with the potential antiprotozoal agents.

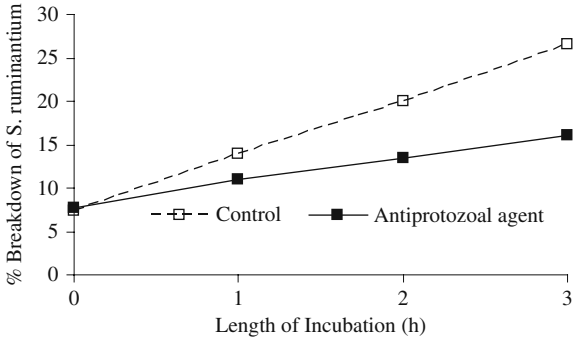

Fig. 4.1 The effect of an antiprotozoal agent on the breakdown of *S. ruminantium*

The antiprotozoal agent has thus reduced the breakdown of *S. ruminantium* by 50% in Fig. 4.1.

Screening of Antiprotozoal Agents Based on Their Ability to Inhibit the Uptake of ^{14}C-Choline in Mixed Rumen Fluid

In the rumen, choline is taken up specifically by ciliate protozoa and not apparently, by the bacteria; as such, ^{14}C-choline has been used to label rumen protozoa for use in in vivo studies [7]. Campbell et al. [3] suggested that the antiprotozoal effects of surfactants could be assessed by measuring the uptake of methyl-^{14}C-choline into protozoa in mixed rumen fluid.

Basis of the Method

Strained rumen fluid is incubated with methyl ^{14}C-choline under anerobic condition; uptake of the label is measured in protozoa recovered by low speed centrifugation.

1. Rumen fluid is collected via a rumen cannula or by stomach tube or at slaughter and strained through a double layer of muslin and stored under CO_2 at 39°C.
2. Strained rumen fluid (5 mL) is added to a Hungate tube (Bellco Glass Inc., Vineland, NJ, USA) containing the antiprotozoal agent (concentration will need to be determined empirically we have typically used 1 and 10 g/L of ground plant material in our studies).
3. This mixture is pre-incubated for 1 h at 39°C before adding 0.25 μCi methyl ^{14}C-choline. The incubation is continued under CO_2 in a shaking water bath (Grant Instrument Ltd, Cambridge, 80 strokes/min) at 3°C for a further 30 min.
4. At the end of the incubation, the tube is centrifuged at 500*g* for 2 min.
5. The supernatant is discarded and the pellet resuspended in 5 mL formalin saline (formaldehyde (4% w/v) in NaCl (0.9% w/v)).
6. Finally, the pellet is resuspended in 1 mL formalin saline and a sub-sample counted by liquid-scintillation spectrometry. Generally, we add 200 μL of the supernatant to 2 mL of scintillation fluid.
7. Results are expressed as dpm/mL and expressed as percentage of the control (no additive incubation).

Calculations

If the uptake of ^{14}C-choline in the control incubation was 500 dpm and in the sample preincubated with the potential antiprotozoal agents, the count was 250 dpm then it might be assumed that the antiprotozoal agent has reduced protozoal activity by 50%.

Visual Assessment of Protozoal Viability

It is not always possible to have access to facilities to work with ^{14}C compounds. Under such situations, it is possible to access the antiprotozoal action of test materials using a visual assay. However, it should be noted that by its very nature such an assay is subjective and can at best be only semi quantitative.

Basis of the Method

Ciliate activity is assessed against a common scale when examined at low magnification (× 100) using light microscopy.

1 Rumen fluid is collected via a rumen cannula or by stomach tube or at slaughter and strained through a double layer of muslin and stored under CO_2 at 39°C.
2. Strained rumen fluid (5 mL) was added to a Hungate tube (Bellco Glass Inc., Vineland NJ, USA) containing the antiprotozoal agent (concentration will need to be determined empirically but we have typically used 1 and 10 g/L of ground plant material in our studies).
3. This mixture is incubated for 1 h at 39°C before accessing protozoal activity at low magnification (×100) using light microscopy according to the following scale.

Score	Comment
5	All genera active
4.5	Holotrichs active
4	Reduced motility/ciliary activity in holotrich protozoa only
3	No ciliary activity/motility
2	Vacuoles visible in holotrich protozoa
1	Cellular disruption
0	No whole protozoa evident

References

1. Beever, D.E. and R.C. Siddons. 1986. Digestion and metabolism in the grazing ruminant, pp. 479–497. In P. Milligan, W.L. Grovum, and A. Dobson (eds.). The Control of Digestion and Metabolism in Ruminants. Prentice-Hall, New Jersey.
2. Bryant, M.P. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Am. J. Clin. Nutr. **25**:1324–1328.
3. Campbell, A.J., G.J. Cumming, C.A. Graham, and R.A. Leng. 1982. An in vitro assay for compounds toxic to rumen protozoa. NZ J. Agric. Res. **25**:535–540.
4. Harrison, D.G. 1979. The contribution of protozoa to the protein entering the duodenum of sheep. Br. J. Nutr. **41**:521–527.
5. Hobson, P.N. 1969. Rumen bacteria. Methods Microbiol. **3B**:133–159.

6. Hungate, R.E. 1969. A roll tube method for cultivation of strict anaerobes. Methods Microbiol. **3B**:117–132.
7. Leng, R.A. 1982. Dynamics of protozoa in the rumen of sheep. Br. J. Nutr. **42**:399–415.
8. Newbold, C.J., S.M. El Hassan, J. Wang, M.E. Ortega, and R.J. Wallace. (1997) Influence of foliage from African multipurpose trees on activity of rumen protozoa and bacteria. Br. J. Nutr. **78**:237–249.
9. Nolan, J.V., and S. Stachiw. 1979. Fermentation and nitrogen dynamics in Merino sheep given a low-quality-roughage diet. Br. J. Nutr. **42**:63–79.
10. Teferedegne, B., F. McIntosh, P.O. Osuji, A. Odenyo, R.J. Wallace, and C.J. Newbold. 1999. Influence of foliage from different accessions of the sub-tropical leguminous tree, Sesbania sesban, on ruminal protozoa in Ethiopia and Scottish sheep. Anim. Feed Sci. Technol. **78**: 11–20.
11. Wallace, R.J. and C.A. McPherson. 1987. Factors affecting the rate of breakdown of bacterial protein in rumen fluid. Br. J. Nutr. **58**:313–323.
12. Williams, A.G. and G.S. Coleman. 1992. The Rumen Protozoa. Springer-Verlag, London.

Chapter 5
Screening for Anti-proteolytic Compounds

Ellen M. Hoffmann, Natascha Selje-Assmann, Klaus Becker, R. John Wallace, and Glen A. Broderick

Introduction

Dietary protein entering the rumen is broken down in an apparently uncontrolled way, resulting in ammonia formation and subsequent loss of N in the urine. The low efficiency of nitrogen retention represents a major economic loss, causes metabolic stress in the animal, and places a burden on the environment, by way of nitrogen-rich wastes. If a means of slowing the breakdown process at any of the individual steps can be identified, these problems would be decreased.

Many different microbial species, employing a range of proteolytic enzymes, carry out the initial step of protein breakdown. The variety of proteolytic microbes present has made rational manipulation of the initial proteolytic step impossible, and solutions have generally required treatment of the protein before feeding, by heating for example. Tannins have been explored as a means of decreasing protein breakdown, but they frequently impair other aspects of rumen fermentation, including fibre breakdown. New plant materials that bind to proteins and prevent their digestion, or preferably which inhibit the proteinases directly, yet are not otherwise detrimental, would be of enormous benefit to ruminant livestock production globally.

The subsequent processes of peptide and amino acid breakdown are carried out by more defined populations. The only methods available for altering these activities are dietary addition of antibiotics and ionophores, which suppress the growth of the bacteria responsible. Finding substances, which decrease ammonia formation, could lead to more acceptable ways of inhibiting the processes leading to ammonia formation in the rumen.

In this chapter, we describe methods that can be used to measure different aspects of the processes involved in degradation of protein by ruminal microorganisms in vitro. The methods described include the use of diazotized or radio-labelled protein

E.M. Hoffmann (✉)
Institute of Animal Production in the Tropics and Subtropics, University of Hohenheim, D-70593 Stuttgart, Germany
e-mail: Inst480@uni-hohenheim.de

P.E. Vercoe et al. (eds.), *In Vitro Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies*,
DOI 10.1007/978-90-481-3297-3_5,

substrates to assess the first step in proteolysis, a method for screening for anti-proteolytic plant compounds that involves a short-term incubation with a complex, protein rich substrate, and an inhibitor in vitro assay of the rate and extent of ruminal protein degradation.

Screening for Antiproteolytic Plant Compounds Using Diazotized or Radio-Labelled Proteins

Diazotized Proteins

Diazotized or radio-labelled protein substrates can be used to assess the first step in proteolysis, i.e. conversion of polypeptide to smaller, acid-soluble peptides. A longer incubation with other protein substrates is described which, unlike the aforementioned methods, incorporates to a degree any adaptation of the microbial community to additives; both the initial proteolysis and the overall end products are measured, by polyacrylamide gel electrophoresis and ammonia/branched chain fatty acids, respectively.

Preparation of Azoproteins

This method works well for soluble proteins, but less well for insoluble proteins, particularly heterogeneous protein supplements because the surface tends to become labelled more than internal regions of particles (see [32]). The method is also less suitable for grazing animals due to background colour derived from the forage. The preparation of azoproteins is based on the paper of Tomarelli et al. [28]. Alternatively, and recommended, azocasein and azoalbumin can be purchased from Sigma (A2765 and A2382, respectively).

To prepare the azoproteins, dissolve 10 g of substrate protein (casein is usually used, but other proteins such as bovine serum albumin can be used as well) in 100 mL of 4% $NaHCO_3$ in distilled water. Prepare diazotized sulfanilic acid by dissolving 0.26 g of sulfanilic acid in 20 mL of 0.25 M NaOH. Stir on ice and gradually (over 5 min) add 0.35 g sodium nitrite. Then add 2 mL of 5 N HCl, stir for 2 min, and add 2 mL of 5 M NaOH. Within 5 s of adding the NaOH, mix the diazotized sulfanilic acid solution to the substrate protein solution and stir on ice for 1 h. Add to dialysis tubing (e.g. Visking). Dialyze against distilled water for 24 h at 4°C, changing water four times. Freeze and freeze-dry resulting dialyzed solution.
Caution: *The diazotizing solution should not touch the skin – wear gloves! The HCl and NaOH solutions are hazardous.*

Measurement of Proteolysis

Make up 2-mg/mL azocasein in 0.1 M potassium phosphate buffer, pH 7.5. Take sample of rumen liquor and use fresh (This method is not suitable for frozen

samples!). Strain the rumen liquor through 4 layers of muslin cloth. Set up four numbered plastic 10-mL polypropylene centrifuge tubes for each sample that is to be analysed, containing:

2 mL of azocasein solution [1]
2 mL of azocasein solution + 1 mL 25% trichloroacetic acid [2]
2 mL 0.1 M potassium phosphate buffer pH 7.5 [3]
2 mL 0.1 M potassium phosphate buffer pH 7.5 + 1 mL 25% trichloroacetic acid [4]
Equilibrate in water bath adjusted at 39°C

At $t = 0$, add 2 mL of strained rumen digesta to all tubes. Continue to incubate at 39°C. After 1 h[1] at 39°C, add 1 mL 25% trichloroacetic acid to [1] and [3].

Transfer the tubes to ice water and then centrifuge at 5000 g for 30 min. [Or transfer the tubes to a cold room (4°C) for about 48 h and then centrifuge.]

Carefully[2] remove 2.0 mL of supernatant into another tube containing 2.0 mL of 0.5 M NaOH. Measure A_{440} of this solution.

Calculation of Proteolytic Activity

Make up the following tubes for calibration with 0.2-mg/mL azocasein (a 10-fold dilution of the stock solution used as substrate (Table 5.1)):

Table 5.1

0.2 mg/mL azocasein (mL)	Buffer (mL)	0.5 M NaOH (mL)
0	2.0	2.0
0.1	1.9	2.0
0.2	1.8	2.0
0.3	1.7	2.0
0.4	1.6	2.0
0.5	1.5	2.0

Measure A_{440}. Draw a standard curve of A_{440} vs. concentration of azocasein (i.e. 0–0.025 mg/mL). From the best fitting straight line (i.e. linear regression), calculate the extinction coefficient (E) in units of A_{440} of a 1-mg/mL solution of azocasein.

---

[1] Exactly. Time may vary depending on the activity of ruminal liquor. Calculate how much of the azocasein has been digested – if it is more than half, the assay should be repeated for a shorter time. Or, if there is insufficient colour generated, extend the incubation time. Azoalbumin is hydrolysed more slowly.

[2] This is the step where the greatest error can occur. Some of the part-digested azocasein floats on the meniscus in some samples. The pipette should be submerged gently through this layer before drawing the 2-mL volume. When withdrawn, some of the part-digested azocasein often clings to the pipette tip. Take care not to touch the recipient tube with the tip in order to minimize contamination with this material.

Note that different batches of azocasein have different E values

The proteolytic activity of ruminal digesta is then calculated from the absorbance read in the four numbered tubes (see *Measurement of proteolysis*) as follows:

Absorbance of digested azocasein at time t = $[A_1 - A_2 - A_3 + A_4]$
Concentration of azocasein digested (mg/mL NaOH solution) = $[A_1 - A_2 - A_3 + A_4]/E$
Concentration of azocasein digested (mg/mL TCA extract) = $2 \times [A_1 - A_2 - A_3 + A_4]/E$
Dilution of ruminal digesta was 2 mL to a final volume of 5 mL in TCA extract, so:
Concentration of azocasein digested (mg/mL strained rumen digesta) = $2.5 \times 2 \times [A_1 - A_2 - A_3 + A_4]/E$
Therefore, proteolytic activity (mg azocasein hydrolysed/h per mL of ruminal digesta) = $(5 \times [A_1 - A_2 - A_3 + A_4])/(E \times t)$

Radio-Labelled Proteins

The use of radiolabelled proteins overcomes many of the limitations of the azocasein assay, in that there is minimal interference from chromogenic compounds in plant materials, including forages.

Note: *All procedures should be carried out using gloves and protective clothing and in a designated laboratory. Strict rules for the use and disposal of radioactive material should be observed. The half-life of ^{14}C is 5730 years – any spillage or inappropriate disposal will leave a long legacy of hazard.*

Preparation of ^{14}C-Formaldehyde-Labelled Proteins

The method is based on the reductive methylation of protein using formaldehyde and sodium borohydride. The result is a tiny structural modification of the protein that has no effect on its susceptibility to proteolytic digestion. Either ^{14}C-formaldehyde or ^{3}H-sodium borohydride can be used. The latter is much cheaper, but its use is much less widely reported.

For the application of the method to measuring the proteolytic activity of ruminal digesta, see [31].

Note: *This method works well for soluble proteins, but less well for insoluble proteins, particularly heterogeneous protein supplements because the surface tends to become labelled more than internal regions of particles.*

Dissolve 0.1 g of casein sodium (Sigma) in 10 mL of 0.2 M sodium borate buffer pH 9.0. Make up fresh 0.5 mg/mL $NaBH_4$ and a solution of 0.01% (0.1 mg/mL) formaldehyde. The stock solution is 37% (w/v) and has a specific gravity of 1.09 g/mL, so use 0.25 mL/L of water. Chill the casein solution on ice.

Add 10 μL of stock 1 μCi/μL ^{14}C-formaldehyde to 50 μL of the 0.01% formaldehyde (add more radioactivity if required; this method should give about 40,000 dpm per mg casein). On ice, add 0.15 mL of $NaBH_4$ solution to the casein solution, mix, and after a few seconds add the diluted ^{14}C-formaldehyde. Incubate on ice for 30 min, then dialyse overnight at 4°C and freeze dry. Redissolve the freeze-dried material in 10 mL of water and count 50 μl in duplicate and calculate the specific radioactivity (D_S) in dpm/mg. This solution can then be used as the basis of the substrate solution in proteinase assays. You should aim to dilute the radioactive casein with unlabelled casein to give a count of about 40,000 dpm per mL in the solution added to the assay mixture.

Measurement of Proteolysis

This protocol is small-scale in order to minimise the use of radioactivity. On this scale, it may not be suitable for the testing of small quantities of plant samples in powder form. However, it will be excellent for the testing of liquid extracts. For testing solids, it may be necessary to scale-up ten-fold.

Make up a stock 2-mg/mL ^{14}C-labelled casein (approx. 80,000 dpm/mL, but a higher specific activity can be used) in 0.1 M potassium phosphate buffer pH 7.5. Dilute the radio-labelled casein solution with 2 mg/mL unlabelled casein in 0.1 M potassium phosphate buffer pH 7.5. Remove sample of rumen liquor and use fresh. This method is not suitable for frozen samples. Strain through 4 layers of muslin cloth.

Set up two micro-centrifuge tubes for each sample that is to be analysed, containing:-

100 μL of ^{14}C-labelled casein [1]
100 μL of ^{14}C-labelled casein + 50 μL 25% trichloroacetic acid [2]
Equilibrate in water bath at 39°C

At t = 0, add 100 μL of strained rumen digesta to all tubes. Continue to incubate at 39°C. After 1 h[3] at 39°C, add 50 μL 25% trichloroacetic acid to [1]. Transfer the tubes to ice water then centrifuge at 12,000 g for 10 min [Or transfer the tubes to a cold room (4°C) for about 48 h and then centrifuge].

Carefully[4] remove 100 μL of supernatant into a scintillation vial. Add scintillation fluid and measure ^{14}C. In addition, count 2 × 50 μl of the ^{14}C-labelled casein solution added to tubes 1 and 2. Calculate mean as D_S dpm/50 μL = 20 × D_S/mL = 20/2 × D_S dpm/mg casein = 10 × D_S dpm/mg casein.

---

[3] Exactly. Time may vary depending on the activity of ruminal liquor. Calculate how much of the casein has been digested – if it is more than half, the assay should be repeated for a shorter time.

[4] This is the step where the greatest error can occur. Some of the part-digested casein floats on the meniscus in some samples. The pipette should be submerged gently through this layer before drawing the 100 μl volume. When withdrawn, some of the part-digested casein often clings to the pipette tip. Take care not to touch the recipient vial with the tip in order to minimize contamination with this material.

Calculation of Proteolytic Activity

The proteolytic activity of ruminal digesta is then calculated as follows (subscripts to D refer to the respective tube numbers, see *Measurement of proteolysis*):

Dpm of digested casein at time t	$= [D_1 - D_2]$ dpm/100 μL supernatant
	$= 10 \times [D_1 - D_2]$ dpm/mL supernatant
	$= 2.5 \times 10 \times [D_1 - D_2]$ dpm/mL rumen digesta
	$= 25 \times [D_1 - D_2]$ dpm/mL rumen digesta
But specific radioactivity of casein	$= 10 \times D_S$ dpm/mg casein
So, concentration of casein digested	$= 25 \times [D_1 - D_2]/(10 \times D_S)$ mg/mL reaction mixture
Therefore, proteolytic activity of ruminal digesta.	$= (2.5 \times [D_1 - D_2])/(DS)$ mg casein hydrolysed/h per mL

Screening for Antiproteolytic Plant Compounds by Short-Term Incubation with a Complex, Protein Rich Substrate

This screening method is based on short-term batch incubation as described by Mauricio et al. [20]. It is thus small enough to work with small amounts of plant samples (ca. 1.5 g DM) and quick enough to process sets of 15–20 samples per experiment. The plant material to be tested is added to a standardized, protein rich substrate. Over a period of 10–12 h with repeated sampling the disappearance of soluble substrate protein as well as the release of branched SCFA and ammonium are monitored as proteolysis-specific parameters. Total SCFA release gives supplementary information on general fermentation. If undisturbed parallels are included and incubation time is extended, gas production and 24 h digestibility can be optionally determined alongside. As shown in Selje-Assmann et al. [26, 27] this experimental approach is able to detect immediate effects, such as the precipitation of dietary protein by tannins, as well as slower effects mediated by modification of the microbial activity. When monensin, a well-established inhibitor of ruminal proteolysis, was added to this system, the effects described in the literature could be reproduced. On one hand, this validated the experimental approach. On the other hand, monensin could be introduced as an external standard to correct for the variability in biochemical responses associated with variations in the rumen fluid inoculum.

Incubation

Substrates

The substrate was composed to resemble a concentrate rich ruminant diet. Maize silage served as roughage component, barley grain as energy supplement, and a combination of soybean meal (Sigma-S9633) and BSA (bovine serum albumin)

Table 5.2 Substrate composition for batch incubations

Component (mg/bottle)	Negative control	Positive control	Treatment
Maize silage	450	450	300
Barley grain	225	225	225
Soybean meal	150	150	150
BSA	10	10	10
Monensin[a]	–	11.5 μl (ad 3 μM)	–
Test plant in optional parallels	–	–	150
PEG	450	45	450

[a]A stock solution (14 mg/mL) in ethanol is prepared freshly prior to each experiment and is added immediately after filling in the buffered rumen fluid.

(Sigma-A9647) as protein supplement. The proportions are given in Table 5.2. With our materials, the crude nutrient composition of the control substrate was: CA 3.9%, CP 16.9%, EE 1.9%, NDF 31.3%, ADF 23.1% and ADL 3.5% DM.

If entire plant material (i.e. dried, ground (green) biomass) was to be tested, it was added to replace an equivalent amount of maize silage, as this resulted in the lowest changes of crude nutrient composition. Routinely, 150 mg of test material were added; series of 30–200 mg of test material were successfully assayed for dosage effects. If purified extracts are to be evaluated, addition of the extract without reducing the maize silage may be a more suitable strategy [15]. This should be decided by the lowest interference with crude nutrient composition.

A negative control shows the proteolytic activity of a given inoculum under standard conditions. A positive control shows the inhibitory effect of monensin, relative to the negative control. Treatments can be evaluated relative to the negative control, or relative to the effect achieved by monensin.

The contribution of tannins to any observed effects on proteolysis can be evaluated if the experimental design is amended by additional parallels of all controls and treatments including polyethylene glycol (PEG) [26].

All substrate components (except monensin, which is added as stock solution immediately after inoculation) are weighed directly into 100 mL serum bottles, which are then pre-warmed prior to the addition of buffered rumen fluid.

Donor Animals and Preparation of Inoculum

For the screening system described here, the donor animals of rumen fluid (fistulated cattle or sheep) should be adapted to a concentrate-rich diet. If hay-fed animals are used as donors, the kinetics of fermentation are slower and total incubation time and sampling points need to be adapted. Rumen fluid is collected prior to morning feeding by manually squeezing liquid from the feed mat into pre-warmed thermos flasks. Again, if rumen fluid is withdrawn from the liquid phase by pump, kinetics are likely to be slower due to lower microbial density.

Experimental Design

Only incubations run with different inocula can be considered true replicates. This can be achieved by using different donor animals within the same experiment, or by subsequent experiments using the same donor animal. The lowest variation should be expected when the rumen fluid of two or three donors is mixed to provide a single inoculum, and experiments are repeated on different days.

In any case, positive and negative controls as well as blanks (bottles without any substrate) need to be included for every inoculum. Every treatment is incubated in 3 parallels, one of which is kept closed for exact gas readings, while the other two are designated for repeated sampling. Incubations run for 12 h, with samples being taken repeatedly from the same bottle after 1, 6, 8, and 10 h.

Thus, the total number of bottles to prepare is $b = (9 + 3 \times T) \times I$, where I = number of inocula, T = number of treatments respective additives to be tested, and the 9 resulting from triplicates of blank, positive and negative control. Substrates should be weighed into the bottles a day ahead. Collection of rumen fluid, inoculation, incubation (12 h) and processing of samples will add up to a total duration of ca. 16 h for the actual experiment.

Incubation Medium

The incubation medium (Table 5.3) can be considered as "artificial saliva", providing the buffer capacity and minerals to maintain favourable conditions for microbial fermentation. It is prepared freshly before each incubation and is pre-warmed and reduced before rumen fluid is added. Reduction is achieved by bubbling the solution with CO_2-gas for several hours, and by addition of the reducing solution (Table 5.4) shortly before the incubation. Reduction is indicated by the dye Reazurin, which changes from blue (over pink) to colourless.

Table 5.3 Composition of incubation buffer

Components	MW	Final conc.	Amount
Ammonium bicarbonate	60.1	13.5 mM	4.06 g
Sodium bicarbonate	84.0	86.5 mM	36.33 g
Di-Sodium hydrogen phosphate	142.0	5.5 mM	3.91 g
Potassium di-hydrogen phosphate	136.1	9.5 mM	6.46 g
Magnesium sulphate ($\times$ 7 H_2O)	246.5	0.5 mM	0.62 g
Micro-minerals (stock solution[b])		0.020%	1000 µL
Resazurine (1%)		0.001%	500 µL
dH_2O			4190 mL
Reducing solution		6%	310 mL
Total volume			4500 mL
Rumen fluid to be added		10%	500 mL
Total volume[a]			5000 mL

[a]The final volume of 5 L allows for the incubation of ca. 15 treatments plus blanks and controls in triplicates. It can be adjusted to the experimental design.
[b]The composition of the micro-minerals stock solution is given in Table 5.4.

Table 5.4 Reducing solution

Components	MW	Final conc.	Amount
Cysteine HCl	52.9	0.118 M	1.938 g
NaOH (1 M)	40.0	0.040%	12.40 mL
Na_2S	240.2	0.026 N	1.938 g
dH_2O to make total volume			310 mL

Table 5.5 Micro-minerals stock solution

Components	MW	Final conc.	Amount
Calcium chloride $2 \times H_2O$	147.0	0.45 M	3300 mg
Manganese chloride $4 \times H_2O$	197.9	0.25 M	2500 mg
Cobalt chloride $6 \times H_2O$	237.9	0.02 M	250 mg
Ferric trichloride $6 \times H_2O$	270.3	0.15 M	2000 mg
dH_2O to make total volume			50 mL

Note: *For reasons of time management, the buffer, without reducing solution and rumen fluid, can be prepared a day ahead and gassed for 2–3 h with CO_2. It can then be pre-warmed over night. In the next morning, prior to the collection of rumen fluid, the reducing solution is prepared and added, and gassing continues while collecting the rumen fluid.*

Inoculation

When the buffer is completely reduced, one volume of filtered (100-μm) rumen fluid is added to 9 volumes of buffer, to make up the "buffered rumen fluid". Three 1 mL samples of this are taken for subsequent analysis (see "Sampling section below"). Aliquots of 75 mL are then dispensed in the pre-warmed serum bottles containing the substrate. Routinely, all treatments are run in triplicate; three bottles without any substrate serve as blanks (to monitor gas production arising just from the inoculum rather than the incubated substrate). Monensin solution (Table 5.6) is pipetted in the designated controls. The headspace is flushed with CO_2 gas; the bottles are closed with rubber stoppers and incubated at 39°C.

Gas Reading

As fermentation sets on, gas is released into the headspace of the bottle and builds up a pressure. A syringe needle with an attached pressure transducer is inserted through the rubber stopper at regular intervals to measure the current pressure. Once

Table 5.6 Monensin stock solution

Components	Amount
Monensin	3.5 mg
dH_2O	250 μL

the value has been recorded (along with the time of measurement), the transducer is detached and the pressure is released through the open syringe needle. Finally, the needle is removed and pressure can build up again until the next measurement point. Routinely, pressure is measured after 1, 2, 3, 4.5, 6, 8, 10 and 12 h (where applicable: prior to sampling). Further measurements are necessary if incubation time is extended to 24 h. A calibration series is used to convert pressure data to gas volume.

In order to obtain the calibration curve shown in Fig. 5.1, serum bottles were filled with 75 mL water, warmed to 39°C (i.e. the incubation temperature) and closed with rubber stoppers. Defined gas volumes of 5, 10, 15, 20 ... to 40 mL were injected into four parallel bottles each with calibrated, gas-tight syringes. The pressure was then measured as described above. This calibration should be done with the specific equipment available in the lab when introducing the methodology. The same calibration curve can then be used in subsequent experiments.

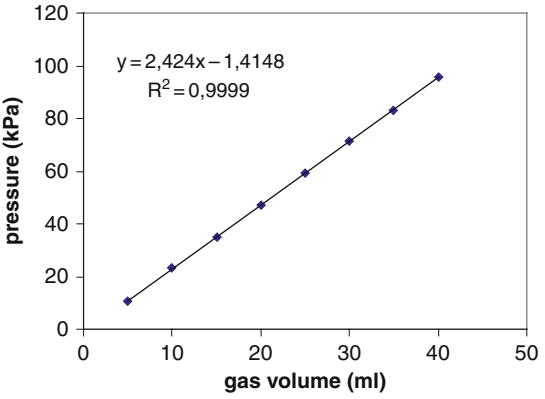

Fig. 5.1 Example of a calibration curve to convert pressure to gas volume

Calculation of Gas Production Rate and Cumulative Gas Production

All pressure readings are converted to mL gas by the calibration curve; the average gas volume produced in the blanks is calculated at each time point, and all measurements are corrected by their corresponding blank.

The exact incubation time since the previous gas reading is determined for each bottle (from time data recorded along with the pressure measurement), and net gas production is related to the hours of incubation to express data as gas production rate (mL/h)

Net gas production is added up over the entire incubation period, and the sum is related to the amount of substrate incubated in the respective bottle and expressed as cumulative gas production (mL/g).

If bottles with and without PEG are incubated, gas production of the sample + PEG is set to 100%, and inhibition in the sample without PEG is expressed relative to this.

Note: *Exact gas measurements can only be taken from undisturbed bottles, which have never been opened for sampling. Gas readings from sampling bottles can only serve as a rough indicator for the speed of fermentation.*

Sampling

After 1, 6, 8, 10 and 12 h samples are withdrawn repeatedly from the designated bottles. After recording and release of gas pressure, the stoppers are removed, and aliquots of 1 mL volume are pipetted into prepared sampling tubes (e.g. 1.5 mL Eppendorf cups) kept on ice to stop the fermentation process. To ensure the withdrawal of homogeneous samples, a stirbar is inserted into the bottle, and contents are vigorously stirred while pipetting; wide bored tips have to be used to avoid plugging by feed particles. The stir bar remains inside the bottle for subsequent samplings. The headspace is flushed again with CO_2 gas, bottles are closed again with the same stopper, and incubation continues.

An overview of the sample processing is given in Fig. 5.2. The samples are centrifuged (10 min, 10,000 g, 4°C) and supernatant and pellet are carefully separated. An aliquot of 50 µl of the supernatant is transferred to a fresh vial and frozen at –20°C for determination of soluble protein under native conditions.

Another 50 µl of the supernatant are mixed with 50 µl of double strength Laemmli-buffer (2 × LBF, Table 5.17) and boiled for 3 min to generate denatured samples of soluble protein. Pellets are re-suspended in 1 mL of Laemmli-buffer (1 × LBF), boiled for 5 min to generate denatured samples of insoluble protein. Both of the denatured samples are backups for protein determination by dot blot (see "Quantitative protein analysis by dot blot assay") and, if desired, subsequent qualitative analysis by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (see "Qualitative protein analysis by SDS-PAGE" below).

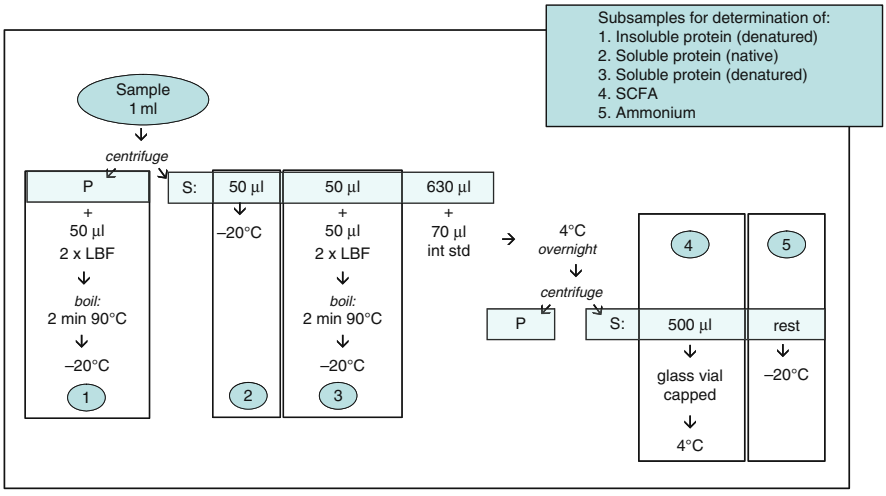

Fig. 5.2 Flow diagram of sample processing

Another aliquot of 630 μl of the supernatant is transferred into a fresh vial and 70 μl of internal standard (int std, Table 5.8) are added. These samples are kept at 4°C over night to precipitate soluble proteins. They are centrifuged again (10 min, 10,000 g, 4°C) to remove the precipitate. 500 μl of the acidified, de-proteinized supernatant are transferred into glass vials, sealed with serum caps, for SCFA analysis. The remaining supernatant is transferred into a fresh vial, frozen at –20°C, and kept for determination of ammonium.

Note: *Due to the complex sampling scheme and the large number of samples to process all cups and vials should be prepared and labelled ahead of the experiment.*

The amount needed is:
sampling bottles $= b_s = (4 + 2 \times t) \times I$
glass vials $= b_s \times t \times a$
sampling cups $= b_s \times t \times 5 \times a$
(where: t = no. of sampling times, a = no. of aliquots, I = no. of inocula)

One set of cups is needed for collecting the original 1 mL aliquots; after centrifugation, the pellet is left in these cups and re-suspended in 1 × LBF. Three sets of cups are needed for sharing the first supernatant as indicated; one of these sets should be prefilled with 2 × LBF. Another set of cups is needed to collect the supernatant after the second centrifugation.

Optional: 24 h Digestibility

If applicable, digestibility is determined in undisturbed incubation bottles set aside for gas reading only. Incubation time is extended to 24 h, which usually requires 1 or 2 additional gas readings to avoid high pressure in the gas space. At the end, all bottles are transferred to an ice bath to stop fermentation, and the contents are emptied quantitatively into pre-weighed nylon bags (50 μm pore size, e.g. from Bar Diamond Inc. Parma, ID, USA). The nylon bags are held by glass beakers to collect the filtrate, any particulate matter is held back in the bags. Analytical samples are withdrawn from the filtrate as described in previous section. After this, the bottles can be rinsed with distilled water and residues are combined in the respective bag. The bags are closed by folding the upper edge, excess liquid is gently squeezed from the bags, and they are hung up using a fold back clamp until drained completely. The bags are dried overnight at 100°C and weighed to determine apparent digestibility. Dry bags are heat-sealed and boiled for 1 h in NDS (Table 5.7), rinsed several times in distilled water and dried again to determine in vitro true digestibility. The mass difference of original residue and NDS-boiled residue can be taken as a rough estimate of microbial mass.

Analytical Procedures

SCFA Analysis by Gas Chromatography

SCFA are determined in an acidified, de-proteinized rumen fluid sample (see "sampling" section above) containing 10% (v/v) of internal standard. The sample is

Table 5.7 Neutral detergent solution (NDS)

Components	MW	Final conc.	Amount
EDTA	372.2	0.050 M	93.0 g
Sodiumtetraborate (10 H_2O)	381.4	0.018 M	34.0 g
SDS		3.0%	150.0 g
Monoglycolether		1.0%	50.0 mL
Sodium dihydrogenphosphate	142.0	0.032 M	22.8 g
dH_2O to make total volume			5000 mL

provided in a 1.5 mL glass vial, closed tightly by a serum cap. Samples are analysed in a gas chromatograph (e.g. GC 14A, Shimazu Corp., Kyoto, Japan) with a stainless steel column packed with GP 10% SP, 1000 1% H_3PO_4, Chromosorb W AW (Supelco Inc. Bellafonte, PA). The method was developed by [13].

To guarantee reliable measurements, internal as well as external standardization is used. Methylvaleric acid, which does not naturally occur in rumen liquid, is used as internal standard (Table 5.8). This serves as reference for the analytical device and needs to be present in every sample measured. Double distilled water containing 10% (v/v) internal standard is used to clear the column in regular intervals. For samples derived from the described incubation, two vials of water are inserted after every 15 vials of samples. An external standard (Table 5.9) is inserted once per run among the second half of the sample set.

The gas chromatography program automatically detects the individual SCFA-peaks and converts the peak area to concentration (μmol/mL). All readings are corrected for SCFA brought in with the inoculum. The net SCFA concentration can then be related to the amount of substrate present in each bottle (mol/g). The sum

Table 5.8 Internal standard for SCFA analysis

Components	Final conc.	Amount
Methylvaleric acid (100%)	1.0%	1.0 mL
Formic acid to make total volume		100 mL

Table 5.9 External standard (10x stock solution)

Components	MW	Final conc.	Amount
100% acetic acid	60.1	60.0 μmol/ml	3.03 g
100% propionic acid	74.1	30.0 μmol/ml	2.22 g
100% butyric acid	88.1	10.0 μmol/ml	0.81 g
100% valeric acid	102.1	1.0 μmol/ml	0.102 g
98% isobutryric acid	88.1	1.0 μmol/ml	0.090 g
100% isovaleric acid	102.1	1.0 μmol/ml	0.102 g
dH_2O to make total volume			100 mL

When diluting the stock solution (1/10) to the final concentration, 10% (v/v) internal standards are added.

of branched SCFA is considered a proteolysis-specific parameter and is expressed, either in absolute terms (μmol/g) or as proportion of total SCFA (%), relative to the negative and positive control.

Ammonium Determination by Phenol Hypochlorite Reaction

The assay is based on the method of Koroleff [17]. In alkaline solution, ammonium ions react with hypochlorite and form an intermediate product, monochloramine. If hypochlorite is in excess and nitroprusside is added as catalyst, this reacts further with phenol to form a dark blue dye, indophenol. The maximum absorbance of this dye occurs around 620–640 nm. Absorbance is proportional to the initial ammonium concentration.

This test has wide application in the analysis of water quality; other trivalent forms of nitrogen do not interfere with the assay. Turbidity or hydrogen sulphide, however, may disturb it. Therefore, when applied to rumen liquid, the sample has to be clarified by centrifugation and hydrogen sulphide has to be eliminated by acidification. Both are achieved in the sample preparation outlined above. The volumes given below were downscaled as compared to the original protocol, and the concentrations are adapted to measurements in 150 μl aliquots in a microplate reader.

A standard curve is prepared according to Table 5.10, using 0.5–5 mM $(NH_4)_2SO_4$ (i.e. 1–10 mM NH_4, respectively). Aliquots of 300 μl of phenol nitroprusside reagent (Table 5.11) are pipetted into a 1.5 mL vial, then 15 μl of standard or sample are added and mixed well. 15 μl of ddH_2O are added for a blank. All treatments should be prepared at least in duplicates. Finally, 300 μl of alkaline

Table 5.10 Pipetting scheme of calibration series for ammonium determination

$(NH_4)_2SO_4$ stock solution 10 mM (μl)	H_2O (μl)	Final conc. (mM)
100	900	1.0
200	800	2.0
400	600	4.0
600	400	6.0
800	200	8.0
1000	0	10.0

Table 5.11 Phenol nitroprusside

Components	Final conc.	Amount
Phenol*	10.0 mg/mL	2.5 g
Sodium nitroprusside	50.0 μg/mL	12.5 mg
dH_2O to make total volume		250 mL

The solution can be stored at 4°C for 1 month.
*Phenol is very hazardous compound. When handling it make sure to wear gloves and protective clothing and always work in a fume hood.

Table 5.12 Alkaline hypochlorite

Components	Final conc.	Amount
NaOH	10.0 mg/mL	2.5 g
Sodium hypochlorite	0.84% (v/v)	2.1 mL
dH_2O to make total volume		

The solution can be stored at 4°C for 1 month.

hypochlorite reagent (Table 5.12) are added and the mixture is incubated for 30 min at 37°C. After the colour has fully developed, absorbance is read at 625 nm.

Absorbance measured in unknown samples is converted to ammonium concentration by the respective calibration curve. In contrast to cumulative parameters such as gas or SCFA concentration, ammonium concentration reflects the current balance between release by fermentation and uptake by microbes.

Quantitative Protein Analysis by Dot Blot Assay

This assay is based on the method of Neuhoff [23], as modified by Hoffmann et al. [14]. It works in the presence of SDS, but due care has to be taken that the SDS concentration of the samples is the same as that in the standards used for calibration. Denaturation is recommended only if qualitative analysis of protein patterns by SDS-PAGE is to follow, or if the insoluble protein pellet is to be quantified.

The protein concentration in the native supernatant can be determined without further processing.

Dot Blot Procedure

A calibration series is prepared with BSA as shown in Table 5.13.

A cellulose acetate membrane (Sartorius 12200) is placed in a holder (Fig. 5.3), i.e. between two plates with an application grid of 84 holes (ca. 1 cm in diameter), and fixed by inserting two pins. The holder can be manufactured from any kind of

Table 5.13 Pipetting scheme of calibration series for protein determination

BSA stock solution 2 mg/mL (µl)	H_2O (µl)	(2×) buffer[a]	Final protein conc.
20	180	200	0.1
40	160	200	0.2
80	120	200	0.4
120	80	200	0.6
160	40	200	0.8
200	0	200	1.0

[a]The components of the RPT-buffer do not affect the staining intensity; therefore water can be used for native calibration standards; for denatured samples, 2× Laemmli-buffer has to be used, as SDS affects the staining with amido black.

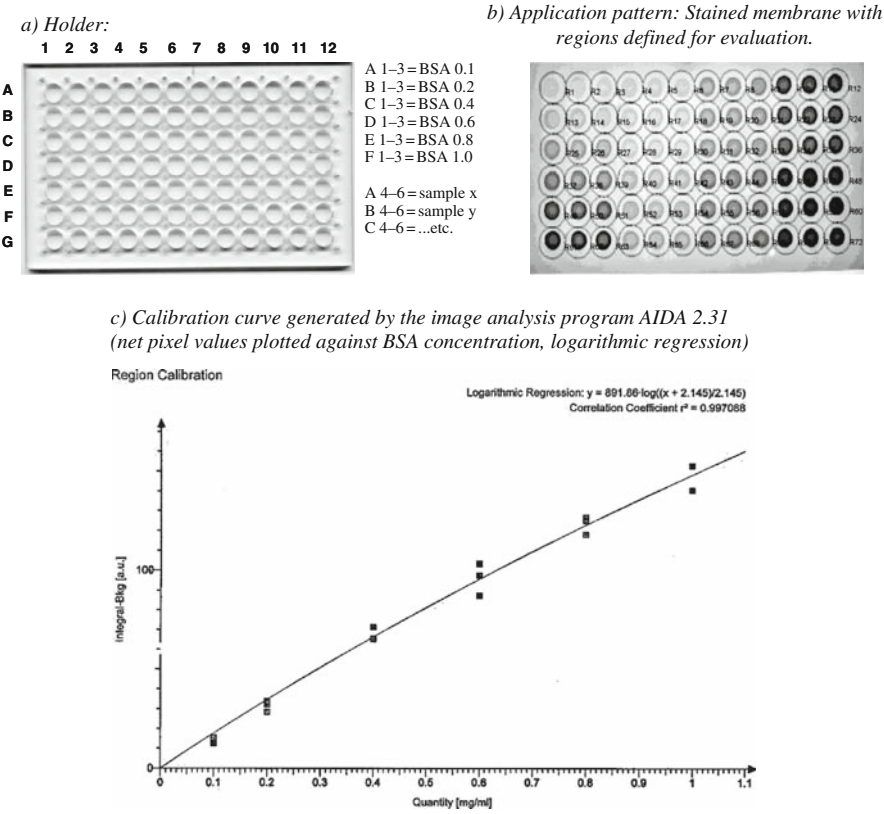

Fig. 5.3 Illustration of holder, application pattern and a stained membrane. (**a**) Holder; **(b)** Application pattern: Stained membrane with regions defined for evaluation; (**c**) Calibration curve generated by the image analysis program AIDA 2.31 (net pixel values plotted against BSA concentration, logarithmic regression)

inert plastic and guarantees, that within the cavities, the membrane does not touch the support and the samples can be quantitatively applied. An application pattern is designed to define each sample by its position on the grid. Triplicates of 2 μl of standard or sample are applied very slowly in the centre of the cavities. The protein concentration should be in the range of 0.1–1.0 mg/mL. Gilson/Eppendorf pipets or 2 μl glass capillaries can be used for sample application. When the sample is completely absorbed by the membrane, there should still be a small margin between the edge of the spot and the cavity wall. The membrane should dry completely at RT before removing it from the holder. If denatured samples are applied, a heat-fixation step is recommended; i.e. the membrane is baked for 3 min at 98°C in a dry heating block (or incubator).

The dry membrane is slowly immersed into the staining solution (Table 5.15) and stained for 3 min with gentle agitation. The staining tray should be closed with a lid

Table 5.14 Methanol-acetic acid

Components	Final conc. (%)	Amount (Ml)
Methanol	90	450
Acetic acid (100%)	10	50
Total volume		500

Stored at 4°C to minimize evaporation.

Table 5.15 Staining solution

Components	Final conc.	Amount
Amido black	0.5%	250 mg
Methanol-acetic acid		50 mL
Total volume		50 mL

Stored at 4°C to minimize evaporation.

Table 5.16 Butanol-methanol-acetic acid

Components	Final conc. (%)	Amount (mL)
Butanol	60	120
Methanol	30	60
Acetic acid (100%)	10	20
ddH_2O to make total volume		200

The solution can be used several times, as long as it stays clear. Stored at 4°C to minimize evaporation.

to avoid the evaporation of methanol. The membrane is then destained for 3 × 5 min and 1 × 15 min in methanol-acetic acid (Table 5.14). Again, the vessels should be closed and the membrane should never fall dry during transfers! If methanol is allowed to evaporate from the membrane, the acetic acid will concentrate and destroy the membrane. Finally, the membrane is equilibrated for 2 min in butanol-methanol-acetic acid (Table 5.16). As acetic acid evaporates faster than butanol, now there is no more danger of destroying the membrane.

A digital picture is taken of the wet membrane, avoiding any air bubbles between the membrane and the support. A video camera system or a flatbed scanner may be used for taking the picture. Any image analysis program able to count pixels values in defined areas (e.g. AIDA 2.31, Raytest GmbH, Straubenhardt, Germany) can be used to convert the staining intensity of the spots to numbers. The net pixel numbers per spot will then be converted to protein concentrations according to the calibration series blotted on the same membrane. Protein concentrations determined in the samples are then plotted against the incubation time to show the degradation kinetics. An exemplary result for positive and negative control is shown in Fig. 5.4. Linear regression has been used to calculate the degradation rates.

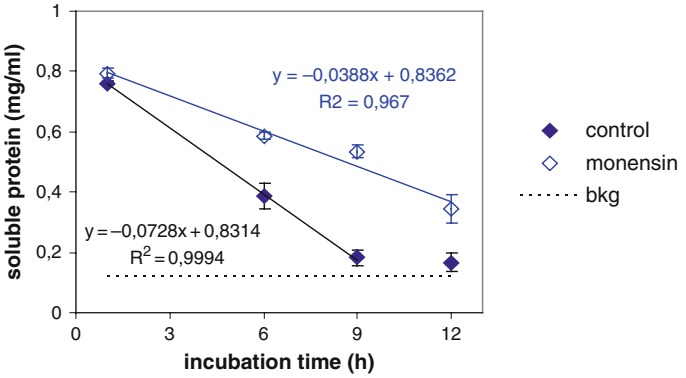

Fig. 5.4 Degradation kinetics of soluble protein in positive and negative controls

Evaluation of Results

The soluble protein concentration at 1 h is taken as reference point for each kinetic. It was shown that 1 h is the point of maximum concentration, due to the slow solubilization of soybean protein [26]. On the background of soybean meal and BSA the inclusion of 150 mg of normal, green plant samples did not significantly increase the measured concentration. (If an unknown sample should be high enough in soluble protein to show up as false positive for anti-proteolytic activity, it can be noted at this point.)

Measurements at later sampling times can be expressed relative to the corresponding 1 h value, or in absolute terms as μg protein degraded. In the latter case, degradation rates (μg/h) can be calculated by linear regression. Both, relative protein concentrations at a given hour, or protein degradation rates can then be evaluated relative to the negative and positive control.

Insoluble protein concentration is of particular interest at 1 h, as increased values will indicate precipitation of dietary proteins, and at the end of 24 h incubation as an estimate of microbial biomass. The sum of soluble and insoluble protein (corrected for respective dilution factors) gives an estimate of total true protein in the sample. However, if proteolysis is inhibited, protein measured at 24 h may still partially constitute undegraded dietary protein. In that case, PAGE analysis is needed to discriminate substrate from microbial protein.

In the negative control (without monensin) the substrate protein was degraded close to background level (bkg) after 9 h, therefore the 12 h value was excluded from regression; the corresponding degradation rate was 72.8 μg/(mL × h). In the presence of monensin, the rate was reduced to 38.8 μg/(mL × h) and degradation was not yet complete after 12 h.

Qualitative Protein Analysis by SDS-PAGE

SDS-PAGE is a technique that separates polypeptides by their molecular mass. The name refers to the detergent sodium dodecylsulphate (SDS), which is used to

Table 5.17 Laemmli buffer (LBF)

Components	Final conc.	Amount
Tris-HCl pH 6.8 (0.5 M)	62.5 mM	12.5 mL
SDS (20%)	2%	10.0 mL
Glycerol (87%)	10%	11.5 mL
2-mercaptoethanol	5%	5.0 mL
Bromophenol blue (0.5%)	0.0025%	0.5 mL
ddH_2O		60.5 mL
To make total volume		100.0 mL

For double strength buffer add only 10.5 mL H_2O *ad* total volume 50 mL. Stored at room temperature.

denature the proteins prior to separation. This also confers a strong negative charge on the proteins, irrespective of their native charge due to amino acid composition. All proteins will thus move towards the anode (+) in an electric field.

The matrix used for electrophoresis is a polyacrylamide gel (PAGE). Small molecules can move faster through this matrix than large ones, thus resulting in separation by molecular mass. A discontinuity in gel concentration and buffer system between the upper "stacking gel" and the actual "separation gel" improves the sharpness of the protein bands and thus the resolution. This system was originally established by Laemmli [18]. It is nowadays a standard method in protein analysis.

Preparation of the Polyacrylamide Gels

Standard protein electrophoresis equipment comprises a setup for casting the gels, a setup for running the gels, and a power supply. Refer to the instructions of the respective manufacturer, how to assemble and use them. The procedure below refers to the Minigel-System of Hoefer (USA) with gel dimensions of 10.1 cm × 8.3 cm × 0.75 mm.

Glass plates and spacers are assembled to form the gel chambers and are tightened by foldback clamps. The lower edge of the chambers is sealed, either by a rubber gasket in the casting stand, by a 1% agarose seal or by an acrylamide plug (see below).

The acrylamide solution for the separating gel (T, Table 5.18) is prepared on ice; immediately before casting the starter compounds (TEMED and ammonium persulphate) are added, carefully mixed, and ca. 4.5 mL of gel solution are pipetted into each chamber to fill it to ca. 2 cm below the upper edge. The gel solution is over layed with water, to get a smooth edge and to exclude oxygen, which would prevent polymerization of the gel in the upper layer. The gels are left undisturbed at room temperature until polymerization is complete; after 1–2 h a sharp interface becomes visible which indicates that the gel has polymerized.

The water layer is removed carefully with a drawn out pipette tip (e.g. gel loader tips), and the surface is rinsed once or twice with water until any unpolymerized residues of acrylamide are removed. Meanwhile the acrylamide solution for the stacking gel (S, Table 5.19) has been prepared on ice; when the separating gels

Table 5.18 PAGE separation gel solution (T)

Components	Final conc.	Amount
1.5 M Tris-HCl pH 8,8[a]	375 Mm	1.50 mL
30% Acrylamide/Bis Solution (37,5:1)	15.0%	3.00 mL
ddH_2O		
10% SDS	0.100%	60.00 µl
TEMED	0.012%	7.20 µl
10% Ammonium persulphate	0.035%	21.00 µl
Total volume (for 1 gel)		6.0 mL

Multiply by the number of gels you intend to cast!
[a]The composition of the buffer stock solution is given in Table 5.21.

Table 5.19 PAGE stacking gel solution (S)

Components	Final conc.	Amount
0.5 M Tris-HCl pH 6,8[a]	125 mM	0.75 mL
30% Acrylamide/Bis Solution (19:1)	3%	0.30 mL
ddH_2O		1.91 mL
10% SDS	0.1%	30.00 µl
TEMED	0.012%	3.60 µl
10% Ammonium persulphate	0.035%	10.50 µl
Total volume (for 1 gel)		3.0 mL

Multiply by the number of gels you intend to cast!
[a]The composition of the buffer stock solution is given in Table 5.20.

are ready, the starter compounds are added. Teflon combs are inserted in the upper space of the gel chamber, leaving sufficient space (ca. 1 cm) between the bottom of the wells and the edge of the separation gel. Then the stacking gel solution is pipetted into the chamber, avoiding any air bubbles, and is left to polymerize. The chamber is filled to the upper edge, and if necessary, more solution can be added carefully during polymerization. After another 1–2 h the gels are ready. The combs are carefully removed and the wells are rinsed once or twice with water. Finally, they are filled with electrophoresis buffer (EP, Table 5.20). Immediate use of the gels is recommended.

Table 5.20 Electrophoresis buffer stock solution (2 × EP)

Components	MW	Final conc.	Amount
Tris	121.1	100 mM	12.1 g
Glycine	75.1	760 mM	57.1 g
10% SDS		0.2%	2 mL
dH_2O *ad* total volume			1000 mL
pH ca. 8.5 *(do not titrate!)*			

Dilute 1 + 1 with dH_2O to get the working solution.

Table 5.21 Stacking gel buffer stock solution (Tris/HCl pH 6.8)

Components	MW	Final conc.	Amount
Tris	121.1	0.50 M	6.06 g
ddH_2O			50 mL
Titrate with HCl to pH 6.8			
ddH_2O to make total volume			100 mL

Store at 4°C.

Gels can be stored overnight at 4°C in a moist chamber; the well should then be filled with 0.125 M Tris-HCl pH 6.8. Before loading, they need to be washed again and filled with electrophoresis buffer.

Note: *If an acrylamide-plug is to be used for sealing the gel chambers, 0.5 mL per gel are taken from the solution and mixed with 10 μl TEMED and 15 μl 10% ammonium persulphate. This solution is carefully and slowly dispensed along the bottom of the gel unit; capillary force should draw the solution into the chamber and form a seal of 1–2 mm width. Because of the high starter concentration, the plug polymerizes very quickly (5 min) and gels can be cast as described above.*

Loading and Running the Gels

An application scheme is prepared for each gel, to define which sample, marker or standard is loaded in which lane. The gel chambers are inserted in the electrophoresis apparatus, and the buffer tanks are filled with electrophoresis buffer. To facilitate sample application the upper can be filled just below the level of the gel edge. With drawn-out tips 6 μl of denatured sample (see "sampling" section above) are carefully pipetted to the bottom of the designated wells, avoiding disturbance by air bubbles. When all lanes are loaded, the buffer tank is filled up above the gel level. The electrodes are connected to the power supply, and the gels are run for 10 min at 80 V and 40 min at 120 V. When the front (visible as a blue line) reaches the bottom of the gel, the run is finished. The apparatus is disassembled; the gels are removed from the chambers and transferred into a fixation bath.

Table 5.22 Separation gel buffer stock solution (Tris/HCl pH 8.8)

Components	MW	Final conc.	Amount
Tris	121.1	1.50 M	18.17 g
ddH_2O			50 mL
Titrate with HCl to pH 8.8			
ddH_2O to make total volume			100 mL

Fixation and Staining

The gels are fixed in 10% sulfosalicylic acid (Table 5.23.) for 30 min with gentle agitation. After that, they are transferred to the staining solution and stained overnight in closed containers with gentle agitation.

Table 5.23 Fixation solution for PAGE gels

Components	Final conc.	Amount
Sulfosalicylic acid	10%	20 g
H_2O		200 mL

Store at RT; repeated use is possible.

Note: *The conventional agent for fixing PAGE gels is trichlor acetic acid (TCA). The use of sulfosalicylic acid instead avoids toxic wastes (halogenated organic solvents).*

The staining solution given in Table 5.24 follows the procedure of Neuhoff et al. [22]. In contrast to common alcoholic staining solutions with CBB-R (Coomassie Brilliant Blue), this provides CBB-G in a colloidal form and thus allows clear background staining at gel concentrations above 9% acrylamide. Furthermore, sensitivity is higher than with CBB-R. The stained gels are briefly rinsed with water to remove dye particles settled on the gel surface (5–15 min, several changes of water). They can then be recorded with a digital camera or a scanner. An exemplary result is shown in Fig. 5.5.

The PAGE-gel in Fig. 5.5 is showing the degradation of individual protein bands during a 24 h incubation of control substrate without and with 3-μM monensin. Lanes are labelled according to the hour of sampling. M = molecular weight marker, high range. Band A (BSA) disappeared after 12 h in the control, but was persistent even after 24 h in the presence of monensin. Band B (an unidentified soybean protein) disappeared after 6 h in the control, and after 9 h with monensin. Band

Table 5.24 CBB-staining solution

Components	Final conc.	Amount
85% Phosphoric acid dissolved in 300 mL dH_2O	2%	10 g
CBB 250 g dissolved in 20 mL dH_2O	0.1%	500 mg
Ammonium sulphate dissolved in 80 mL dH_2O	6%	30 g
– First add CBB to phosphoric acid and stir		
– While stirring, add ammonium sulphate, bit by bit		
– Keep stirring		
Finally add dH_2O to make total volume		500 mL

Store at RT; repeated uses is possible, but avoid acidification due to carry-over of fixation solution, as this will affect the sensitivity of staining. If correctly prepared, the dye is dispensed in a colloidal form, and small particles settle on the bottom of the flask; therefore shake well before use!

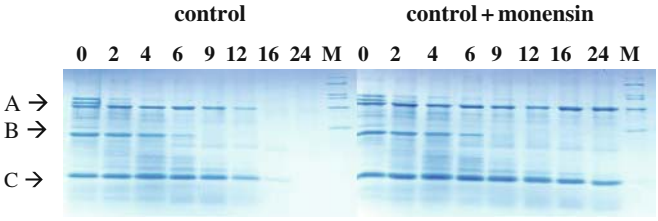

Fig. 5.5 Example of SDS-PAGE analysis of protein degradation in the screening system

C (trypsin inhibitor) disappeared after 16 h in the control, and was still present after 24 h with monensin. The delay of protein degradation due to monensin is thus clearly documented with the described experimental approach.

Calibration and Evaluation

Individual protein bands on a PAGE gel are usually identified/addressed by their molecular mass. Therefore, molecular weight standards should be run on each gel. They are available from various sources; the example shown above (Fig. 5.5) used high range markers from Biorad (161-0303, SDS-PAGE-Standards, high). Broad range markers also cover the lower range down to 6.5 kDa (BioRad #161-0317 SDS-PAGE-Standards, broad). If provided in lyophilized form, marker proteins are reconstituted in water, denatured by adding the same volume of 2 × LBF, and finally diluted to yield suitable band intensity.

Individual protein bands can be quantified, if suitable equipment for densitometry or image analysis is available. In our experiments, we used the same software as in the evaluation of dotblot membranes to quantify selected bands (AIDA 2.31, Raytest GmbH, Straubenhardt, Germany). For calibration BSA standards were prepared at 0.05, 0.1 and 0.2 mg/mL in LBF, and 6 μl of these were loaded on the same gel as the samples to be quantified. The intensities of the target bands could thus be converted to protein concentrations. If the major protein bands of a given lane are quantified like this and added up, the sum correlates quite well with the total protein content determined by dotblot.

Conclusion

Based on an incubation of 12 h, the described screening system allows the detection of slow effects in ruminal protein metabolism, that require microbial growth, in addition to immediate ones mediated e.g. by the precipitation of dietary protein. Yet, it is still short enough to be considered a high throughput system; 15–20 samples per incubation can be handled conveniently. The amount of plant material required is small, with ca. 1.5 g DM for three independent incubations of triplicates. The composition of the standard substrate is optimized not only to sustain high proteolytic activity, but also to reveal the response in proteolysis by straightforward and reliable measurements in the parameters investigated, avoiding extensive dilution

or purification of samples. Nevertheless, analyses can proceed to various degrees of detail. For a rough screening, it may be sufficient to determine total protein by dotblot, SCFA, and ammonium, which can be accomplished in ca. 3 days. To acquire further information on the nature of anti-proteolytic effects, the experimental design can be amended by incubations with and without PEG, to discriminate tannin related activities. PAGE analysis of supernatant and pellet, especially of the 1 h samples, can be an alternative to, or a further validation of this. While the overall influence of a plant additive on general fermentation can already be seen from gas and total SCFA-production, this aspect can be deepened if incubations are extended to 24 h to determine digestibility or microbial protein synthesis by the respective methodologies of nutrition research.

Inhibitor In Vitro Assay of Rate and Extent of Ruminal Protein Degradation

Rationale and Overview

Rates and extents of ruminal degradation of feed proteins are required in a number of systems of ruminant ration formulation. Lack of reliable data on protein degradation can cause farmers to under- or over-feed protein to their livestock. To avoid problems due to either under or over-feeding of protein, routine methods that are both accurate and rapid are needed to allow timely characterization of protein degradation of common feeds. We have devoted a number of years to developing an inhibitor in vitro (IIV) method for assessing protein degradation [2; 3; 4; 5; 7]. With this approach, substrate limiting amounts of protein (i.e., under first-order conditions) are incubated with ruminal inocula to which metabolic inhibitors have been added to allow quantitative recovery of protein breakdown products. Degradation rate (kd) is derived from the time-course of net (i.e., blank-corrected) appearance of degraded protein in the form of total free amino acids plus ammonia. Extent of degradation is computed using this rate and an assumed ruminal passage rate (kp), typically 0.06/h, from the ratio: kd/(kd + kp) [30]. This IIV procedure successfully predicted differences in milk and protein yield of dairy cows fed solvent and expeller soybean meal [8], characterized the ruminal degradability of different species of legume forages [4] and several protein concentrates [11], identified the optimal extent of heating required for protecting protein in roasted soybeans [12], and served as the basis of a solubility test [16] and a near infrared spectrometric calibration [29] to estimate protein degradability in roasted soybeans. Other workers have employed the IIV inoculum, but quantified extent of degradation from net release to total N remaining soluble in the presence of protein precipitants [24, 25].

The following protocol describes the method as used in our laboratory for routine estimation of protein degradation rate and escape, including a shorthand method version that may be useful when assaying large numbers of samples.

Incubation

The basis of the IIV technique is that inhibitors of microbial amino acid and ammonia incorporation, hydrazine sulphate (HS) and chloramphenicol (CAP), when added to in vitro inocula containing mixed ruminal organisms, allow all or virtually all of the protein degradation products to be recovered as ammonia and amino acids. In short-term incubations (less than 6-h long), extent of protein degradation is not underestimated due to microbial uptake of the degradation products. In incubations longer than 6-h, proteolytic activity begins to decline, possibly due to autolysis of microbial enzymes and build-up of end products.

Feed samples are analyzed for dry matter (DM) and total N. Enough sample to provide 1.875 mg N is weighed into each incubation tube (50-mL plastic centrifuge tubes). Each sample is "soaked" in 5 mL McDougall's [21] buffer at 39°C for 1 h prior to starting the incubation. The inoculum is prepared from strained ruminal fluid (SRF) obtained by straining solids from rumen cannulated donor cows through two then four layers of cheesecloth. Pre-incubation of the SRF with soluble carbohydrates is done at 39°C for 3–4 h to reduce background concentrations of ammonia and total amino acids (TAA). The inoculum is a mixture of SRF and McDougall's [21] buffer containing 1.5 mM HS and 45 μg/mL of CAP. Tubes are inoculated with 10 mL of the inoculum (final concentrations = 1.0 mM HS and 30 μg/mL of CAP). Tubes are flushed with CO_2, capped with Bunsen valves (see supplies below) and incubated at 39°C for various time-points up to 6 h. Water baths and incubator ovens and rooms have all been used successfully for this purpose. In the shorthand version of the assay, time-points of only 0- and 4-h are used when large sample numbers are to be studied in the same incubation. This method is described below. Microbial activity is stopped by adding trichloroacetic acid (TCA) to a final concentration of 5% (w/v).

The fraction degraded, and remaining undegraded, at each time-point is computed from net (i.e., blank corrected) release of N in the form of ammonia and total free amino acids (TAA), which are assayed using automated equipment. Formally, TAA were determined by ninhydrin assay, including a correction for ammonia contribution to total colour that was adapted to a continuous-flow system [5]. However, this method has been replaced by a fluorimetric procedure based on orthophthaldialdehyde (OPA) adapted to flow-injection [6]. A variation on the original colorimetric ammonia assay [5] is still used but it is also conducted by flow-injection [6]. The net N released as TAA is computed from the ratio TAA/mg N determined for each protein source being studied after hydrolysis in 6 N HCl. This value is added to N released as ammonia and the fraction degraded is computed based on the amount of protein-N added to each tube (usually 1.875 mg). Fractions degraded and undegraded may be corrected for acid detergent insoluble nitrogen (ADIN) but this has been found to have little effect on rate except for samples that have been extensively heated. Rate of degradation is estimated as the slope of the linear regression of the log of the fraction remaining undegraded on time. Equations used for these computations are described below in detail. Casein and two standard soybean meal samples of known in vivo ruminal degradability are included in each incubation to assess day-to-day variation in activity.

Materials

Chemicals

McDougall's buffer (4 L batch) [21]

- 39.2 g $NaHCO_3$
- 37.1 g $Na_2HPO_4.12H_2O$
  - 2.28 g KCl
  - 1.88 g NaCl

  Dissolve in 4 L of distilled water, and then add

- 0.52 g $MgCl_2.6H_2O$
- 0.182 g $CaCl_2$, anhydrous

Mix until dissolved and then bubble with CO_2 for 2 h (until pH reaches 6.8). Rebubble with CO_2 as necessary to bring pH back to 6.8.

- 3 N NaOH
- TCA solution – 65% wt/vol trichloroacetic acid in distilled water.
- Antifoam 204 (Sigma A-6426)
- Maltose (Sigma M-2250)
- Starch (Sigma S-2004)
- Xylose (Sigma X-1500)
- Pectin (Sigma P-9135)
- Mercaptoethanol (Sigma M-6250) (noxious odour – keep and use in hood)
- Hydrazine sulphate (Sigma H-7394) (toxic)
- Chloramphenicol (Sigma C-0378) (toxic)

Standard Proteins

- Casein (Sigma C-5890)
- Solvent soybean meal (SSBM)
- Expeller soybean meal (SSBM; "SoyPlus")
- Ammonium sulphate (Fisher Scientific A938-500; "Primary Standard")
- Leucine (Sigma L-8000)
- 0.1 N HCl
- 6 N HCl

Supplies

- 1 L thermos
- Large diameter funnel
- Cheese cloth (#58706-4325: American Wipers and Supplies, Milwaukee, WI USA)

- Large graduated cylinders
- 2-l and 5-l Bottles
- 50-mL Polyethylene centrifuge tubes (Nalgene # DS3112-0050; Fisher Scientific, Itasca, IL USA)
- 4 50-mL tubes are dried at 60°C overnight and weighed (see below)
- 5-mL, 12×75 mm, Centrifuge tubes (# 55.5266: Sarstedt, Newton, North Carolina USA)
- Stoppers with Bunsen valve: # 5.5 one-hole rubber stopper; 3/16" outside dimension (O.D.) glass tube; rubber policeman (w/slit) (# 53801-0087: VWR, St. Paul Minnesota, USA).

Inoculum

Steps are conducted under CO_2 to "protect" microbes as much as practical from contact with air. Collect whole rumen contents (liquid plus solids) from 2 rumen cannulated lactating dairy cows just prior to the morning feeding. At the barn, squeeze enough whole rumen contents through 2-layers of cheesecloth into a thermos (warmed previously using 39°C tap water) to yield the required volume of SRF, collecting about half of the total SRF from each cow. Discard squeezed whole contents in the gutter. Back at the laboratory, strain the SRF through 4-layers of cheesecloth into an appropriately sized graduated cylinder that had been flushed with CO_2 and rinsed with warm water. Measure pH and transfer to the pre-incubation flask.

Pre-incubation

A 3 h pre-incubation is carried out in a water bath at 39°C to decrease the background ammonium concentration in the inoculum. In a few cases, ammonia has remained in excess of 2 mM after 3 h and incubating for 4 h was useful to reduce concentration to less than 1 mM. However, the 3 h pre-incubation usually reduces ammonia to < 0.5 mM. For each litre of final inoculum, add 800 mL of SRF, 2.5 g $NaHCO_3$ (dissolved in 50 mL McDougall's [21] buffer), and 0.16 mL antifoam 204 (see above).

Then add the following carbohydrates:

- 6.4 g Maltose
- 3.2 g Starch
- 3.2 g Xylose
- 3.2 g Pectin dissolved in 100 mL warm McDougall's [21] buffer overnight.

Flush flask continuously with CO_2 during pre-incubation. At 0 h and once each h for the total 3 h, take two 3-mL samples and transfer in 12 × 75 mm sampling tubes with 0.25 mL 65% (w/v) TCA solution; hold on ice for 30 min. Also, monitor pH (by inserting electrode into pre-incubating inoculum) and temperature: as necessary,

adjust inoculum pH back up to 6.4 by adding 3 N NaOH. Record volume of NaOH used (total used over 3-h is a crude index of fermentative activity).

After pre-incubation is complete, add (per 950 mL of pre-incubated inoculum), 0.39 mL mercaptoethanol; this should be done in the fume hood because of the noxious odour. Then add the inhibitors: 0.3252 g hydrazine sulphate (dissolved in 25 mL McDougall's [21] buffer) and 0.075 g chloramphenicol (dissolved in 25 mL water). This gives a total volume of 1 L. Mix inoculum with these reagents at 39°C for 20 min before starting the incubation.

Sample Preparation

Samples should be ground through a 1-mm screen. Concentration of N in samples must be determined. Duplicate tubes are used for each sample at each time-point. Empty tubes are used for blanks at each incubation time-point; additional blanks are used if sample set is large. Within each run, at least 3 standard proteins (casein, SSBM, ESBM) are incubated.

Label all incubation tubes and centrifuge tubes with indelible marker.
Weigh into each of 50 mL tube an amount of sample equivalent to 1.875 mg of N.
"Hydrate" these samples for 1 h prior to the incubation by adding 5 mL of warm McDougall's [21] buffer into each tube. Hold at 39°C in the incubator.
Add 1.25 mL 65% w/v TCA (before inoculum to prevent any degradation) to the 0-h-incubation tubes and place these in the ice bath.

Incubation

Add to each tube 10 mL of the inoculum using a Cornwall, re-pipette (or similar rapid dispenser). Start with timed incubations and end with 0 h incubations. Flush the tubes with CO_2 and close them with a stopper fixed with bunsen valve, and incubate for prescribed times with shaking at 150 rpm. We have also found that swirling tubes by hand every 60 min provides satisfactory agitation and mixing over the incubation. After completing inoculation of tubes to be incubated, add 10 mL of inoculum to each of 4 labelled pre-weighed, dry 50-mL centrifuge tubes for the determination of inoculum DM. When incubation time is complete, add 1.25 mL 65% w/v TCA to each tube to kill microbial activity. After each tube at each time-point has received TCA, hand swirl the tubes to mix and place in ice bath for 30 min.

Sampling

For ammonium and total amino acid analyses

- All tubes with TCA are kept on ice for at least 30 min.
- Mix contents of tubes treated with TCA either by hand swirling or using a Vortex mixer and pour an aliquot into a labelled 5-mL 12 × 75 mm centrifuge tube.

- Centrifuge at 10,000*g* for 10 min.
- Pour supernatants into a second set of labelled 5-mL 12 × 75 mm centrifuge tubes, cap them and label cups, and store at 4°C until analysis.

Analytical Procedures

Inoculum Dry Matter Determination

Centrifuge samples taken for inoculum DM determination at 30,000*g* for 15 min. Discard supernatant and dry the pellet at 60°C for at least 48 h. After 48 h, cool these tubes in a desiccator to room temperature for at least 2 h and weigh.

Determination of Ammonium and TAA

Samples from 0-h and other time-point incubations are analyzed for ammonium (based on phenol-hypochlorite reagent) and TAA (based on OPA-fluorimetry) using assays adapted to the flow-injection system [6]. Calibration/recovery is conducted by either standard solutions of ammonium sulphate and leucine in 0.1 N HCl, or using the method of standard additions (MOSA), adding ammonium sulphate and leucine directly to the inoculum matrix [9, 10]. Standards or MOSA standards are placed at the beginning and end of the sample series; additional sets of standards/MOSA standards are used if sample number is large. Standards with appropriate concentration ranges are prepared using ammonium sulphate and leucine in 0.1 N HCl.

Calculations

- Dry matter (DM) of the inoculum in g/l is calculated as:

$$[\text{Weight of tube plus dry pellet in mg} - \text{Weight of empty tube in mg}]/10$$

Most of the DM in the high-speed pellet from SRF is bacterial; variation in inoculum DM content from run to run accounts for some of the variation in degradative activity observed.

- Fraction degraded at each time-point (FDt) is computed from net (i.e., blank corrected) release of N in the form of NH_3 and TAA (in leucine equivalents) using the equation:

$$\text{Fraction degraded (FDt)} = [[(\mu\text{mol } NH_3 \times 0.014007) + (\mu\text{mol TAA}/(\mu\text{mol TAA/mg N})]/\text{mg N}]$$

where μmol TAA/mg N is the AA content (per unit total N) of each protein source determined (after acid-hydrolysis) by OPA-fluorimetry; and mg N is the amount of

protein-N added to each tube (usually 1.875 mg). The FDt may also be computed using the equation:

$$\text{Fraction degraded (FDt)} = [(\text{mg NH3} - \text{N}) + (\text{mg TAA} - \text{N}/0.7003)]/\text{mg N}$$

where "0.7003" is the average proportion of alpha-amino N in total N determined for a number of protein sources and "mg N added" is the amount of feed-N weighed into each tube (usually 1.875 mg N/15 mL incubation mixture). The ratio 0.7003 derives from a mean of 50-mmol total amino acids/mg total N found for a number of feed proteins after acid hydrolysis. This value can differ somewhat between feed proteins because of variation in AA composition. To determine the actual TAA/N ratio for each protein, duplicate protein samples are hydrolyzed for 24 h at 105°C in sealed vials under a N_2 atmosphere in 6 N HCl containing 0.1% wt/vol phenol [19] using a ratio of 1 mg sample N/5 mL of acid [1]. After hydrolysis, samples are cooled, HCl removed by vacuum evaporation, and the residues re-dissolved in 0.1 N HCl. These protein hydrolysates are then analyzed for TAA using the same OPA-fluorimetry assay (with leucine as standard) that is used for samples deriving from in vitro incubations. Response in μmol Leu equivalents/mg N for each protein is then used to compute the value for net TAA release into the amount of degraded protein N. The fraction undegraded is computed:

$$\text{Fraction Undegraded (FUDt)} = 1 - \text{FDt},$$

When individual time-points are used, the natural log of FUDt is regressed on time using linear regression function in Excel; the slope of this line is the fractional degradation rate and has the units "/h". When the shorthand version of the method is used (time-points only at 0- and 4-h only), degradation rate is computed:

- $\text{Degradation Rate (kd), / h} = [\ln(\text{FUD4}) - \ln(\text{FUD0})]/4$

where ln is the natural log and 4 is the incubation time in h. Degradation rate will be negative (reflecting the decreasing amount of intact protein). The potentially degradable fraction (fraction B) is computed using the equation:

$$\text{Fraction B, \%} = [1 - \text{FD0})] \times 100$$

Fraction escaping the rumen (the "bypass" value) may be estimated assuming passage rate (kp) = 0.06/h:

$$\text{Estimated Ruminal Protein Escape, \%} = \text{B} \times [\text{kd} / (\text{kd} + \text{kp})].$$

References

1. Block, R. J. and K. W. Weiss. 1956. The Amino Acid Handbook. Thomas, Springfield, IL.
2. Broderick, G. A. 1978. In vitro procedures for estimating rates of ruminal protein degradation and proportions of protein escaping the rumen undegraded. J. Nutr. **108**:181–190.

3. Broderick, G. A. 1987. Determination of protein degradation rates using a rumen in vitro system containing inhibitors of microbial nitrogen metabolism. Brit. J. Nutr. **58**:463–475.
4. Broderick, G. A. and K. A. Albrecht. 1997. Ruminal in vitro degradation of protein in tannin free and tannin-containing forage legume species. Crop Sci. **37**:1884–1891.
5. Broderick, G. A. and J. H. Kang. 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. J. Dairy Sci. **63**:64–75.
6. Broderick, G. A., P. Udén, M. L. Murphy, and A. Lapins. 2004. Sources of variation in rates of in vitro ruminal protein degradation. J. Dairy Sci. **87**:1345–1359.
7. Broderick, G. A., M. L. Murphy, and P. Udén. 2004. Effect of inhibitor concentration and end-product accumulation on estimates of ruminal in vitro protein degradation. J. Dairy Sci. **87**:1360–1371.
8. Broderick, G. A., D. B. Ricker, and L. S. Driver. 1990. Expeller soybean meal and corn by-products versus solvent soybean meal for lactating dairy cows. J. Dairy Sci. **73**:453–462.
9. Cardone, M. J. 1986. New technique in chemical assay calculations. 1. A survey of calculational practices on a model problem. Anal. Chem. **58**:433–438.
10. Cardone, M. J. 1986. New technique in chemical assay calculations. 2. Correct solution of the model problem and related concepts. Anal. Chem. **58**:439–445.
11. England, M. L., G. A. Broderick, R. D. Shaver, and D. K. Combs. 1997. Comparison of in situ and in vitro techniques for measuring ruminal degradation of animal by-product proteins. J. Dairy Sci. **80**:2925–2931.
12. Faldet, M. A., and L. D. Satter. 1991. Feeding heat-treated full fat soybeans to cows in early lactation. J. Dairy Sci. **74**:3047–3054.
13. Hoeltershinken, M., U. Plitt, F. C. Tammen, P. Hoffmann, and H. Scholz. 1997. Influence of mouldy grass on fermentation and thiamine metabolism in bovine rumen fluid (in vitro). Deutsche Tierärztliche Wochenschrift **104**:17–22.
14. Hoffmann, E. M., S. Muetzel, and K. Becker. 2002. A modified dot-blot method of protein determination applied in the tannin-protein precipitation assay to facilitate the evaluation of tannin activity in animal feeds. Br. J. Nutr. **87**:421–426.
15. Hoffmann, E. M., N. Selje-Assmann, and K. Becker. 2008. Dosage studies on the anti-proteolytic effect of a methanol extract from *Knautia arvensis* in ruminal fermentation in vitro. Anim. Feed Sci. Technol. **145**: 285–301
16. Hsu, J. T. and L. D. Satter. 1995. Procedures for measuring the quality of heat-treated soybeans. J. Dairy Sci. **78**:1353–1361.
17. Koroleff, F. 1976. Determination of ammonia. In: Methods of Seawater Analysis, 126–133. (K. Grasshoff, ed.) Verlag Chemie, Weinheim.
18. LaemmLi, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680–685.
19. Mason, V. C., S. Bech-Andersen, and M. Rudemo. 1979. Hydrolysate preparation for amino-acid determinations in feed constituents.1. Stability of bound amino-acids to oxidation with performic acid hydrogen peroxide reagents. Z. Tierphysiologie Tierernaehrung Futtermittelkunde **41**:226–235.
20. Mauricio, R. M., F. L. Mould, M. S. Dhanoa, E. Owen, K. S. Channa, and M. K. Theodorou. 1999. A semi-automated in vitro gas production technique for ruminant feedstuff evaluation. Anim. Feed Sci. Technol. **79**:321–330.
21. McDougall, E. I. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochem. J. **43**:99–109.
22. Neuhoff, V., R. Stamm, and H. Eibel. 1985. Clear background and highly sensitive protein staining with Coomassie Brilliant Blue dyes in polyacrylamide gels: a systematic analysis. Electrophoresis **6**:427–448.
23. Neuhoff, V., K. Philipp, H. G. Zimmer, and S. Mesecke. 1979. A simple, versatile, sensitive and volume-independent method for quantitative protein determination which is independent of other external influences. Z. Physiol. Chemie **360**:1657–1670.

24. Neutze, S. A., R. L. Smith, and W. A. Forbes. 1993. Application of an inhibitor in vitro method for estimating rumen degradation of feed protein. Anim. Feed Sci. Technol. **40**:251–265.
25. Newbold, J. R., B. DeWannemaeker, and P. Gerardy. 2001. Use of an inhibitor in vitro method to determine protein degradability coefficients in the NRC (2001) protein evaluation system. J. Dairy Sci. **84**(Suppl. 1):363 (Abstr).
26. Selje-Assmann, N., E. M. Hoffmann, S. Muetzel, R. Ningrat, R. J. Wallace, and K. Becker. 2007. Results of a screening proramme to identify plants or plant extracts that inhibit ruminal protein degradation. Br. J. Nutr. **98**:45–53.
27. Selje-Assmann, N., E. M. Hoffmann, and K. Becker. 2008. A batch incubation assay to screen additives for their ability to inhibit rumen protein degradation. Anim. Feed Sci. Technol. **145**:302–318.
28. Tomarelli, R. M., J. Charney, and M. L. Harding. 1949. The use of azoalbumin as a substrate in the colorimetric determination of peptic and tryptic activity. J. Lab. Clin. Med. **34**:428–433.
29. Tremblay, G. F., G. A. Broderick, and S. M. Abrams. 1996. Estimating ruminal protein degradation of roasted soybeans using near infrared reflectance spectroscopy. J. Dairy Sci. **79**:276–282.
30. Waldo, D. R., L. W. Smith, and E. L. Cox. 1972. Model of cellulose disappearance from the rumen. J. Dairy Sci. **55**:125–129.
31. Wallace, R. J. 1983. Hydrolysis of ^{14}C-labelled proteins by rumen micro-organisms and by proteolytic enzymes prepared from rumen bacteria. Br. J. Nutr. **50**:345–355.
32. Wallace, R. J. and J. Kopecny. 1983. Breakdown of diazotised proteins and synthetic substrates by rumen bacterial proteases. Appl. Environ. Microbiol. **45**:212–217.

Chapter 6
Screening for Compounds Enhancing Fibre Degradation

Devki N. Kamra, Neeta Agarwal, and Tim A. McAllister

Introduction

Ruminants have a unique capacity to utilize ligno-cellulosic feeds as a major component of their diet to get energy for their survival. They are also able to utilize non-protein nitrogen sources for the synthesis of microbial protein in the rumen. These tasks are accomplished in the rumen by a complex consortium of rumen microbes that live in an ecto-symbiotic relationship with the host animal. This microbial eco-system consists of bacteria, archaea, protozoa, fungi, mycoplasmas and bacteriophages. These microbes are a mixture of micro-aerophilic, facultative and obligate anaerobes that utilize a minimal amount of feed energy for their survival and conserve more than 85% of gross energy intake in the form of volatile fatty acids which are used by the host as a source of energy. The rumen contains microbes that represent a rich pool of a large number of highly active fibrolytic enzymes. The extraction of energy from the ligno-cellulosic feed is only partial and a substantial amount of dietary energy remains unutilized due to resistance of ligno-cellulose to digestion. Digestion of ligno-cellulose may be limited by the ability of enzymes to gain access to target substrates or by direct inhibition arising from the presence of anti-nutritional factors. Cereal straws, sugarcane tops, green forages and a variable amount of cereal grains are commonly used as feeds for ruminants. Enhancing the digestibility of ligno-cellulosic feeds will be beneficial to that population of ruminants that rely on high-fibre agricultural by-products as staple feed for production.

The physical and chemical characteristics of ligno-cellulosic feeds, such as degree of crystallinity of cellulose, extent of lignification and levels of other anti-nutritional factors, vary considerably. The microbial consortium of the rumen is highly sensitive to the type of diet fed to the animal and therefore the enzyme

D.N. Kamra (✉)
Centre of Advanced Studies in Animal Nutrition, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122, India
e-mail: dnkamra@rediffmail.com (D.N. Kamra); neetaagarwal_1@yahoo.co.in (N. Agarwal); mcallistert@agr.gc.ca (T.A. McAllister)

P.E. Vercoe et al. (eds.), *In Vitro Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies*,
DOI 10.1007/978-90-481-3297-3_6,

profile changes. Tajima et al. [18] reported a decrease in cellulolytic bacteria (*Ruminococcus flavefaciens* and *Fibrobacter succinogenes*), hemi-cellulolytic bacteria (*Eubacterium ruminantium)* and an increase in starch-utilizing bacteria (*Prevotella ruminicola,* 7 fold and *P. bryantii,* 263 fold) by shifting the diet from hay to grain. The variations in rumen microbial and enzyme profile with changing ratio of concentrate to roughage have been reported by Kamra et al. [9] and Agarwal et al. [2]. Their results indicated increased carboxymethylcellulase (CMCase) and xylanase activities by increasing the level of roughage in diet of buffalo. In another in vitro experiment, Agarwal et al. [1] reported that the activities of CMCase and xylanase were significantly higher with maize than with lucerne as substrate, which corresponded to the higher fibre content in maize. Similarly, Hristov et al. [7] and Martin and Michalet-Doreau [13] observed a decrease in cellulase and xylanase activities and increase in amylase activity by shifting the diet from forage to high grain. Increased lignin and nitrogen contents in the diet stimulated cellulase activity in the rumen [15]. It appears from various studies that the enzyme profile of the rumen is dependent on diet composition and that the nature of this profile changes depending on the digestibility of feed. Consequently, alteration in the activity of enzymes and their profile can be used as an indicator of the effect of various feed additives on the digestion process. In screening experiments for improving fibre degradation, enzyme activities of a group of selected enzymes (Table 6.1) responsible for degradation of ligno-cellulosic feed or those responsible for the hydrolysis of bonds between lignin and carbohydrates can be determined. Additionally, in vitro true degradability and degradation of radio-labelled substrates can be used as additional parameters for such screening experiments.

Table 6.1 Major enzymes in the degradation of ligno-cellulosic feeds in the rumen

Substrate	Enzyme	Enzyme code
Carboxymethylcellulose	Endo-β-1,4-glucanase	EC 3.2.1.4
Crystalline cellulose (avicel)	Exo-β-1,4-glucanase	EC 3.2.1.91
Cellobiose, *p*-nitrophenyl-β-D-glucopyranoside	β-1,4-glucosidase	EC 3.2.1.21
Xylan from oat spelt or birchwood	Xylanase	EC 3.2.1.8
p-Nitrophenyl-β-D-xylopyranoside	β-1,4-xylosidase	EC 3.2.1.37
Mannan	β-1,4-mannanase	EC 3.2.1.78
p-Nitrophenyl-β-D-mannopyranoside	β-1,4-mannosidase	EC 3.2.1.25
p-Nitrophenyl-α-L-arabinofuranoside	α-L-arabinofuranosidase	EC 3.2.1.55
p-Nitrophenyl-α-D-galactopyranoside	α-galactosidase	EC 3.2.1.22
2-0-(4-O-Methyl-α-D-glycopyrano-xyluronic acid)-xylobiose	α-glucuronidase	EC 3.2.1.3
Acetylated xylan	Acetyl xylan esterase	EC 3.2.1.72
Starch free wheat bran	Ferulic esterase	EC 3.1.1.73

Fibre-Degrading Enzymes

Extraction of Enzyme

The fibre-degrading enzymes are extra-cellular but mostly cell bound. Therefore, the enzymes are extracted from the microbes by cell lysis for estimation of enzyme activity [3, 7].

Reagents

1. 0.1 M phosphate buffer, pH 6.8.
   Stock solutions
   a) 0.2 M monobasic sodium phosphate (NaH_2PO_4) 27.8 g/1000 mL
   b) 0.2 M dibasic sodium phosphate ($Na_2HPO_4.7H_2O$) 53.65 g/1000 mL
   Mix 51 mL of solution (a) and 49 mL of solution (b) and make volume up to 200 mL to provide a 0.1 M buffer, pH 6.8.
2. Lysozyme (0.4%): Dissolve 0.4 g lysozyme in 100 mL 0.1 M phosphate buffer, pH 6.8. Make fresh before use or store in small aliquots at –20°C.
3. Carbontetrachloride.

Procedure

1. Incubate feed (200 mg) for 24 h in in vitro gas production test (method described in this laboratory manual: Chapter 7). The substrate used is either hay or a mixture of wheat straw and concentrate mixture in the ratio of 1:1. The compounds to be tested can be added as such in the substrate. To test the plants containing secondary metabolites, the plant parts are either added after drying and grinding or extracts are prepared using different solvents. A required amount of plant extract (10–500 μL/30 mL reaction mixture) is pipetted into the syringe before the buffer (containing the rumen liquor) is added. While testing of extracts, syringes containing respective solvents are to be prepared which serve as control. For each treatment, a minimum of 4–6 syringes should be used. Along with controls and treatments, 2–3 syringes should be prepared as blank (without substrate) to estimate the contribution of inoculum to the production of gas and other fermentation products, which should be subtracted from the respective observations from the treatment syringes. Every set of syringes should also contain two syringes of standard (a feed with known gas production) to check the reproducibility of the system. We use maize hay as a standard in all our experiments.
2. After the incubation, transfer the contents of each syringe to a 100 mL beaker.
3. Add 5 mL lysozyme solution and 5 mL carbon tetrachloride.
4. Incubate for 3 h at 39°C.
5. Stop reaction by placing it in ice bath.

6. Sonicate (50 mV) the sample for 6 min with 30 s pulses and intermittent cooling for 30 s/min. During sonication, the sample should be immersed in ice bath.
7. Centrifuge the sample at 27,000*g*, at 4°C for 20 min.
8. Collect supernatant and determine enzyme activity.

Cellulases

Carboxymethylcellulase (Endo-1,4-β-glucanase, EC 3.2.1.4)

Endoglucanase (endo-1,4-β-D-glucan-4-glucanohydrolase EC 3.2.1.4) hydrolyses β-1,4-glycosidic bonds of the cellulose chain at random sites, resulting in a decrease in chain length and an increase in reducing ends of the polymer. To estimate the enzyme activity, amorphous celluloses such as carboxymethylcellulose or hydroxymethylcellulose are used as substrate. With these substrates the action of exoglucanase (exo-1,4-β-D-glucan-4-cellobiohydrolase EC 3.2.1.91) is limited therefore it represents only endoglucanase activity. During incubation of substrate with the enzyme at suitable temperature for 1 h, the reducing sugars are released and then are estimated spectrophotometrically using method described by Miller [14]. The amount of reducing sugars (glucose) released per unit of time during incubation represents the enzyme activity.

Reagents

1. 0.1 M phosphate buffer, pH 6.8.
2. Carboxymethylcellulose (1%): Place 1 g of carboxymethylcellulose in a 250 mL Erlenmeyer flask and add 100 mL distilled water. Stir on magnetic stirrer until a homogenous viscous solution is obtained. Store at 4°C.
3. Dinitrosalicylic acid (DNS): Dissolve 10 g sodium hydroxide pellets in 500 mL distilled water. Add 10 g DNS and 2 g phenol and dilute to 1 L with distilled water. Sodium sulphite (0.05%, w/v) is added just before use. Store it in an amber coloured bottle at room temperature. Without sodium sulphite, it can be stored for a period of 1 month.
4. Rochelle salt (40%): Dissolve 40 g of Rochelle salt (sodium-potassium tartrate) in distilled water and make volume up to 100 mL. Store at room temperature.
5. Standard glucose (0.1%): Dissolve 100 mg of glucose in 100 mL distilled water.

Procedure

1. Prepare tubes as follows

   - *Test*: Combine 1.0 mL phosphate buffer, 0.5 mL sample and 0.5 mL carboxymethylcellulose in a test tube and mix well. Incubate the tubes for 20 min at 39°C. Stop the reaction by adding 3 mL DNS.

Table 6.2 Plotting a calibration curve for estimation of glucose

Tube No.	Blank	1	2	3	4	5	6
Distilled water (mL)	2.00	1.75	1.50	1.25	1.00	0.75	0.50
Standard glucose (mL)	0.00	0.25	0.50	0.75	1.00	1.25	1.50
Glucose concentration (μg)	0.00	250	500	750	1000	1250	1500
DNS reagent (mL)	3.00	3.00	3.00	3.00	3.00	3.00	3.00

- *Control:* Mix 1.0 mL phosphate buffer, 0.5 mL sample in a test tube. Add 3 mL DNS and followed by 0.5 mL carboxymethylcellulose solution (DNS to be added before the substrate so that enzyme does not get a chance to react with the substrate).

Note: Control tube is prepared to account for the reducing sugars present in the enzyme sample. It also takes care of colour imparted by the sample, if any.

- *Standard*: Prepare tubes in duplicate with the standard glucose solution and distilled water to plot a calibration curve as described in Table 6.2.

2. Keep all the tubes in boiling water bath for 10 min.
3. Add 1 mL Rochelle salt in each tube and cool to room temperature.
4. Read absorbance at 575 nm against blank (the samples may be diluted to 10 mL with distilled water, if it reads very high absorbance, but do not forget to draw a standard curve with volumes made to 10 mL).
5. Prepare calibration curve by plotting absorbance against glucose concentration.

Calculation

Change in absorbance A = Absorbance of Test – Absorbance of Control

Read ΔA on the calibration curve to determine the μg glucose released during incubation where:

$$\text{Enzyme activity (Units)} = \mu\text{mol glucose/mL/h} = (\mu\text{g glucose})/\text{T} \times \text{S} \times 180,$$

and T = Incubation time (h), S = volume of sample (mL), and 180 = molecular weight of glucose.

Avicelase (Exo-β-1,4-glucanase, EC 3.2.1.91)

Avicel (from Fluka BioChemika, catalogue number 11365) is a microcrystalline cellulose and when used as substrate it represents the activity of all the three enzymes (exoglucanase, endoglucanase and β-glucosidase) with majority of exoglucanase. The procedure for estimation of avicel-degrading activity is similar to that for endo-l,4-β-D-glucanase, but with the following differences:

1. Avicel (1%): Suspend 1 g avicel in 100 mL of 0.1 M phosphate buffer (pH 6.8) and incubate at 4°C for 48 h for proper swelling of the substrate.
2. Assay mixture: 1 mL of 1% avicel suspension (pipette with continuous shaking) and 1 mL enzyme sample.
3. Incubation time: 1 h at 39°C with continuous shaking.
4. Before measuring absorbance, filter or centrifuge the contents to remove unhydrolyzed avicel crystals.

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21)

This enzyme catalyses the hydrolysis of cellobiose and other water-soluble cellodextrins to release glucose. For the estimation of enzyme activity, commonly used substrates are cellobiose and *p*-nitrophenyl-β-D-glucopyranoside (PNPG). With PNPG as a substrate, the enzyme activity is determined by measuring the amount of *p*-nitrophenol released during incubation of substrate with the enzyme as described by Shewale and Sadana [16].

Reagents

1. 0.1 M phosphate buffer, pH 6.8.
2. PNPG (0.1%): Dissolve 100 mg of PNPG in 100 mL of the phosphate buffer. Store in amber-coloured bottle at 4°C.
3. *p*-Nitrophenol (0.01%): Dissolve 10 mg of *p*-nitrophenol in 100 mL distilled water.
4. Sodium carbonate (2%): Dissolve 2 g sodium carbonate in 100 mL distilled water.

Procedure

1. Prepare the tubes as follows:

   - *Test:* Mix 0.1 mL enzyme and 0.9 mL PNPG. Incubate the tubes for 10 min at 39°C. Stop the reaction by adding 1 mL sodium carbonate. Read absorbance at 400 nm against blank.
   - *Control:* Mix 0.1 mL enzyme and 1 mL sodium carbonate. Add 0.9 mL PNPG solution. (Sodium carbonate is to be added first so that enzyme does not get chance to react with the substrate).

Note: Control tube will take care of colour of the sample, if any.

   - *Standard:* Prepare tubes of graded concentration of *p*-nitrophenol in duplicate as described in Table 6.3.

2. Read absorbance at 400 nm against blank.

Table 6.3 Plotting a calibration curve for estimation of *p*-nitrophenol

Tube no.	Blank	1	2	3	4	5	6	7
Distilled water (mL)	1.00	0.98	0.96	0.94	0.92	0.90	0.85	0.80
p-nitrophenol (mL)	0.00	0.02	0.04	0.06	0.08	0.10	0.15	0.20
p-nitrophenol (μg)	0.00	2.0	4.0	6.0	8.0	10.0	15.0	20.0
Sodium carbonate (mL)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

3. Prepare a calibration curve by plotting absorbance against concentration of *p*-nitrophenol.

Calculations

Change in absorbance A = Absorbance of Test – Absorbance of Control
Read ΔA on calibration curve to get the amount (μg) of *p*-nitrophenol released.

$$\text{Enzyme activity (Units)} = \mu \text{ mol } p\text{-nitrophenol/mL/min} = (\mu\text{g } p\text{-nitrophenol})/\text{T} \times \text{S} \times 139.11$$

where T = time (minutes), S = volume of sample taken (mL), and 139.11 = molecular weight of *p*-nitrophenol

Xylanases

Endoxylanase (β-1,4-xylan xylanohydrolase; Endo-β-1,4-xylanase, EC 3.2.1.8)

This enzyme breaks the backbone of xylan, producing both substituted and non-substituted shorter oligomers, xylobiose and xylose. Activity of the enzyme is determined by estimating spectrophotometrically the amount of reducing sugars released in terms of xylose during incubation of enzyme with substrate.

Procedure

The procedure for estimation of xylanase is similar to that of endoglucanase

1. 0.25% xylan: Combine 250 mg xylan (from Birchwood, catalogue number X-0502 SIGMA) and 100 mL distilled water. Warm at 70°C for 10 min with continuous shaking.
2. Prepare standard curve using graded concentration of xylose.
3. The assay mixture contains 1 mL phosphate buffer, 0.5 mL sample and 0.5 mL 0.25% xylan.

4. Incubation time 30 min.
5. Enzyme activity Units/mL = μ mol xylose/mL/min = (μg xylose)/T × S × 150,

where T = time (minutes), S = volume of sample taken (mL), and 150 = the molecular weight of xylose.

β-Xylosidase (β-1,4-D xylan xylohydrolase: Exo-β-1,4-D xylosidase, EC 3.2.1.37)

Hemicellulose is the predominant xylo-glucan polymer in plant tissues which on hydrolysis releases small chain xylose polymers and xylose. β-Xylosidase further hydrolyses these small polymers to release xylose. Using *p*-nitrophenyl-β-D-xylopyranoside as a substrate, the activity of enzyme is determined spectrophotometrically by measuring the amount of *p*-nitrophenol released during incubation of the enzyme with substrate.

Procedure

Procedure for β-xylosidase estimation is similar as that for β-glucosidase with the only difference that the substrate is 0.1% *p*-nitrophenyl-β-D-xylopyranoside.

Acetyl Esterases (EC 3.1.1.6)

These enzymes hydrolyze acetylated carbohydrates like acetyl xylose, acetyl mannose, acetyl glucose, acetyl maltose, acetyl cellobiose, etc. The enzyme activity is estimated by measuring the amount of *p*-nitrophenol released during reaction of enzyme with *p*-nitrophenyl acetate. Method of Huggins and Lapides [8] is described with some modifications.

Reagents

1. Phosphate buffer (0.1 M), pH 6.8.
2. *p*-Nitrophenyl acetate (2 μmol/mL): Dissolve 36 mg *p*-nitrophenyl acetate in minimum volume of dimethyl sulfoxide and dilute to 100 mL with the buffer. For the dilution, tip of the pipette containing the substrate is dipped in the buffer and dispensing is carried out with continuous shaking of volumetric flask otherwise precipitates are formed.

Note: The substrate is unstable; it should be used within 30 min of its preparation.

Procedure

1. Mix 1.0 mL buffer, 0.9 mL *p*-nitrophenyl acetate and 0.1 mL enzyme sample.
2. Incubate at 39°C for 10 min.
3. Read absorbance at 410 nm.
4. A reagent blank is prepared that includes all the reagents except enzyme.
5. If enzyme is coloured, prepare an enzyme blank with all the ingredients except substrate.
6. Prepare a calibration curve using graded concentration of *p*-nitrophenol.

Feruloyl and p-*coumaryl Esterases*

These esterases selectively hydrolyse the ester bond between L-arabinosyl residues of xylan and ferulic or *p*-coumeric acid. The enzyme releases ferulic acid or *p*-coumaric acid from the substrate (starch-free wheat bran) when the enzyme is incubated with substrate. The ferulic acid or *p*-coumaric acid thus released is quantified using HPLC [12].

Reagents

1. Phosphate buffer: 0.1 M, pH 6.8.
2. Starch free wheat bran: Prepare starch-free wheat bran by treating it with excess volume of potassium acetate (0.25% w/v) at 95°C for 10 min. In our laboratory, we suspend 10 g wheat bran in 200 mL potassium acetate (0.25% w/v). Wash thoroughly the treated wheat bran until the washings are neutral, which indicates removal of potassium acetate completely. Dry it in oven at 80°C and store at room temperature.

Procedure

1. Combine 100 mg starch-free wheat bran, 1.0 mL buffer and 1.0 mL enzyme in a test tube.
2. Incubate the tubes for 30 min at 39°C.
3. Stop the reaction by boiling for 3 min.
4. For control tube, add enzyme sample after denaturation by boiling for 3 min.
5. Centrifuge the tubes to remove residual substrate.
6. Filter the supernatant through a 0.2-μm membrane before loading onto the HPLC.
7. The quantification of ferulic acid and *p*-coumaric acid is done by using the following conditions:

   - Column reverse phase C18 (Octadecylsilane)
   - Column temperature 40°C

Mobile phase: Use either

(i) 10 mM NaOH solution, adjusted to pH 3.0 by adding formic acid (88%, v/v). Mix this NaOH solution and methanol (HPLC grade) in 79:21 ratio.

or

(ii) Mix 750 mL water, 250 mL acetonitrile, 40 mL of tetramethyl ammonium hydroxide (25%,v/v), and 5.7 mL of orthophasphoric acid (88%, v/v; pH 3.0).

- Flow rate 1.5 mL/min.
- Ferulic acid or *p*-coumaric acid (1μg/mL) is used as a standard.
- A Unit of enzyme activity is expressed as μg ferulic acid or *p*-coumaric acid released/mL/h.

Note: The linearity of the enzyme reaction was assessed with respect to time and enzyme concentration for all the enzymes assays. It is suggested that the workers confirm this for the enzyme preparation they obtain.

Protein Estimation

Estimating Protein Quantities

Protein estimation as described by Lowry et al. [11] is carried out by the formation of copper protein complex in alkaline medium. This complex then reduces phosphomolybdic-phosphotungstate reagent to yield intense blue colour.

Reagents

1. Standard solution of bovine serum albumin (0.06% BSA): It is prepared in distilled water to contain 0.6 mg BSA/mL.
2. Trichloroacetic acid (20% TCA): Dissolve 20 g TCA in distilled water and make the final volume to 100 mL.
3. Solution A: Dissolve 2 g sodium carbonate in 100 mL of 0.1 N NaOH.
4. Solution B: Dissolve 1 g sodium-potassium tartrate in 100 mL distilled water. Add to it 0.5 g copper sulphate and keep it overnight at room temperature. Filter to remove the precipitate, if any. Solution A and B can be stored at room temperature.
5. Solution C: Mix 50 mL solution A and 1 mL solution B just before use.
6. Solution D: Mix 1 mL of 2 N Folin and Ciocalteu's phenol reagent and 2 mL distilled water just before use.

Procedure

1. Mix 1.0 mL sample with 1.0 mL 20% TCA and leave it overnight.
2. Centrifuge at 5000 g for 5 min. Discard the supernatant.

3. Dissolve the precipitate in 1 mL of 1 N NaOH.
4. Combine 0.1 mL sample and 0.4 mL distilled water in a test tube in duplicate.
5. Prepare tubes of standard BSA in duplicate as described in Table 6.4.
6. Add 5 mL solution C in all tubes and leave for 10 min at room temperature.
7. Add 0.5 mL solution D and mix it immediately and vigorously.
8. After 10 min record absorbance (A) against blank at 600 nm.
9. Prepare calibration curve by plotting absorbance against BSA concentration.
10. Calculate protein concentration mg/mL by reading absorbance of sample on standard curve.

Table 6.4 Plotting a calibration curve for estimation of protein

Tube no.	Blank	1	2	3	4	5	6
BSA (mL)	0.0	0.05	0.10	0.20	0.30	0.40	0.50
Distilled water (mL)	0.50	0.45	0.40	0.30	0.20	0.10	0.00
BSA (μg)	000	30	60	120	180	240	300

Precautions

Folin and Ciocalteu's reagent is only stable in the acidic medium. Care should be taken while adding it to the alkaline medium. It should be well mixed immediately to reduce the solution in step 7 above before it becomes degraded in the alkaline medium.

The protein content in the sample is used to express enzyme activity in terms of specific activity as follows:

$$\text{Specific activity (units/mg protein)} = \text{Activity (in units/mL)/mg protein/mL}$$

In Vitro True Digestibility

Reagents

Neutral detergent solution

1. Sodium lauryl sulphate (SLS) 30.00 g
2. Disodium ethylene diamine tetra-acetate (EDTA) 18.61 g
3. Sodium borate decahydrate (Borax) 6.81 g
4. Disodium hydrogen phosphate anhydrous 4.56 g
5. 2-Ethoxy ethanol (purified grade) 10.00 mL

Take weighed amount of EDTA and borax in a beaker (2000 mL capacity) and add 500 mL distilled water. Heat to dissolve ingredients and add weighed amount of SLS and 2-ethoxy ethanol. In another beaker dissolve disodium hydrogen phosphate in 100 mL distilled water. Mix well the two solutions and check pH. If properly prepared, the pH should be 6.9–7.1. Adjust pH if required. Make volume to 1000 mL.

Procedure

Incubate feed (200 mg) for 24 h in in vitro gas production test (method described in this laboratory manual in Chapter 7) and transfer the of the syringe is to a spoutless beaker by repeated washing with 100 mL neutral detergent solution. Reflux the content of the beaker is refluxed for 1 h and filter through pre-weighed Gooch crucibles (Grade G1). After drying for 24 h at 80°C, weigh the crucible to obtain NDF content of the residue. In vitro true digestibility of feed is calculated as described by Van Soest and Robertson [19]:

$$\%\text{True digestibility (TD)} = \{(\text{Initial DM of feed} - \text{NDF in residue}) \times 100\}/(\text{Initial DM of feed})$$

The enhancement in fibre-degrading activity due to inclusion of a compound can be assessed by comparing the results of samples tested (enzyme activity and in vitro true digestibility) with those obtained in control reactions. If the extracts are tested, comparisons are made with the controls containing respective solvents used for the extraction of the compound. An increase in the activity of fibre-degrading enzymes and in vitro true digestibility will indicate fibre degradation-enhancing activity of the compound.

Isotopic Labelling of Plant Cell Walls

Isotopic labelling of plants has also been used as a method of defining the extent of microbial hydrolysis of plant cell walls and to assess the digestibility of forages by ruminants. Early studies almost exclusively utilized ^{14}C as a means of labelling plant tissues [6, 10], but more recent studies have used the stable isotope ^{13}C for this purpose [17]. Others have focused on specifically labelling lignin through the introduction of ^{14}C–phenylalanine into the plant [4]. Degradation of lignin is of particular focus in many studies due to its role in limiting the rate and extent of overall cell wall digestion. Increased restrictions on the use of radioactive isotopes in the laboratory and in animal feeding experiments have encouraged the use of stable isotopes to assess the rate and extent of ligno-cellulose degradation. There is also evidence that ^{13}C results in more uniform labelling of plant tissues as well. Both of these procedures require the availability of sophisticated

laboratory equipment such as solid-state oxidizers and liquid scintillation counters for radioactive isotopes and an isotope ratio mass spectrometer for stable isotopes. The sophistication of the analytical equipment required and the need for specialized training in the handling of radioactive isotopes may limit the number of laboratories that can utilize isotopes to characterize the degradation of plant cell walls.

^{14}C- and ^{13}C-Labelling of Plant Cell Walls as a Means of Assessing Ligno-Cellulose Degradation

Equipment Required

Sealed growth chamber with circulation fans
Combustion analyzer and scintillation counter; ^{14}C
Isotope mass ratio spectrometer; ^{13}C:^{12}C ratios

Reagents

1. 99% atom% $^{13}CO_2$
2. Pee Dee Belemnite standard for ^{13}C
3. $^{14}CO_2$ in the form of $Ba^{14}CO_3$ or $NaH^{14}CO_3$ (150 μCi)
4. Phosphoric or sulphuric acid for liberation of $^{14}CO_2$
5. L-[U-^{14}C] phenylalanine

^{14}C or ^{13}C Procedure for Whole Plant Labelling

1. Place plants in a transparent gas tight chamber equipped with circulation fans. Figure 6.1 illustrates a simple chamber system designed by Fallon and Pfaender [6] consisting of a Plexiglas chamber equipped with fluorescent lights and a fan to circulate the air in the chamber. Temperature of the overall system can be controlled by placement of this unit in a larger temperature-controlled chamber or by running tap water at a controlled temperature down the walls of the chamber. Include a dehumidifier in the chamber if labelling is going to be conducted over a prolonged period [10]. Fertilize plants prior to placement in the chamber and ensure adequate light.
2. For ^{14}C, directly infuse $^{14}CO_2$ into the chamber or liberate $^{14}CO_2$ via the addition of acid into an aqueous solution of $Ba^{14}CO_3$ or $NaH^{14}CO_3$.
3. Monitor levels of $^{14}CO_2$ in the chamber via collection of gas samples and their subsequent absorption into 2 N NaOH and measuring counts using a liquid scintillation counter.

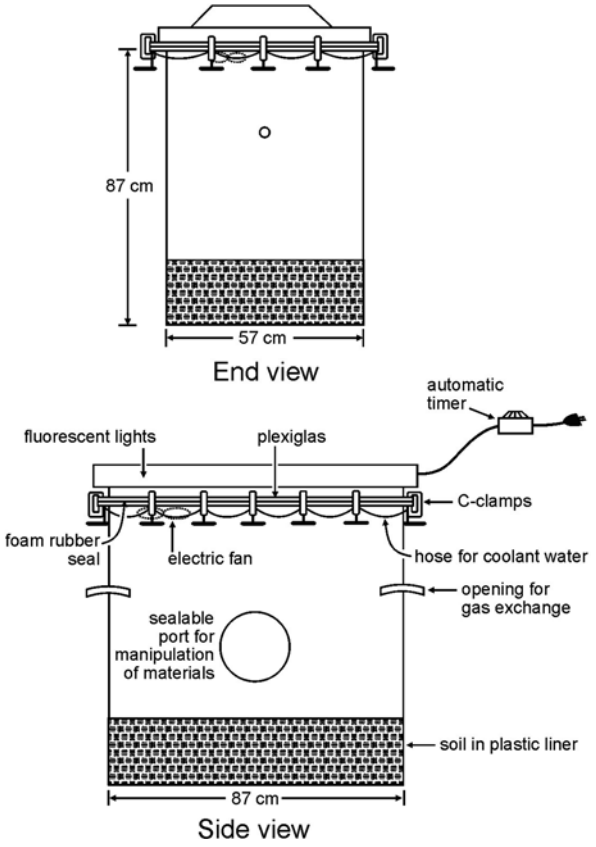

Fig. 6.1 A simplistic growth chamber for the labelling of plants with $^{14}CO_2$ or $^{13}CO_2$ for digestion studies. Adapted from [10]

4. For ^{13}C, infuse 99 atom% $^{13}CO_2$ directly into the chamber to achieve a total CO_2 concentration that is at least 640 ppm above ambient CO_2 concentration.
5. Retain plants in the presence of labelled CO_2 for a minimum of 40 min. Uniform labelling of all C fractions within the plant may require exposure periods greater than 8 h.
6. If more uniform labelling of cell wall fractions is desired, the harvest time can be extended to a week or longer. Complete uniform labelling of the cell wall may require that the plant be exposed to $^{14}CO_2$ from the seedling stage onward, as the turnover of C in the cell wall fractions occurs at a very slow rate.
7. After harvest, air-dry plants at 25–30° C for a period of 3–5 days.
8. Dissect plants into component parts (e.g., leaves, stems, roots) to examine digestion of specific components or grind the whole plant (1–4 mm screen) to assess total plant cell wall digestion.

9. Utilize labelled plant material in in vitro or in in situ digestibility procedures as described in the present manual.
10. Estimate the liberation of $^{14}CO_2$ or $^{13}CO_2$ from plant cell wall or the amount of labelled C in the residue after digestion as a means of estimating the extent and rate of degradation of the plant cell walls. Plant dry matter can be further fractionated into components as described by Alexander et al. [5] and as shown in Fig. 6.2.

Once generated ^{14}C- and ^{13}C-labelled forages can then be employed in a variety of digestive procedures that will result in the liberation of C from the labelled substrate. This could include the use of the in vitro digestibility procedure as described above. The in vitro procedure can be performed with collected digestive fluids (e.g., ruminal, intestinal, faecal), defined enzyme cocktails or pure cultures of microorganisms. Substrates are incubated with the digestive solution for a defined period (e.g., 4, 24, and 48 h).

Labelled substrates can also be employed in the in situ procedure where nylon bags of known pore size and a weighted amount of substrate are incubated within the rumen and removed at predetermined time points. Remaining residue is subsequently collected and the level of label remaining is assessed.

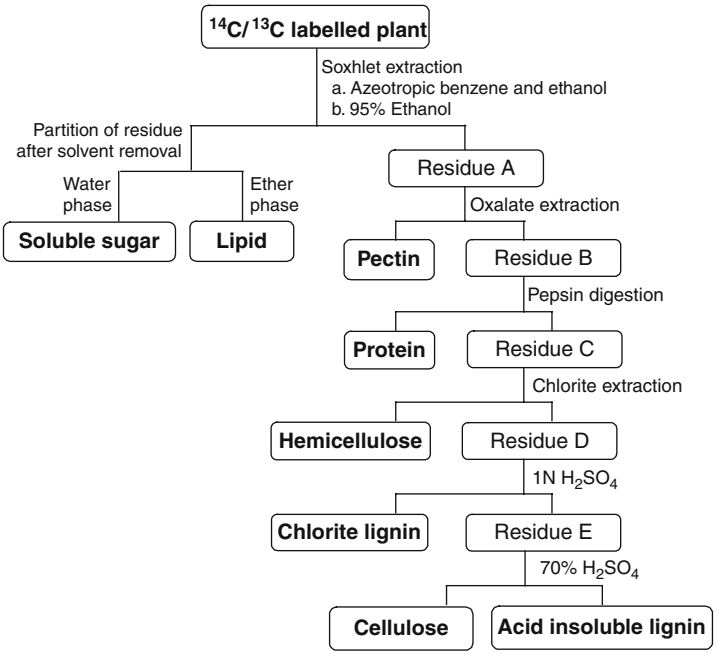

Fig. 6.2 Scheme for the chemical fractionation of labelled plant material in order to study specific degradation of soluble sugar, lipid, pectin, protein, hemicellulose, lignin and cellulose components of plants. Adapted from [5]

^{14}C-Labelled Plant Substrates

Collection of Liberated $^{14}CO_2$

Liberated $^{14}CO_2$ arising from digestion in incubation flasks is trapped by passing liberated gases through sparging tubes containing 2 N NaOH during the incubation. In prolonged incubations (>48 h), NaOH solutions are renewed every 48 h to ensure that saturation of the NaOH solution with CO_2 does not occur. Incubations can be terminated with 0.5 M H_2SO_4 at quantities to ensure that the pH of the incubation fluid is <2, with care taken to ensure that because of acidification, the released CO_2 is passed through the sparging tubes. A solution of $NaH^{14}CO_3$ of known radioactivity can be placed in the incubation vial and used to estimate the yield of $^{14}CO_2$ from acidified fermentation liquids. NaOH along with trapped $^{14}CO_2$ are introduced into counting vials. Radioactivity in the liquid is determined by liquid scintillation using a PCS solubilizer (Amersham Corp., Arlington Heights, IL) or other suitable scintillation liquid such as Hydrofluor or Picofluor 30 containing 1% Carbosorb (Packard, Stockholm, Sweden). Counting solutions should be allowed to standard for at least 1 h prior to counting in a liquid scintillation spectrophotometer. Degradation data can be expressed as $^{14}CO_2$ liberated per unit time (e.g., h, day) and expressed as a percentage of the total radioactively labelled ^{14}C substrate that was included in the incubation. Samples are corrected for background radioactivity and counting efficiency as determined by using an external standard such as ^{226}Ra.

Estimation of Retained ^{14}C in Plant Material

Retained ^{14}C in the remaining residual solid substrate is estimated by combustion of the residue in an oxidizer such as a Packard Tri Carb sample oxidizer. The liberated $^{14}CO_2$ is trapped in a mixed scintillation liquid consisting of a mixture of Carbosorb: Permafluor V at 9:13. In in vitro incubations, differential centrifugation of the culture liquid (i.e., 2500*g* for 5 min, 5°C followed by 10,000*g* for 30 min, 5°C) results in the isolation of a microbial fraction. The microbial fraction is washed/centrifuged 3 times using phosphate buffered saline and the microbial fraction dried at 60°C. The isolated microbial fraction can then be subjected to combustion in order to estimate incorporation of ^{14}C into microbial matter.

^{13}C-Labelled Plant Substrates

^{13}C-Labelled plant material can be prepared in a manner similar to that described above with the exception that $^{13}CO_2$ as opposed to $^{14}CO_2$ is provided to the plants for photosynthesis. $^{13}C/^{12}C$ ratios are determined either in trapped CO_2 or within the residual substrate using stable isotope ratio mass spectrometry. By determining the natural abundance of ^{13}C in non-labelled plants, the excess ^{13}C attributable to the labelling procedure can be determined. $^{13}C/^{12}C$ ratios are determined in the

sample and standards and reported relative to the international PeeDee Belemnite standard and expressed as parts per thousand.

Indicators of Digestive Activity

In in vitro incubations, enhanced liberation of ^{13}C- or ^{14}C-labelled CO_2 is indicative of increased digestion. Fractionation of plant material (as illustrated in Fig. 6.2) and incubation of these fractions with microbial cultures separately is a powerful approach to defining the rate and extent of degradation of various plant fractions. Conversely, conservation of labelled ^{13}C or ^{14}C in the residue remaining after digestion is indicative of those substrates that were not readily subject to enzymatic liberation by microbial enzymes. In the in situ procedure, analysis of the residue remaining after incubation is the only method of estimating the degree of degradation of cell wall fractions as trapping of any liberated $^{14}CO_2$ or $^{13}CO_2$ is not practical under these conditions. Remaining residue can still be subjected to the fractionation scheme illustrated in Fig. 6.2 as a means of assessing the extent of degradation of various chemical components within plants.

^{14}C-Labelled Lignin Using L-[U-^{14}C] Phenylalanine

1. Label lignin by administration of [L-U-^{14}C] phenylalanine through the last node using the stem infusion method. Plants are cut under water at the last node and placed in tubes containing water. Water is removed and a radioactive solutions containing L-[U-^{14}C] phenylalanine is introduced and retained with the plants until uptake of the labelled lignin precursor is complete. Once uptake is complete, a standard plant nutritive solution is added and after a period of at least 6 h in the light, plants are allowed to metabolise for a minimum of a 96 h in the dark at 25°C while being retained in the nutritive solution.
2. Grind the upper and lower halves of the apical internodes in liquid nitrogen.
3. Remove labelled proteins by incubating ground powder in pronase (2250 units/g) in 50 mL of 0.1 M phosphate buffer (pH 7) for 2 h at 35°C.
4. Wash residue in distilled water and dry at 60°C for 48 h.
5. Prepare cell wall residues by refluxing the cell wall material in 1:2 ethanol-toluene followed by 95% ethanol until the extracts become colourless.
6. Labelled phenolic residues are then used as substrates in the in vitro procedures described above.
7. Liberated $^{14}CO_2$ is trapped in sparging tubes or ^{14}C in the residue remaining residue can be determined as described above. The use of radiolabelled phenylalanine and sinapic acid results in a more targeted labelling of the phenolic elements of lignin as opposed to the use of $^{14}CO_2$, which results in the labelling of all carbon fractions within the plant.

Indicators of Digestive Activity

The level of radioactivity is examined in the solid (remaining residual) liquid (solubilised phenolic acids) and $^{14}CO_2$ fractions (liberated C) as described above. Those incubations that produce the highest fraction of $^{14}CO_2$ are indicative of high lignin degradation whereas high levels of ^{14}C in the residue reflect a low degree of lignin degradation. Label in the liquid fraction is indicative of solubilized, but undegraded phenolic acids.

References

1. Agarwal N., D.N. Kamra, P.N. Chatterjee, R. Kumar, and L.C. Chaudhary. 2008. In vitro methanogenesis, microbial profile and fermentation of green forages with buffalo rumen liquor as influenced by 2-bromoethanesulphonic acid. Asian-Aust. J. Anim. Sci. **21**: 818–823.
2. Agarwal, N., J. Saxena, S. Saha, L.C. Chaudhary, and D.N. Kamra. 2004. Changes in fermentation characteristics, microbial populations and enzyme profile in the rumen of buffalo as affected by roughage level in the diet. Bubalus bubalis. **III**:81–90.
3. Agarwal, N., I. Agarwal, D.N. Kamra, and L.C. Chaudhury. 2000. Diurnal variations in the activities of hydrolytic enzymes in different fractions of rumen contents of Murrah buffaloes. J. Appl. Anim. Res. **18**:73–80.
4. Agosin, E., J.-J. Daudin, and E. Odier. 1985. Screening of white-rot fungi on (^{14}C) lignin-labelled and (^{14}C) whole-labelled wheat straw. Appl. Microbiol. Biotechnol. **22**:132–138.
5. Alexander, C.L., R.M. Meyer, and E.E. Bartley. 1969. Rumen removal rates of some chemically defined fractions of ^{14}C-labelled alfalfa. J. Anim. Sci. **29**:746–756.
6. Fallon, R.D., and F.K. Pfaender. 1976. Production and fractionation of $^{14}CO_2$ labelled smooth cordgrass Spartina alterniflora. Chesapeake Sci. **17**:292–295.
7. Hristov, A.N., T.A. McAllister, and K.J. Cheng. 1999. Effect of diet, digesta processing, freezing and extraction procedure on some polysaccharide degrading activities of ruminal contents. Can. J. Anim. Sci. **79**:73–81.
8. Huggins, C. and J. Lapides. 1947. Acetyl esters of *p*-nitrophenol as substrate for the colorimetric determination of esterase. J. Biol. Chem. **170**:467–482.
9. Kamra, D.N., S. Saha, N. Bhatt, L.C. Chaudhary, and N. Agarwal. 2003. Effect of diet on enzyme profile, biochemical changes and *in sacco* degradability of feeds in the rumen of buffalo. Asian-Aust. J. Anim. Sci. **16**:374–379.
10. Kieth, C.L., E.E. Bartley, E.L. Sorensen, L.R. Fina, and D.W. Stanton. 1963. Inexpensive technique for production of C14 alfalfa for animal feeding experiments. J. Dairy Sci. **46**: 745–749.
11. Lowry, O.H., N.J. Rosenbrough, A.R. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
12. MacKenzie, C.R., D. Bilous, H. Schneider, and K. Johnson. 1987. Induction of cellulolytic and xylanolytic enzyme systems in *Streptococcus* spp. Appl. Environ. Microbiol. **53**:2835–2839.
13. Martin, C., and B. Michalet-Doreau. 1995 Variations in mass and enzyme activity of rumen microorganisms: Effect of barley and buffer supplements. J. Sci. Food Agric. **67**:407–413.
14. Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determining reducing sugar. Anal. Chem. **31**:426–428.
15. Nogueira Filho, J.C.M., M. Fondevila, A. Barrios Urdaneta, and M. González Ronquillo. 2000 In vitro microbial fermentation of tropical grasses at an advanced maturity stage. Anim. Feed Sci. Technol. **83**:145–157.
16. Shewale, J. G., and J. C. Sadana. 1978. Cellulase and β-glucosidase by a basidomycete species. Can. J. Microbiol. **24**:1204–1216.

17. Svejcar, T.J., T.W. Boutton, and J.D. Trent. 1990. Assessment of carbon allocation with stable carbon isotope labeling. Agron. J. **82**:18–21.
18. Tajima, K., R.I. Aminov, T. Nagamine, H. Matsui, M. Nakamura, and Y. Benno. 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Appl. Environ. Microbiol. **67**:2766–2774.
19. Van Soest P.J. and J.B. Robertson. 1988. A laboratory manual for animal science 612. Cornell University, USA.

Chapter 7
In Vitro Screening of Feed Resources for Efficiency of Microbial Protein Synthesis

Harinder P.S. Makkar

Introduction

Recent advances in ration balancing include manipulation of feed to increase the quantity and quality of protein and energy delivered to the small intestine. Proportionally high conversion of feed degraded in the rumen into microbial mass, i.e. a high efficiency of microbial mass production, will lead to efficient utilization of feed nitrogen and carbon. Selection of fibrous feeds based on high efficiency of microbial protein synthesis in the rumen along with high dry matter and fibre digestibility; and development of feeding strategies based on high efficiency as well as high microbial protein synthesis in the rumen will lead to higher supply of protein post-ruminally. This will decrease both the need for supplementing rumen undegradable feed protein and the flow of feed carbon flow to fermentative carbon dioxide and methane (environment pollutants). The prediction of feed-dependent differences in efficiency of microbial protein synthesis is, therefore, of considerable interest in feed analysis. In addition, as a result of strong pressure from consumers to phase out antibiotics and other chemicals from feeds, because of the risk to human of chemical residues in food and of antibiotics resistance being transferred to human pathogens, intensive efforts are being made to identify plants, plant extracts or plant compounds which could substitute antibiotics and growth promoters. In quest for this, plants are being screened for properties that could lead to the use of plants or plant products for enhancing efficiency of rumen fermentation. In this context, how these plants or plant extracts when used as additives to diets affect the efficiency of microbial protein synthesis becomes important.

In this chapter, a number of approaches for measuring efficiency of microbial protein synthesis in vitro using the gas method are presented. In addition, variants of the gas method enabling measurement of fibre degradability and methane

H.P.S. Makkar (✉)
Institute of Animal Production in the Tropics and Subtropics, University of Hohenheim, D–70593 Stuttgart, Germany
e-mail: makkar@uni-hohenheim.de

P.E. Vercoe et al. (eds.), *In Vitro Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies*,
DOI 10.1007/978-90-481-3297-3_7,

production are also dealt with. The theory behind the methods and explanations of the procedure by giving examples are also provided.

In Vitro Feed Evaluation Methods

In vitro methods for laboratory estimations of degraded feeds are important for ruminant nutritionists. An efficient laboratory method should be reproducible and the values obtained should correlate well with those actually measured in vivo. In vitro methods have the advantage of being less expensive, less time-consuming, and more ethical (minimizes the number of animals); and they allow maintaining experimental conditions more precisely than do in vivo trials.

The methods based on the digestion by rumen microbes are more meaningful since microorganisms are more sensitive to factors influencing the rate and extent of digestion than are chemical methods [50]. Four major techniques, based on the digestion by rumen microbes, are currently available for determining the nutritive value of ruminant feeds:

- in situ incubation of samples in nylon bags in the rumen [41],
- digestion with rumen microorganisms as in [49], and
- in vitro gas methods such as [18, 42, 44, 48],
- a modified in vitro gas method: gas production with concomitant microbial mass measurement [4, 8, 35].

Nylon Bag Technique

The nylon bag technique has been used for many years to provide estimates of both the rate and extent of disappearance of feed constituents. This technique provides a useful mean to estimate rates of disappearance and potential ruminal degradability of feedstuffs and feed constituents whilst incorporating effects of particulate passage rate from the rumen. The disadvantage of the method is that only a small number of forage samples can be assessed at any one time, and it requires at least three fistulated animals to account for variations due to animal. It is therefore of limited value in laboratories undertaking routine screening of a large numbers of samples. It is also laborious, and requires large amounts of samples. Substantial error could result in values obtained at early stages of digestion due to a low weight loss; and for poor quality roughages, adherence of microbes at early stages can even lead to higher weights and thus distortion of results if kinetic modelling does not incorporate the lag-phase [19, 40].

Tilley and Terry Technique

The technique [49] is used widely because of its convenience, particularly when large-scale testing of feedstuffs is required. This method is employed in many forage evaluation laboratories and involves two stages in which forages are

subjected to 48 h fermentation in a buffer solution containing rumen fluid, followed by 48 h of digestion with pepsin in an acid solution. The method was modified by Goering and Van Soest [26], in that the residue after 48 h incubation was treated with neutral detergent solution to estimate true dry matter digestibility. Although the method of Tilley and Terry [49] has been extensively validated with in vivo values [50], the method appears to have several disadvantages. The method is an end-point measurement (gives only one observation) thus, unless lengthy and labour-intensive time-course studies are made, the technique does not provide information on the kinetics of forage digestion; the residue determination destroys the sample and therefore a large number of replicates are needed. The method is therefore difficult to apply to materials such as tissue culture samples or cell-wall fractions.

Both the Tilley and Terry and nylon bag methods are based on residue determinations and may result in overestimation of dry matter digestibility for feeds rich in plant secondary metabolites, such components are solubilized in both these systems but may or may not contribute to nutrient supply to animals [29, 30].

In Vitro Gas Method

General Description

A number of gas devices have been used to measure the gas volume in in vitro gas methods [20]. In this chapter, use of the in vitro gas method developed by Menke et al. [42] and based on incubation in calibrated syringes, is discussed; since, it is easier to use and a large set of values obtained using this method have been validated with the in vivo work. In the method of Menke et al. [42], fermentations are conducted in 100-mL capacity calibrated glass syringes containing feedstuff and a buffered rumen fluid. The gas produced on incubation of 200 mg feed dry matter after 24 h of incubation together with the levels of other chemical constituents are used to predict digestibility of organic matter determined in vivo and metabolisable energy of feeds. Aiple et al. [1] compared three laboratory methods (enzymatic, crude nutrient and gas measuring technique) as predictors of net energy (as estimated by equations based on in vivo digestibility) content of feeds and found that for predicting net energy content of individual feeds, the gas method was superior to the other two methods.

More recently, the increased interest in the efficient utilisation of roughage diets has led to an increase in the use of this technique due to the advantage in studying fermentation kinetics [4, 5, 15]. Gas measurement provides a useful data on digestion kinetics of both soluble and insoluble fractions of feedstuffs. Several gas measuring techniques and in vitro gas methods are in use by several groups. Advantages and disadvantages of these methods are discussed by Getachew et al. [20]. The in vitro gas method based on syringes [7, 42] appears to be the most suitable for use in developing countries. Other in vitro methods such as Tilley and Terry and nylon bag methods are based on gravimetric measurements which follow disappearance of the substrate (the components which may or may not necessarily contribute to fermentation), whereas gas measurement focuses on the appearances of

fermentation products (soluble but not fermentable products do not contribute to gas production). In the gas method, kinetics of fermentation can be studied on a single sample and therefore a relatively small amount of sample is required or a larger number of samples can be evaluated at a time. The in vitro gas method is more efficient than the *in sacco* method in evaluating the effects of tannins or other anti-nutritive factors. In the *in sacco* method these factors are diluted in the rumen after getting released from the nylon bag and therefore do not affect rumen fermentation appreciably. In addition, in vitro gas methods can better monitor nutrient-antinutrient and antinutrient-antinutrient interactions [34].

Origin of Gas and Stoichiometry

On incubation of a feedstuff with buffered rumen fluid in vitro, the carbohydrates in the feed are fermented to produce short chain fatty acids, gases and microbial cells. Gas production is the result of fermentation of carbohydrates to acetate, propionate and butyrate. Gas production from protein fermentation is relatively small as compared to carbohydrate fermentation. The contribution of fat to gas production is negligible. When 200 mg of coconut oil, palm kernel oil and/or soybean oil were incubated, only 2.0–2.8 mL of gas were produced while a similar amount of casein and cellulose produced about 23.4 and 80 mL gas in 24 h.

The gas produced in the gas technique is the direct gas produced as a result of fermentation plus the indirect gas produced from the buffering of short chain fatty acids. For roughages, when bicarbonate buffer is used, about 50% of the total gas is generated from buffering of the short chain fatty acids and the rest is evolved directly from fermentation. At very high molar propionate, the amount of CO_2 generated from buffering of short chain fatty acids is about 60% of total gas production. Gas is produced mainly when substrate is fermented to acetate and butyrate. Substrate fermentation to propionate yields gas only from buffering of the acid and, therefore, relatively lower gas production is associated with propionate production. The gas that is released with the generation of propionate is only the indirect gas produced from buffering. The molar proportion of different short chain fatty acids produced is dependent on the type of substrate. Therefore, the molar ratio of acetate to propionate has been used to evaluate substrate related differences. Rapidly fermentable carbohydrates yield relatively higher propionate as compared to acetate, and the reverse takes place when slowly fermentable carbohydrates are incubated. Many workers reported more propionate and thus lower acetate to propionate ratio in the ruminal fluid of cows fed a high grain diet. If fermentation of feeds leads to a higher proportion of acetate, there will be a concomitant increase in gas production compared with a feed with a higher proportion of propionate. In other words, a shift in the proportion of short chain fatty acids will be reflected by changes in gas production.

The gas produced on incubation of cereal straws [4], a wide range of feeds including many dairy compound feeds and their individual feed components whose protein and fat contents vary greatly [11], and tannin containing browses [25] in absence or presence of polyethylene glycol (a tannin complexing agent) in the buffered rumen

fluid was closely related to the production of short chain fatty acids as per Wolin [51] stoichiometry, which is as follows:

$$\text{Fermentative CO2 (mmol)} = A/2 + P/4 + 1.5B \tag{7.1}$$

where A, P and B are moles of acetate, propionate, and butyrate respectively.

$$\text{Fermentative CH4 (mmol)} = (A + 2B) - CO2 \tag{7.2}$$

where A and B are mmol of acetate and butyrate respectively and CO_2 is mmol of CO_2 calculated from Eq. (7.1). Using these equations one can calculate, the waste products of fermentation: carbon dioxide and methane from acetate, butyrate and propionate produced during the fermentation.

In the in vitro gas method, the "expected gas volume" can also be calculated from acetate, butyrate and propionate produced during the fermentation.

Total volume of gas (mmol), calculated from short chain fatty acids production = (FG + BG)

FG = fermentative gas (mmol) (CO_2 + CH_4); calculated using Eqs. (7.1) and (7.2)
BG = gas volume (mmol) from buffering of short chain fatty acids
CF = correction factor for altitude and pressure

For the determination of BG, one requires the amount of short chain fatty acids (acetate + butyrate + propionate) produced during the fermentation. One mmol of short chain fatty acids releases one mmol of CO_2 from the bicarbonate-based buffer in the incubation medium, and is described as buffering CO_2. Therefore, mmol of buffering CO_2 is equal to mmol of total short chain fatty acids generated during incubation.

The total gas produced (mmol), which is the addition of mmol FG and mmol BG, can be converted to volume (mL) by the following equation:

$$\text{Gas volume at mean sea level} = \text{mmol of gas} \times \text{gas constant (R)} \times T$$

Where R = the ratio between molar volume of gas to temperature (Kelvin zero; K) i.e.

$(22.41\,l/273 = 0.0821)$,T = incubation temperature(Kelvin); $273 + 39^{\circ}C = 312\,K$

So the volume of 1 mmol of gas at 39°C at sea level would be: $1 \times 0.0821 \times 312 = 25.6$ mL.

The volume of gas depends on the altitude of a place, and hence a correction factor is required. If the incubation is conducted at Hohenheim, Germany, which is at an altitude of 400 m, the correction factor is 0.953 [11]. The volume of 1 mmol of gas at 39°C in Hohenheim would therefore be: $1 \times 0.082 \times 312/0.953 = 26.5$ mL.

The total volume of gas as mmol, calculated from mmol of FG and BG, can be converted to volume (mL) by multiplying with 26.5 for a place with an altitude of 400 m and by multiplying with 25.6 for a place at mean sea level. The detailed information on behaviour of gases at different temperature (as temperature increases, the volume increases) and pressure (as altitude increases, pressure decreases) can be obtained from a textbook on physical chemistry. The origin and stoichiometry of gas production, given above, have been described in details in [11] and [20].

The in vitro gas production measured after 24 h of incubation of tannin containing browses in the presence or absence of polyethylene glycol was strongly correlated with the gas volume stoichiometrically calculated from short chain fatty acids. The relationship between short chain fatty acid production (mmol) and gas volume (mL) after 24 h of incubation of browse species with a wide range of crude protein (5.4–27%) and phenolic compounds (1.8–25.3% and 0.2–21.4% total phenols and total tannins as tannic acid equivalent respectively) was [25]:

- In the absence of polyethylene glycol:

$$\text{Short chain fatty acids} = 0.0239 \times \text{Gas} - 0.0601;$$
$$\text{R2} = 0.953; \quad \text{N} = 39; \quad \text{P} < 0.001$$

- In the presence of polyethylene glycol

$$\text{Short chain fatty acids} = 0.0207 \times \text{Gas} + 0.0207;$$
$$\text{R2} = 0.925; \quad \text{N} = 37; \quad \text{P} < 0.001$$

- Overall (pooling the data)

$$\text{Short chain fatty acids} = -0.00425 + 0.0222 \times \text{Gas};$$
$$\text{R2} = 0.94; \quad \text{N} = 76; \quad \text{P} < 0.001$$

These relationships are similar to those obtained for wheat straw [6].

The short chain fatty acid production could be predicted from gas values using the above relationship. The level of short chain fatty acids is an indicator of energy availability to the animal. Since short chain fatty acid measurement is important for relating feed composition to production parameters and to net energy values of diets, prediction of short chain fatty acids from in vitro gas measurement will be increasingly important in developing countries where laboratories are seldom equipped with modern equipments to measure short chain fatty acids.

A modified in vitro gas method: gas production with concomitant microbial mass measurement

General Description

A simple in vitro approach is described here which is convenient and fast, and allows a large number of samples to be handled at a time. It is based on the quantification of substrate degraded or microbial protein produced using internal or external markers,

and of gas or short chain fatty acid production in the in vitro rumen fermentation system based on syringes [42]. This method does not require sophisticated equipment or the use of a large number of animals (one or preferably two fistulated animals are required) and helps selection of feeds or feed constituents based not only on the dry matter digestibility but also on the efficiency of microbial protein synthesis.

The method of Menke et al. [42] was modified by Blümmel and Ørskov [4] in that feeds were incubated in a thermostatically controlled water bath instead of a rotor in an incubator. Makkar et al. [35] and Blümmel et al. [8] modified the method further by increasing the amount of sample from 200 to 500 mg and increasing the amount of buffer two-fold, as a result the incubation volume increased from 30 mL in the method of Menke et al. [42] to 40 mL in the modified method. In the 30 mL system, the linearity between the amount of substrate incubated and the amount of gas produced is lost when the gas volume exceeds 90 mL because of the exhaustion of buffer of the medium resulting from short chain fatty acid production; and in the 40 mL system, the linearity is lost when the gas volume exceeds 130 mL [21]. The exhaustion of the buffer decreases pH of the incubation medium; consequently the fermentation is inhibited. If the amount of gas production exceeds 90 mL using the 30 mL system [42] and 130 mL using the 40 mL system [7, 35], the amount of feed being incubated should be reduced.

The main advantages of the modified method (the 40 mL system and incubation in a water bath) are:

a) an increase in amount of sample from 200 to 500 mg reduces the inherent error associated with gravimetric determination needed to determine concomitant in vitro organic matter degradability (see below),
b) almost no drop in temperature of the medium during the period of recording gas readings (when compared to the incubation of syringes in an incubator at 39°C as in the original method of Menke et al. [42]). This is useful for studying the kinetics of fermentation where gas volume must be recorded at various time intervals, and
c) drastic drop in the temperature of the incubation is prevented in case of power breakdown for a short duration because of large volume of water in the water bath and its higher temperature holding capacity.

Determination of Microbial Mass

In vitro gas tests are attractive for ruminant nutritionists since it is very easy to measure the volume of gas production with time, but the measurement of gas only implies the measurement of nutritionally wasteful and environmentally hazardous products (CO_2 and CH_4). In most studies, the rate and extent of gas production have been wrongly considered equivalent to the rate and extent of substrate (feed) degradation. Current nutritional concepts aim at high microbial efficiency, which cannot be achieved by measurement of gas only in in vitro gas methods. In vitro gas measurements reflect only short chain fatty acid production. The relationship between short chain fatty acids and microbial mass production is not constant and

the explanation for this resides in the variation of microbial mass production per unit ATP generated. This can impose an inverse relationship between gas volume (or short chain fatty acid production) and microbial mass production particularly when both are expressed per unit of substrate truly degraded. This implies that selecting roughages by measuring only gas using in vitro gas methods might result in a selection against the maximum microbial mass yield. Blümmel et al. [7, 8] have demonstrated how a combination of in vitro gas production measurements with a concomitant quantification of the truly degraded substrate provides important information about partitioning of fermentation products.

Partitioning Factor and Efficiency of Microbial Protein Synthesis

The partitioning factor is defined as the ratio of organic matter degraded in vitro (mg) to the volume of gas (mL) produced by it. A feed with higher partitioning factor means that proportionally more of the degraded matter is incorporated into microbial mass, i.e., the efficiency of microbial protein synthesis is higher. Different in vitro partitioning factor values are also reflected by in vivo microbial protein synthesis as estimated by purine derivatives (the higher the partitioning factor, the higher the excretion of urinary purine derivatives [12]), in methane production by ruminants (the higher the partitioning factor, the lower the methane output [12, 15]), and in predicting the dry matter intake (the higher the partitioning factor, higher the dry matter intake [8, 9]). These results show that the partitioning factor calculated in vitro provides meaningful information for predicting the dry matter intake, the microbial mass production in the rumen, and the methane emission of the whole ruminant animal.

The procedures for the determination of truly degraded substrate and organic matter degraded, and the calculation of the stoichiometrical factor; stoichiometrical relationship between short chain fatty acids and gas volume; and relationship between short chain fatty acid production, ATP production and microbial mass yield are given in Blümmel et al. [7] and Getachew et al. [20]. For roughages, partitioning factor values from 2.75 to 4.45 mg/mL approximately reflects ^{Y}ATP from 10 to 32. It may be noted that these procedures and relationships are valid for substrates consisting predominantly of structural carbohydrates, and the findings might not extend to substrates such as those high in soluble carbohydrate, protein, fat or starch. Rymer and Givens [47] have shown that, as observed by [7], good quality feeds (grass silage, wheat, maize, molasses sugar-beet feed and fishmeal) which produce large amounts of gas and short chain fatty acids yield small amounts of microbial mass per unit of feed truly degraded.

It seems therefore justified to suggest that feeds or feed ingredients should be selected that have high in vitro organic matter degradability but low gas production per unit organic matter degraded.

Microbial mass production in in vitro can be calculated as [7]:

$$\text{Microbial mass (mg)} = \text{mg substrate truly degraded} - (\text{mL gas volume} \times \text{stoichiometrical factor}) \tag{7.3}$$

The above equation becomes:

$$\text{Microbial mass (units)} = \text{gas volume (partitioning factor} - \text{stoichiometrical factor)} \quad (7.4)$$

For roughages, the stoichiometrical factor was 2.20.

The derivatization of these two equations, based on balancing of substrate supply and product formation in the in vitro gas method, is given in [7].

The partitioning factor reflected efficiency of microbial protein synthesis and microbial mass determined using the approach presented here (Eqs. 7.3 and 7.4) was found to be in good agreement with ^{15}N studies [8, 13]. This concept of partitioning factor analysis demands a close stoichiometric relationship between short chain fatty acids and gas production, and a reliable determination of organic matter degradability of the substrate. The partitioning factor based approach for prediction of efficiency of microbial protein synthesis is simpler compared to that suggested by the Cornell net carbohydrate and protein system [13].

The Partitioning Factor as a Reflection of Efficiency of Microbial Protein Synthesis for Tannin-Containing Feeds

Unfortunately, the method based on the gas method and the detergent system of fibre analysis to calculate the microbial mass produced for fibrous feeds (the method outlined above) did not work for tannin-rich feeds. The partitioning factor for tannin-rich feeds (calculated as mg truly degraded substrate needed to produce one-mL gas) ranged from 3.1 to 16.1 [22], which is well above the theoretical partitioning factor range of 2.75–4.41 [7]. The high partitioning factor could be due to:

a) solubilization of tannins from the feed. These tannins would make no contribution to gas or energy in the system but would contribute to dry matter loss,
b) the cell solubles contributing to dry matter loss but not to gas production because the gas production is inhibited by tannins or
c) a combination of a) and b).

In addition, the appearance of tannin-protein complexes as artefacts in the true residue also makes the gravimetric approach of quantification of microbial mass redundant [36–38].

For the in vitro evaluation of tannin-rich feeds, the microbial mass should be quantified using diaminopimelic acid or purines as markers, or by ^{15}N incorporation into the microbes [34, 39], and the partitioning factor for tannin-rich feeds can be expressed as the microbial mass determined by these markers per mL of gas produced (or per mmol short chain fatty acids produced).

Using diaminopimelic acid, purines and ^{15}N approaches for measuring microbial mass it has been shown that for tannin-rich feeds the presence of polyethylene glycol (Molecular weight 4000 or 6000) – a tannin-inactivating agent, increased feed degradability and microbial mass production, but decreased the efficiency of

microbial protein synthesis [3, 23, 34, 39]. Similar results have also been obtained by following other approaches based on the gas method in which the rate of ammonia uptake [45] is taken as the efficiency of microbial protein synthesis [39] or microbial protein is determined by the nitrogen balance approach [23]. Conversely, efficiency of microbial protein synthesis is expected to be higher in the presence of tannins. The net microbial mass production would depend on the balance between the extents of "decrease in dry matter degraded" and "higher microbial mass produced per unit of dry matter degraded" in presence of tannins.

Incubation Time and Partitioning Factor

Another study [10], in addition to once again describing the importance of measuring microbial mass, has highlighted the importance of the fermentation time at which the microbial mass should be measured. In this study, substrate degradation and kinetics of in vitro partitioning of three hays, with similar in vivo digestibility, into short chain fatty acids, microbial mass yield, and ammonia, carbon dioxide and methane production were examined after 8, 12, 18 and 24 h of incubation in the gas method under both low and adequate nitrogen levels. Microbial synthesis was quantified gravimetrically [7], by nitrogen balance [23] and by purine analysis [31]. The short chain fatty acids and gas production were positively correlated ($P < 0.0001$) and cumulative at all times of incubation under both low and adequate nitrogen levels. On the other hand, microbial mass, microbial nitrogen and microbial purine yields declined after 12 h of incubation while ammonia production increased. Per unit of substrate degraded, gas and short chain fatty acid production were always inversely ($P < 0.05$) related to microbial mass yield regardless of incubation time and medium (low or adequate nitrogen). At later incubation times, continuously more short chain fatty acids or gas and less microbial mass were produced reflecting microbial lysis and probably increasing microbial energy spilling. All three hays differed ($P < 0.05$) consistently in how the degraded substrate was partitioned into short chain fatty acids, gas and microbial mass in both the low and adequate nitrogen medium. Purine analysis indicated substantial differences in microbial composition across treatments, which might be one explanation for these different microbial efficiencies [10].

For tannin containing feeds [33], the efficiency of microbial growth was higher for 16 h of incubation than 24 h when these were incubated in presence or absence of polyethylene glycol, a tannin-inactivating agent. Additional nitrogen in the medium also affected the efficiency of microbial protein synthesis from tannin-tannins feeds, both after 16 and 24 h [22]. For proper characterisation of feed and feed ingredients, approaches need to be developed for measuring the partitioning factor for the incubation time at which the lysis of microbes is minimal. Some possible simple approaches worth investigating to identify this incubation time are:

a) the time at which half of the maximum gas is produced (t/2), and
b) the inflection point at which the rate of gas production is maximum (the rate increases up to a certain incubation time and thereafter decreases as the

incubation progresses), both these parameters can be mathematically calculated from the gas profiles. Some efforts have been made to measure partitioning factor at t/2, 16 and 24 h for various feed resources and to correlate the values obtained with the measured in vivo efficiency of microbial protein synthesis [13,14].

In this chapter, the method for measuring partitioning factor, a reflection of efficiency of microbial protein synthesis is presented. Based on the available information in the literature, the suggested incubation times are: 16 h for concentrate based feeds/diets and 24 h for roughages. These incubation times would adequately serve the purpose of screening a large number of samples for efficiency of microbial protein synthesis. In addition, an error which could arise in the determination of truly degraded substrate through dissolution of the undegraded substrate in the neutral detergent solution (and thus overestimating truly degraded substrate) is expected to be minimal for most of the feed resources except those rich in starch such as maize grains [13]. Determination of truly degraded substrate required for determination of partitioning factor at incubation times lower than 16 h could substantially overestimate truly degraded substrate and the partitioning factor, leading to incorrect reflection of efficiency of microbial protein synthesis.

Method for Measuring Partitioning Factor

Sample Preparation

Dried sample should be passed through a 1 mm sieve.

Reagents

1. *Bicarbonate buffer solution:* Dissolve 35 g sodium bicarbonate ($NaHCO_3$) and 4 g ammonium carbonate (NH_4HCO_3) in approximately 500 mL distilled water and then make up the volume to 1 L with distilled water.
2. *Macromineral solution:* Dissolve 6.2 g potassium dihydrogen phosphate (KH_2PO_4), 5.7 g disodium hydrogen phosphate (Na_2HPO_4), and 0.6 g magnesium sulphate ($MgSO_4.7H_2O$) in approximately 500 mL distilled water and then make up the volume to 1 L with distilled water.
3. *Micromineral solution:* Dissolve 10 g manganese chloride ($MnCl_2.4H_2O$), 13.2 g calcium chloride ($CaCl_2.2H_2O$), 1 g cobalt chloride ($CoCl_2.6H_2O$), 8 g ferric chloride ($FeCl_3.6H_2O$) in approximately 50 mL distilled water and then make up the volume to 100 mL with distilled water.
4. *Resazurine:* Dissolve 0.1 g resazurine in 100 mL distilled water.
5. *Reducing solution:* Dissolve 996 mg sodium sulphide ($Na_2S.9H_2O$) in 94 mL distilled water and then add 6 mL of 1 N sodium hydroxide solution (dissolve 4 g sodium hydroxide in 100 mL distilled water for 1 N sodium hydroxide).

Weighing of Samples and Preparation of Syringes

Tear a specially made scoop (approximately 4 cm in length and 1 cm in depth/radius; standard sodium hydroxide-containing plastic container can be cut horizontally to half to form the scoop) on an analytical balance. Weigh 500 mg of air-dry feed sample in the scoop and then insert a 5 mL capacity pipette or a glass rod into the narrow end of the scoop and transfer the sample from the scoop into 100-mL calibrated glass syringes. The feedstuffs are incubated at least in triplicate. The blank syringes do not contain feed.

Preparation of In Vitro Rumen Fermentation Buffer Solution and Incubation

Collect the rumen fluid and particulate matter before the morning feed from two cattle, fed a diet of the type similar to that of the samples being analysed in vitro. Mix the contents taken from two cattle, homogenize, strain and filter them through four layers of cheesecloth. Keep all glassware at approximately 39°C and flush these with carbon dioxide before use. Carbon dioxide is heavier than air and hence it remains in the glassware for a reasonable period provided the container is not inverted up side down. The strained rumen fluid is kept at 39°C under carbon dioxide and should be prepared just before start of the incubation. As the amount of feed taken is 500 mg, composition of the medium is according to [49]. Menke et al. [42] reduced the rumen buffer volume per syringe by half as they used 200 mg of the substrate because of the limited volume of the syringes and the inconvenience of emptying the syringes. Here, besides recording the gas volume, we are interested to use the fermented material for various analyses; therefore, the amount of substrate taken is 500 mg [8, 35]. There is an inherent error associated with gravimetric determination of the fermented residue which (error) is large if 200 mg feed is taken in place of 500 mg.

Medium Composition

(According to [35])

Rumen buffer solution (bicarbonate buffer) 630 mL
Macromineral solution 315 mL
Micromineral solution 0.16 mL
Resazurine 1.6 mL
Distilled water 945 mL
Freshly prepared reducing solution 60 mL
The rumen fluid 660 mL
(see above for collection and preparation)

The above volume is sufficient for 60 syringes (40 mL/syringe) plus 10% extra.

Incubation Procedure

Mix, in the order mentioned above, all the above-mentioned solutions, except the reducing solution and rumen fluid, in a 3 or 5 L capacity glass container. The container is kept in a water bath adjusted at 39°C. This water bath is a plastic rectangular container (approximately 400 cm × 300 cm × 200 cm) filled with water, the temperature of which is adjusted at 39°C using a portable thermostat suspended from the top of the plastic container in water. This plastic water bath is kept on a magnetic stirrer. The contents are flushed with carbon dioxide and kept stirred using a magnetic stirrer. After about 5 min, add the reducing solution and keep the mixture stirring and flushing with carbon dioxide at 39°C. When the mixture has been reduced (blue colour of the dye changes to pink and then to colourless; it takes about 15–20 min for the reduction process to complete and during this time we generally homogenized and strained the rumen liquor and the particulate material collected from cattle), add 660 mL of the rumen fluid. Keep this mixture stirring and flushing with carbon dioxide for another 10 min. Transfer a portion (40 mL) of the rumen-fluid medium into each syringe using a dispenser, and incubate in a water bath at 39°C. After some practice, filling 60 syringes should take 30–35 min. After completion of the filling-up process, shake the syringes well and transfer them to the water bath. Shake all the syringes every hour for the first 4 h and then after every 2 h.

Generally, the incubation is started at about 7.30–8.0 a.m. and after 12 h of incubation, the syringes are not shaken until the termination of the incubation (24 h). When 500 mg of the air-dry sample is incubated with 40 mL of the medium containing rumen microbes, invariably the amount of gas in 24 h exceeds the capacity of 100-mL capacity syringes. After 8–10 h of incubation (depending on the fermentability of the feed), the amount of gas produced is registered and the piston is pushed back to 40 or 45 mL mark on the syringe (after pushing back the piston to 40 or 45 mL mark, shake the syringe contents after about 30 min; this will prevent sticking of feed particles on bottom of the piston, which otherwise will not get fermented). At 24 h, again the position of the piston is recorded. The addition of these two sets of values gives the total amount of gas production in 24 h (see an example below). The blanks (at least three in number) contain only the rumen-fluid medium and no feed material. For blanks, there is no need to push back the piston.

An Example

Suppose, at 0 h of incubation the piston was at 41-mL mark. After 8 h of incubation, the piston reached the 86-mL mark and it was pushed back to the 45-mL mark. The following morning after 24 h of incubation the piston was at 67-mL mark. Total gas produced during 24 h = (86 – 41) + (67 – 45) = 77 mL.

The operational aspects of the gas method are available at: http://www.iaea.org/programmes/nafa/d3/mtc/invitro-slideshowapr01.pdf

Notes:

1. *The procedure given here suggests termination of the incubation after 24 h; however, for concentrate-based feeds the incubation should be terminated after 16 h (see Incubation time and partitioning factor). Generally, the incubation is started in the morning (7.30–8.0 a.m.) and termination of the incubation after 24 h is convenient from the practical point of view. On the other hand, for 16 h of incubation (around mid-night), after recording the gas, the syringes can be kept in ice for determination of undegraded organic matter the following day; or better the syringe contents and the syringe washings (see* "Organic matter degradability*" and* "Preparation of apparent undegraded residue"*) could be transferred into a beaker for digestion the following day. For 16 h of digestion, the incubation could also be initiated at around 4 p.m. so that the incubation could be terminated at 8 a.m. the following day; however, variation in the activity of the rumen liquor taken at this hour could be higher than that of the rumen liquor taken before the morning feed. When methane proportion is to be measured in the gas (see Determination of other parameters), the syringes should not be kept in ice, since the solubility of methane and carbon dioxide is different at different temperature (solubility of carbon dioxide in water/buffered medium is higher than methane).*
2. *When the objective is to evaluate the effect of an additive on partitioning factor of a feed, a set of three syringes containing the feed, the additive and 40 mL of the incubation medium form a test set, and the corresponding blank contains a set of three syringes with the additive and 40 mL of the incubation medium (and no feed).*
3. *The 40 mL of the incubation medium consists of 10 mL each of rumen contents and bicarbonate buffer, 5 mL of macro-mineral solution (0.002 mL of which is micro-mineral solution) and 15 mL of distilled water. The nitrogen content in a syringe, delivered from the buffer is approximately 7 mg.*

Net Gas Production

The gas volume is recorded after 24 h in test and blank syringes for roughages and after 16 h for concentrate-based feeds. The net gas production is calculated by subtracting values of the blank from that of the test syringe. Let this net gas value be *x* mL.

Organic Matter Degradability

The syringe contents are digested with neural detergent solution to dissolve the microbes, leaving the undegraded residue. This residue is ashed to obtain undegraded organic matter (undegraded residue *minus* ash).

Reagents

Neutral detergent solution: Mix the Reagents 1 and 2, and dilute to 4 L with distilled water. Check the pH. It should be between 6.9 and 7.1.

Reagent 1: Weigh 74.4 g EDTA (disodium ethylenediamine tetraacetate dehydrate) and 27.2 g sodium teraborate ($Na_2B_4O_7.10.H_2O$) and place together in a large beaker, add distilled water (approximately 2 L) and dissolve with gentle heating, add to this solution 120 g sodium lauryl sulphate (also called as sodium dodecyl sulphate) and 40 mL of 2-ethoxyethanol (ethylene glycol -monoethylether) and mix.

Reagent 2: Weigh 18.24 g disodium hydrogen phosphate (anhydrous), add approximately 1 L of distilled water and dissolve with gentle heating.

Procedure

After measuring the gas volume after 24 h (or after 16 h for concentrate-based feeds), transfer the contents of the syringe quantitatively in a beaker (in practice, after emptying the syringe contents in a beaker, we rinse the syringe twice, each time with approximately 20 mL of double strength neutral detergent solution; each time the syringe is shaken to remove residual feed particles) and digest it with the neutral detergent solution for 1 h using the heating and refluxing unit generally used for estimation of neutral detergent fibre in feeds. The purpose of this treatment is to solubilize the microbes from the syringe contents and obtain only the undegraded feed. Filter the contents of the beaker through a crucible (porosity 2) and wash the residue on the crucible with hot water till the residue is free of the detergent. Dry the crucibles at 130°C for 2 h or at 100°C for 10 h (overnight). Record weight of the crucibles after transferring them to a desiccator. This weight minus weight of empty crucible gives the weight of undegraded feed in that particular syringe.

Note: This method of measuring undegraded residue does not work satisfactorily for tannin-containing feeds/samples (presence of tannin-protein complexes as artefacts in the residue) and for starch-rich feeds (some starch might not be degraded by microbes up to 16 h of incubation but it would get solubilized in the neutral detergent solution leading to underestimation of truly undegraded residue). It may be noted that this method of measurement of undegraded residue should not be applied at the initial hours of incubation (before 16 h of incubation) since during this period a portion of the feed, which is undegraded by microbes could be solubilized in the neutral detergent solution.

Let the weight of this residue (undegraded feed) be *a* mg. Now transfer the crucibles containing this residue to a Muffle furnace (550°C) and ash the sample. The organic matter will disappear leaving the ash. After transferring the crucibles to a desiccator, weigh them and subtract from this weight the weight of the empty crucible to obtain the weight of ash (called as *b* mg). Subtract *b* from *a* to obtain undegraded organic matter *(a–b)* in mg.

For determination of organic matter degraded in 24 h, one needs *(a–b)* value and organic matter in 500 mg of the air-dried sample incubated in the syringe.

Organic matter weighed into the syringe
= [500 × Feed DM content in percentage/100] minus [500 × (Feed DM content in percentage/100) × (Feed Ash content in percentage/100)]

or (500 × Feed DM content in percentage/100) × (1 – Feed Ash content in percentage/100)

Let this value be *c* in mg (ash content of the feed could be determined by burning in a Muffle furnace, 550°C)

$$\text{Organic matter degraded (mg)} = c - (a - b)$$

Determination of Partitioning Factor

For determination of partitioning factor, organic matter degraded and net gas values are required

$$\text{Partitioning factor} = (\text{mg organic matter degraded})/\text{mL gas}$$

or

Partitioning factor = *c – (a–b)* divided by mL net gas (*x*) produced in the syringe for which *c – (a–b)* has been calculated. Higher is this factor, higher the efficiency of microbial protein synthesis.

An Example

Scenario 1. Screening feeds for efficiency of microbial protein synthesis

Two samples (500 mg each) of Feed A and Feed B containing 95% and 93% dry matter (DM) and 5% and 4% ash respectively were incubated for 24 h in the in vitro gas method.

Organic matter weighed into the syringes, *c*:
Feed A = (500 × 95/100) (1 – 5/100) = 451 mg
Feed B = (500 × 93/100) (1 – 4/100) = 446 mg
Gas production at 24 h for blank syringes: 5 mL (average of three syringes)
Gas production at 24 h for Feed A (syringe 1): 115 mL
Gas production at 24 h for Feed B (syringe 1): 105 mL
Net gas production, *x*
Feed A = 115 – 5 = 110 mL
Feed B = 105 – 5 = 100 mL
Undegraded organic matter, *(a–b)*
Feed A (syringe 1) = 144 mg
Feed B (syringe 1) = 164 mg
Organic matter degraded (mg) = *c – (a–b)*
Feed A = 451 – 144 = 307 mg
Feed B = 446 – 164 = 282 mg
Partitioning Factor
Feed A = 307/110 = 2.79
Feed B = 282/100 = 2.82

On one day, three syringes are incubated for each feed, so one has three values of partitioning factor for each Feed A and Feed B.

Conclusion: Efficiency of microbial protein synthesis is higher for Feed B than Feed A. It may be noted that in this example, both the gas production and organic matter degradability are higher in Feed A. Therefore to, the efficiency of microbial protein synthesis may not follow the same pattern as the organic matter degradability or the net gas production. These results should not be looked in isolation. Ideally, a feed with higher organic matter degradability and higher partitioning factor should be preferred when the objective is to use the feed for increasing livestock productivity. On the other hand, in situations such as extreme cold where the aim is to keep the body generating heat and protect the animal from dying, or for draught animals where the energy requirement is high, a feed with higher organic matter degradability and lower partitioning factor could be preferred, since for this feed a higher proportion of the substrate would be partitioned to short chain fatty acids (main energy source for ruminants) and lower to microbial mass. In addition, in feeding situations below the maintenance requirements, high partitioning factor values might not be beneficial.

Some Data from the Literature

Values for partitioning factor and related parameters are presented in Table 7.1, showing the importance of measuring partitioning factor in an in vitro gas method. Sodium hydroxide and ammonia treatments of straws not only increased the true substrate degradability and gas production but also the efficiency of microbial protein synthesis. The efficiency of microbial production (as reflected by partitioning factor) was inversely related to methane production/kg digestible organic matter in vivo [15]. The reader is suggested to refer to [13] for partitioning factor of another set of roughages and mixed diets and the importance of partitioning factor values in predicting efficiency of microbial protein synthesis in vivo.

Table 7.1 In vitro gas production (GP), truly degraded substrate (TDS) and partitioning factor (PF) of untreated and NaOH- and ammonia-treated wheat straw and oat after 24 h of incubation (Partitioning factor derived from data of [15])

	TDS (mg)	GP (mL)	PF
Winter wheat variety Pastiche, untreated	240	98	2.45
Winter wheat variety Pastiche, NaOH-treated	307	110	2.79
Winter wheat variety Pastiche, NH_3-treated	280	103	2.72
Oat, untreated	235	91	2.58
Oat, NaOH-treated	298	108	2.76
Oat, NH_3-treated	279	102	2.74

TDS is not truly degraded organic matter since it has not been corrected for the ash content. This approach of calculating partitioning factor based on TDS could be used for comparative assessment of feeds (or of treatments on a feed) provided the ash content is similar in the samples tested. In the present case the presence of NaOH in the incubated material would lead to an overestimation of TDS and hence of partitioning factor.

Table 7.2 In vitro gas production (GP), truly degraded substrate (TDS) and partitioning factor (PF) of hybrid and local varieties of maize stover leaves (data from [12])

	TDS (mg)	GP (mL)	PF
Hybrid maize stover leaves	328.7	107.2	3.1
Local maize stover leaves	327.3	114.9	3.3.

Table 7.2 is from the data of [12]. It gives fermentation parameters, including partitioning factor of hybrid and local varieties of maize stover leaves. True substrate degradability of the two varieties at 24 h is the same; however, the gas production for the local varieties is lower, suggesting higher microbial mass production for this variety. The partitioning factor of local varieties was higher. This was reflected in vivo when microbial protein synthesis was estimated [17, 32] by urinary excretion of purine derivatives. Higher in vivo efficiency of microbial protein synthesis and higher microbial protein supply to the animal was recorded for local varieties.

Scenario 2. Study of the effect of an additive (water/buffer soluble) on efficiency of microbial protein synthesis

A sample (500 mg) of a feed containing 95% dry matter and 5% ash was incubated for 24 h in the in vitro gas method, without and with the additive (in three syringes each, with corresponding blanks)

Organic matter weighed into the syringes, *c*:
Feed = (500 × 95/100) (1 – 5/100) = 451 mg
Gas production after 24 h in blank syringes (without additive): 5 mL (average of three syringes)
Gas production after 24 h in blank syringes (with additive): 7 mL (average of three syringes)
Gas production after 24 h for feed, without additive (syringe 1): 95 mL
Gas production after 24 h for feed, with additive (syringe 1): 102 mL
Net gas production, *x*
Feed, without additive = 95 – 5 = 90 mL
Feed, with additive = 102 – 7 = 95 mL
Undegraded organic matter, *(a–b)*
Feed, without additive (syringe 1) = 140 mg
Feed, with additive (syringe 1) = 135 mg
Organic matter degraded (mg) = *c – (a–b)*
Feed, without additive = 451 – 140 = 311 mg
Feed, with additive = 451 – 135 = 316 mg
Partitioning Factor
Feed, without additive = 311/90 = 3.45
Feed, with additive = 316/95 = 3.32

On one day, three syringes are incubated for each feed, so one has three values of partitioning factor each for Feed, without additive and Feed, with additive.

Conclusion: The additive has increased gas production and true degradability but decreased the efficiency of microbial protein synthesis. Some relevant information from the literature is given in Table 7.2.

Note: The theoretical range for partitioning factor is 2.74–4.41. Any value above or below this range should be critically evaluated. For tannin-rich samples, the partitioning factor is normally above 4.41. The higher partitioning factor (for example 7.2) would mean that 7.2 mg of the truly degraded organic matter produce one mL of the gas. The reasons for the high values for tannin-rich feeds are given in the section above "The partitioning factor as a reflection of efficiency of microbial protein synthesis for tannin-containing feeds". For tannin-containing feeds, the syringe contents are not digested after incubation with the neutral detergent solution to determine truly undegraded organic matter. Instead, the syringe contents are taken for the determination of purines and/or ^{15}N incorporation studies (see section below "Methods for measurement of microbial mass, microbial-nitrogen, microbial purines and ^{15}N incorporation in microbes and determination of efficiency of microbial protein synthesis").

Determination of Other Parameters

From the above analyses, the following additional information can also be obtained:

i) Organic matter degradability (%) = $(c - (a–b))\ 100/c$
In the above two examples, organic matter degradability for:
Scenario 1

$$\text{Feed A} = (307) \times 100/451 = 68.1\%$$
$$\text{Feed B} = (282) \times 100/446 = 63.2\%$$

Scenario 2

$$\text{Feed, without additive} = (311) \times 100/451 = 69\%$$
$$\text{Feed, with additive} = (316) \times 100/451 = 70.1\%$$

ii) Neutral detergent fibre (NDF) degradability (or fibre degradability)
NDF degradability (fibre degradability) (%) = [{500 × (Feed DM content in percentage/100) × (Feed NDF content in percentage/100)} – {*(a–b)*}] 100/{500 × (Feed DM content in percentage/100) × (Feed NDF content in percentage/100)}
(Neutral detergent fibre content of the feed could be determined by cooking the sample for 1 h in neutral detergent solution, followed by filtration through a crucible of porosity 2)
Scenario 1
Let, neutral detergent fibre values for Feed A and Feed B are 50 and 55% respectively.
NDF degradability (fibre degradability) (%)

$$\text{Feed A} = (\{500 \times (95/100) \times (50/100)\} - \{135\}) \times 100/\{500 \times (95/100) \times (50/100)\}$$
$$= (237.5 - 135) \times 100/237.5 = 43.2$$

$$\text{Feed B} = (\{500 \times (93/100) \times (55/100)\} - \{142\}) \times 100/\{500 \times (93/100) \times (50/100)\}$$
$$= (255.8 - 142) \times 100/255.8 = 44.5$$

Scenario 2

Similarly, the effect of addition of additive on neutral detergent fibre degradability (fibre degradability) could be determined, once the neutral detergent fibre content of the feed incubated in the syringes is known.

iii) Estimated microbial mass

For most conventional feed resources (not the tannin-containing feeds or starch-rich feed ingredients), microbial mass production can be estimated at the time of termination of the incubation, using Eq. (7.3).

mg microbial mass production = ({*c – (a–b)*} – (net gas in mL x 2.2)); *c – (a–b)* is in mg and 2.2 is the stoichiometric factor

For the above example of Feeds A and B:

$$\text{mg microbial mass production for Feed A} = 307 - 110 \times 2.2 = 307 - 242 = 65\,\text{mg}$$
$$\text{mg microbial mass production for Feed B} = 282 - 100 \times 2.2 = 282 - 220 = 62\,\text{mg}$$

iv) Efficiency of microbial mass production = (({*c – (a–b)*} – (2.2 × net gas in mL)) × 100)/{*c – (a–b)*}

These values for Feed A and Feed B are: 65 × 100/307, and 62 × 100/282, or 21.2 and 22% respectively. The order for the efficiency of microbial protein synthesis calculated by this method is the same as obtained with partitioning factor (partitioning factor for Feed B is higher than Feed A).

Note: Based on the Cornell Net Carbohydrate and Protein System, a maximum incorporation of feed carbohydrate into microbial mass of 50% and 40% in the absence and presence of protozoa respectively has been suggested [46 *].*

v) Methane production

In our laboratory, we measure methane content of the gas in the syringe using an infrared-based methane analyser (0–30% range methane analyser from Pronova Analysentechnik GmbH & Co KG, Berlin, Germany). The methane analyser is calibrated against 10.6% or 12% standard methane. A gas chromatograph can also be used for methane measurement; however, the use of the infrared-based methane analyser is simple, convenient and takes less time and resources.

After measuring the total gas volume, the tubing of the syringe outlet is inserted into the inlet of the methane analyser; the piston is pushed to insert the accumulated gas into the analyser. The methane as percent of the gas is displayed on the methane analyser. This value is used for calculation of methane in the total gas volume. When the feed incubated is 500 mg, the volumes of total gas and methane are measured while pushing back the piston after 8 or 10 h of incubation, and similarly volumes of gas and methane are measured after 24 h. These two values are added to obtain the total methane production in 24 h of

incubation. Generally, we have observed that percent methane level in a syringe is lower for the period 0–8 or 10 h of incubation than for the period 8 or 10–24 h of incubation. If pushback of the piston is to be avoided, 200 mg of the sample can be incubated and the volumes of gas and methane can be measured after 24 h of incubation. These volumes could be multiplied by 2.5 to obtain the volumes corresponding to 500 mg of the sample. However, for determination of organic matter degraded in 24 h (required for expressing methane production based on per unit of organic matter degraded), incubation of 500 mg of air-dried sample is suggested.

The results on methane production can be expressed as:

1. Methane as percent of the total gas (on volume basis)
2. Methane (mL) produced/100 mg of organic matter degraded
   (Methane in the corresponding blank should be subtracted from that in the test syringe).

The in vitro gas method is a useful tool for screening various plants/plant extracts/plant compounds or any other additive having potential to reduce methane emission from ruminants. Lower is the methane produced per unit organic matter degraded, better the feed. In addition, effect of various supplementation strategies could also be evaluated for reducing methane production, enhancing efficiency of microbial protein synthesis or for achieving higher fibre degradability.

If the facilities for methane measurement do not exist, methane production can be calculated from the net short chain fatty acid production (after subtracting short chain fatty acids in the blank), using stoichiometric relationships. Using a gas chromatograph, short chain fatty acids are measured in the supernatant of the fermentation medium after 24 h (see section "Preparation of apparent undegraded residue"). High correlations between stoichiometrically calculated gas and actually observed values have been observed by many workers [20]. This forms the basis for the determination of carbon dioxide and methane from the amount and molar proportion of short chain fatty acids.

For example, for Feed 1 (roughage), net production of short chain fatty acids in 24 h is 1.2 mmol with a molar proportion of: 0.75 acetate, 0.19 propionate and 0.06 butyrate.

Using Eq. (7.1), fermentative CO_2 from 1 mmol short chain fatty acids for Feed 1$= 0.75/2 + 0.19/4 + 1.5 \times 0.06 = 0.5125$ mmol.

Using Eq. (7.2), fermentative CH_4 from 1 mmol short chain fatty acids for Feed 1 $= (0.75 + 2 \times 0.06) - 0.5125 = 0.3575$ mmol.

Total fermentative CH_4 from 1.2 mol short chain fatty acids for Feed 1 $= 0.3575 \times 1.2 = 0.429$ mmol.

Volume of methane = mmol methane × gas constant × temperature in Kelvin/atmospheric pressure.
$= 0.429 \times 0.0821 \times 312$(atmospheric pressure taken as 1 at mean sea level)
$= 10.99$ mL

If the experiment has been conducted at, for example, Hohenheim, Stuttgart which has an altitude of 400 metres, the p = 0.953, the volume of methane in the syringe would be 10.99/0.953 = 11.53 mL.

Let us assume that in in vitro the organic matter degraded in 24 h for Feed 1 is = 250 mg.

Estimated methane production (mL)/g organic matter degraded = (11.53/250) × 1000 = 46.12 mL

From the in vitro gas system, estimated methane production (mL)/kg organic matter degraded = 46.12 L.

If intake of the Feed 1 is known, using the values obtained from the in vitro gas method, methane excretion in vivo can be estimated [15].

If the organic matter intake (dry matter intake *minus* ash) of Feed 1 was 946 g/day in a sheep. Using the organic matter degradability value obtained after 24 h in the in vitro gas method (see section "Organic matter degradability"), the expected digestible organic matter in vivo could be calculated. Let us assume that the organic matter degradability (as directed in "Organic matter degradability") is 55%.

Expected digestible organic matter in vivo = 946 × 0.55 = 520 g.
Estimated methane production/day in vivo = (520 × 46.12)/1000 = 23.98 L.

From the amount of short chain fatty acids and their molar proportions, other parameters such as ATP produced, substrate required for microbial mass, total substrate required for formation of products (short chain fatty acids, microbial mass and fermentative gases), and partitioning factor can be calculated. The procedure for their calculation at ^{Y}ATP of 10 and 20 is given in Table 1 and Figure 1 of Getachew et al. [20].

Note: For Feed 2 (for example concentrate based), net production of short chain fatty acids in 24 h is 1 mmol with molar proportion: 0.46 acetate, 0.465 mmol propionate and 0.075 butyrate. For this feed, methane production would be only 3.4 mL at 1 atmospheric pressure. It may be noted that for the same total short chain fatty acid production of 1 mmol for Feed 1 and Feed 2, methane production could differ substantially, depending on the molar proportion of short chain fatty acids. Therefore, besides intake and digestibility, the nature of fermentation product formed from the digested feed determines the proportion of methane production. In the rumen, partitioning of the digested feed into microbial mass and short chain fatty acids, and within short chain fatty acids, the molar proportion of short chain fatty acids determines methane production.

Methods for Measurement of Microbial Mass, Microbial-Nitrogen, Microbial Purines and ^{15}N Incorporation in Microbes and Determination of Efficiency of Microbial Protein Synthesis

This section deals with measurement of efficiency of microbial protein determination for tannin-containing feeds, since the concept of determination of partitioning factor based on the determination of organic matter degraded does not hold true for

such feeds rich in tannins, saponins or in any other plant secondary compounds. However, the approaches listed below could be used for any feed resource.

The ratio of mg Microbial-N to mL net gas production in 24 h of incubation, as an index of efficiency of microbial protein synthesis

Microbial-N (MN) could also be measured after incubation by following two nitrogen balance approaches [23, 30]. The first approach is:

$$\text{MN} = \text{TN} - (\text{NDF-N} + \text{Ammonia-N}_{24\,\text{h}}) \tag{7.5}$$

where TN is total N i.e., feed N + N in buffered rumen fluid in the syringe before incubation (at 0 time), NDF-N is the N bound to neutral detergent fibre fraction following incubation (24 h) and Ammonia-N is the ammonia-N in the supernatant following the incubation (24 h). In a closed system, the total N present at the start of the incubation can be in microbial mass, NDF-N, ammonia-N and amino acids during any time of the incubation. Negligible amounts of amino acids and peptides are present in the supernatant during fermentation and therefore these can be ignored in calculation of microbial-N.

For determination of TN at time 0, one required nitrogen content of the feed on dry matter basis. For a syringe containing 500 mg sample of 95% dry matter and nitrogen content of 4% on dry matter basis, the feed-N at time 0 will be: $(500 \times 0.95) \times 4/100 = 19$ mg. To this value, N in buffered rumen fluid in the syringe before incubation needs to be added. For determination of N in the buffered medium, we collect approximately 50 mL of the buffered medium while the syringes are being filled at time 0. It is centrifuged at approximately 20,000*g* for 20 min at 4°C to obtain the supernatant which is free of microbes. The supernatant is kept frozen until analysis for ammonia-N. The supernatant is thawed and an aliquot (10 mL) of this medium is taken in a Kjeldahl flask and to it is added 3 mL of 1 N sodium hydroxide and immediately steam distilled to liberate ammonia. The ammonia is absorbed in 2% boric acids solution and titrated with 0.1 N sulphuric acid. The volume of sulphuric acid (mL) used is converted to mg ammonia-N by multiplying with 1.4. This analysis in triplicate and average of the three values gives mg ammonia-N in 10 mL of the medium. This is multiplied by 4 to obtain mg ammonia-N in 40 mL syringe contents. The total-N is the sum of this ammonia-N and the feed-N incubated in the syringe.

Ammonia-$N_{24\,h}$ is calculated by centrifuging (20,000*g* for 20 min at 4°C) the contents of the syringe after 24 h of incubation and determination of ammonia-N as described for time 0.

For determination of neutral detergent fibre fraction for NDF-N after incubation, the syringe contents at 24 h of incubation (after recording the gas volume) are transferred into a 600 mL beaker and the syringes are washed twice with a total of 50 mL neutral detergent solution and emptied into the beaker. The contents are refluxed for 1 h, and then filtered through pre-tarred filter crucibles (porosity 2). The crucibles are dried overnight at 100°C and weighed. The residue after neutral detergent solution treatment (neutral detergent residue, NDF) on the crucibles is subjected to micro-Kjeldahl digestion for determination of nitrogen, NDF-N.

Equation (7.5) is used for determination of microbial-N.

The second approach for determination of microbial-N (MN) is:

$$\text{MN} = \text{APUR-N} - \text{NDF-N},$$

where APUR-N is N bound to apparent undegraded residue after incubation. The preparation of apparent undegraded residue is given in section "Preparation of apparent undegraded residue"). N bound to this fraction is determined in a manner similar to NDF-N determination, using the micro-Kjeldahl method.

The ratio of microbial-N (measured by any of the two methods) to the net gas produced in a syringe is a reflection of efficiency of microbial protein synthesis.

The ratio of microbial-N to net short chain fatty acids produced in 24 h is also a reflection of efficiency of microbial protein synthesis (using a gas chromatograph, short chain fatty acids are measured in the supernatant of the fermentation medium after 24 h; see section "Preparation of apparent undegraded residue").

On incubation of six tannin-rich browses in the presence or absence of polyethylene glycol, a strong correlation ($R^2 = 0.98$) occurred between the two N balance methods. In addition, the pattern observed using these methods was similar to that observed with purines [23].

Another approach for determination of microbial mass is the difference between the apparent undegraded residue after incubation and truly undegraded residue [8, 12]. The former contains undegraded feed and microbes and the latter only the undegraded feed since the microbes have been digested by the neutral detergent solution. Mathematically this could be described as:

$$\begin{aligned}\text{Microbial mass (mg)} = {} & ((\text{mg apparent undegraded residue of the test} - \text{mg apparent} \\ & \text{undegraded residue of the blank at 0 h}) - \text{mg truly} \\ & \text{undegraded residue}) = [(\text{y} - \text{y'}) - \text{a}]\end{aligned}$$

For determination of *y* and *a* see sections "Preparation of apparent undegraded residue" and "Organic matter degradability" respectively. The determination of *y'* is similar to that of *y*, except that 40 mL aliquot of the buffered medium containing rumen liquor which is added into the blank syringes at 0 h of incubation is centrifuged (20,000*g*, 20 min, 4°C) and the pellet washed, centrifuged and lyophilized, as for the 24 h sample (see "Preparation of apparent undegraded residue").

The ratio of the microbial mass to the net gas (or short chain fatty acids) produced in a syringe or $((y - y') - a) \times 100/c - (a–b)$ is the efficiency of microbial protein synthesis, as percent of organic matter degraded.

Since the values for apparent undegraded residue and truly undegraded residue are distorted by the presence of tannins [29, 30], the approach based on the difference between the apparent undegraded residue after incubation and the truly undegraded residue should not be used for tannin-rich feeds [36–38].

Determination of Microbial Protein Production and Efficiency of Microbial Protein Synthesis Using Purines as a Marker

This method involves the determination of purines in apparent undegraded residue left after fermentation (According to [31]).

Preparation of Apparent Undegraded Residue

After 24 h of fermentation, centrifuge (20,000*g*, 20 min, 4°C) the contents of the syringe (volume 40 mL) and discard the supernatant (if one has to measure short chain fatty acids and ammonia-N, retain this supernatant; generally we freeze it at –20°C till analysis). Wash the syringe three times with distilled water, by dispensing each time 15 mL through the spike into the syringe, shaking it to remove residual particles and transferring the contents to the centrifuge tube. After completing rinsing of the syringe, repeat the centrifugation (20,000*g*, 20 min, 4°C) and discard the supernatant. Wash the pellet with distilled water (see *Notes* below) followed by centrifugation (20,000*g*, 20 min, 4°C). Lyophilize the pellet, which consists of undegraded feed and microbial mass. If a lyophilizer is not available, dry the pellet in a vacuum over at approximately 50°C. The lyophilized residue is the apparent undegraded residue. The weight of this residue should be determined: (weight of centrifuge tube plus the residue) *minus* weight of empty centrifuge tube). This weight (let it be *y*) needs to be taken into account in the calculations at a later stage. Use a representative sub-sample of this preparation for analyses. In order to obtain a representative sub-sample, it is advised to grind the entire residue in a pestle and mortar or in a small rotating-ball grinding mill.

Notes:

1. *Saline solution instead of water has also been used for washing the pellet. In such a situation the pellet after drying contains sodium chloride, which will distort the value for apparently undegraded residue.*
2. *In our laboratory, short chain fatty acid determination is done using the procedure described in [27]. In brief, 1.8 mL sample and 0.2 mL formic acid containing internal standard (1 mL 2-methylvaleric acid dissolved in 99 mL formic acid) are incubated overnight at 4°C. The samples are centrifuged at approximately 20,000 g for 10 min at 4°C and about 1 mL of the supernatant is pipetted into 2 mL gas chromatograph vials. Short chain fatty acids are determined with a gas chromatograph (GC-14A, Shimazu Corporation) fitted with a flame ionization detector. Separation is carried out with Chromosorb WAW (100/200 mesh) containing a stainless column packed with GP 10% SP 1000 1% H_3PO_4. The analytical conditions are: N_2 60 mL/min, injection temperature 170°C, detection temperature 220°C, oven temperature with temperature program of 130–165°C with 2°C/min.*

Preparation of Lyophilized Rumen Microbial Fraction

Two hour after the morning feed, collect about 1 L rumen liquor from a cow fed a diet of the kind being analysed in the in vitro gas method. Pass the liquor through two layers of muslin cloth and then keep at 4°C for 30 min in a carbon dioxide-flushed cylinder of 1 L capacity. Separate the rumen fluid devoid of heavy and light particles by pipetting the liquor from the cylinder between the heavy particles that settle down and the light particles, which float on the top. This method for collection of rumen fluid is adapted from [52]. Centrifuge (20,000*g,* 20 min, 4°C) several portions (each 30–35 mL) of this liquor. Wash the pellets with distilled water followed by centrifugation (20,000*g,* 20 min, 4°C). Repeat this washing step two more times, lyophilize the pellets and pool them. Use a sub-sample of one preparation. The nitrogen content of this fraction should be approximately 7.7% [43]. In the lyophilized rumen, microbial fraction prepared in our laboratory in the manner as described above, the nitrogen content was 7.7–8%.

Spectrophotometric Method for Determination of Purines (Marker for Microbial Mass/Protein) ([53], With Some Modification as Described in [13])

Reagents

1. *Ammonium dihydrogen phosphate (0.2 M):* Dissolve 23 g ammonium dihydrogen phosphate in about 700 mL distilled water and then make up the volume to 1 L with distilled water.
2. *Sodium hydroxide (10 M):* Dissolve 40 g NaOH in approximately 70 mL distilled water and then make up the volume to 100 mL with distilled water.
3. *$AgNO_3$ (0.4 M):* Dissolve 1.6987g $AgNO_3$ in approximately 15mL distilled water and then make up the volume to 25 mL with distilled water. Protect the solution from light. Store in a brown bottle and surround the bottle with black paper.
4. *HCl (0.5 M):* Dilute 10 mL HCl (37%) to 240 mL with distilled water.
5. *Ammonium dihydrogen phosphate (0.2 M):* Dissolve 23 g ammonium dihydrogen phosphate in approximately 700 mL of distilled water and then make up the volume to 1 L with distilled water.
6. *Ammonium dihydrogen phosphate (28.5 mM):* Measure 100 mL of the above 0.2 M solution of ammonium dihydrogen phosphate and make up to 700 mL with distilled water.

Procedure

Weigh (25–75 mg) of the apparent undegraded residue (see above) or the lyophilized microbial fraction (see above) in 25 mL screw-cap tubes and add 2.5 mL of 0.6 M

perchloric acid (commercially available 70% phosphoric acid is 12 N). Incubate the mixture in a water bath at 90–95°C for 1 h. After cooling, add 7.5 mL of 28.5 mM ammonium dihydrogen phosphate and return the tubes to a water bath (90–95°C) for 15 min. After cooling, centrifuged (3000*g*, 10 min) the contents and collect the supernatant. Add an aliquot (0.25 mL) of the supernatant to 4.5 mL of 0.2 M ammonium dihydrogen phosphate and adjust the pH between 2 and 3 (generally to 2.5) using 10 M NaOH. After the pH adjustment, add 0.25 mL of $AgNO_3$ (0.4 M) and keep the mixture overnight at 5°C in the dark. Centrifuge (3000*g*, 10 min) the contents and discard the supernatant. Take care not to disturb the pellet. Wash the pellet with 4.5 mL distilled water adjusted to pH 2 (with sulphuric acid) followed by centrifugation. Suspend the pellet in 5 mL of 0.5 M HCl, vortex thoroughly and transfer to the 90–95°C water bath for 30 min after covering the tubes with marbles. Centrifuge (3000*g*, 10 min) the tubes and record absorbance of the supernatant at 260 nm against 0.5 M HCl. For studies with RNA in the range of 25–75 mg (instead of lyophilized microbial preparation or the apparent undegraded residue), read the absorbance at 260 nm after 1:10 dilution of the supernatant. Without adjustment of the pH (which is generally 3.4) to between 2 and 3 before addition of the $AgNO_3$ solution, the recovery of purine basis from yeast RNA (Sigma) is generally lower (80–90% vs. 94–99%), suggesting the importance of the pH-adjustment step in obtaining satisfactory recoveries. Addition of the $AgNO_3$ solution does not change the pH of the solution. Use o-phosphoric acid for adjustment of pH to 2.7.

Express results either based on RNA or lyophilized microbial preparation.

The value for microbial protein (as RNA equivalent or directly as adenine *plus* guanine content) or microbial mass (as lyophilized microbial preparation) obtained from an amount (say 25 mg of the apparent undegraded residue) is used for calculating purine basis in the total amount of the apparent undegraded residue (in *y*, see section "Preparation of apparent undegraded residue") obtained from the syringe (purine bases after 24 h in the syringe).

Similarly, microbial mass is calculated in the total apparent residue obtained by centrifuging (20,000*g*, 20 min, 4°C) 40 mL of the 0 h medium containing rumen liquor (used for filling the syringes for initiating the fermentation), washing with distilled water, followed by re-centrifugation (20,000*g*, 20 min, 4°C) and lyophilisation (purine bases at 0 h in the syringe).

Microbial protein produced in 24 h (as purine basis) = (purine bases in the apparently undegraded residue after 24 h – purine bases in the apparent residue at 0 h). It is assumed that microbial lysis is negligible during this period.

Microbial protein as RNA equivalent or (or microbial mass) produced after 24 h = (microbial protein (or microbial mass) in the apparently undegraded residue after 24 h – microbial protein (or microbial mass) in the apparent residue at 0 h). It is assumed that microbial lysis is negligible during this period.

Microbial protein as RNA equivalent (or microbial mass) produced after 24 *divided by* net gas produced (or short chain fatty acids produced) in 24 h is a measure of efficiency of microbial protein synthesis.

Some Data on Purines [31]

Purine determination (as A260 nm) using spectrophotometric method

	Absorbance at 260 nm	
	Mean	S.D.
25 mg LRM	0.215	0.006
50 mg LRM	0.456	0.005
75 mg LRM	0.675	0.005
25 mg RNA*	0.210	–
50 mg RNA*	0.414	–

LRM, lyophilized rumen microbes
A260 nm = (0.009207) mg LRM - 0.01178 ($r^2 = 0.99$; n = 3)
*Average of two values and after 1:10 dilution

High Performance Liquid Chromatograph (HPLC) Method for Determination of Purine Bases (Adenine and Guanine) ([2], with some modifications as described in [31])

Equipment, reagents, HPLC conditions and analysis: The HPLC equipment that we use consists of a Merck Hitachi L-7100 HPLC pump, an L-7450 photo diode array detector, an L-7200 autosampler, a D-700 interphase module and an LC organiser.

Analytical column: Reverse phase C18 (LiChrospher 100, endcapped 5 μm) 250 mm × 4 mm I.D. (Lichrocart; Merck, Darmstadt, Germany) protected by a guard column containing the material as in the main column.

HPLC solvent A: 10 mM $NH_4H_2PO_4$ and adjust pH to 6 with 10% NH_4OH.

(Dissolve 11.503 g $NH_4H_2PO_4$ in about 500 mL distilled water and then make the volume to 1 L with distilled water. It is 100 mM solution. Pipette 100 mL of this solution and dilute to 1 L to obtain 10 mM solution)

HPLC solvent B: Add 150 mL of acetonitrile to 600 mL of 12.5 mM $NH_4H_2PO_4$ (75 mL of 100 mM solution plus 525 mL of distilled water) and adjust pH to 6 with 10% NH_4OH.

(Filter solvents A and B through a 0.45 μm filter and degas by ultrasonication and by application of vacuum).

Purine bases and internal standard solution for converting integration units to the concentration: Prepare 1 mM stock solution. Put a measuring flask (250 mL capacity) containing approximately 50 mL distilled water on a magnetic stirrer fixed with a hot plate. Add to the flask 100 μl of 10 M sodium hydroxide solution. Heat at about 90°C and then transfer 33.77, 37.77 and 34.03 mg of adenine and guanine and allopurinol respectively to the flask. Wait (generally 30 min) until the contents dissolve. Cool the contents and make up the

volume to 250 mL with distilled water. This solution can be stored at 4°C for 10 days. Dilute this stock solution 12.5 times; pipette 2 mL of the stock solution into 25 mL measuring flask and make up the volume to 25 mL with buffer A of the HPLC. Inject 40 μl of this solution into the HPLC.

Preparation of 3 mM allopurinol solution: Take 100 mL measuring flask and weigh-in 40.83 mg allopurinol. Add approximately 50 mL of distilled water and 20 μl of 12 N (70%) perchloric acid. Heat the contents to approximately 90°C with stirring on a magnetic stirrer. Cool the contents to room temperature and make up the volume to 100 mL with distilled water.

Preparation of 8 mM caffeine solution: Dissolve 155.36 mg caffeine in 80 mL of distilled water and then make up the volume to 100 mL with distilled water.

Gradient: A 30-min linear gradient from 0 to 100% solvent B. After 40 min, increase solvent A to 100% in the following 5 min and equilibrate the column to the starting condition (100% A) in the next 15 min before injecting the next sample (Table 7.3).

Table 7.3 Gradient used for the HPLC

Time (min)	Solvent A (%)	Solvent B (%)
0.0	100	0
30	0	100
40	0	100
45	100	0
60	100	0

Detection wavelength: 254 nm with a full scale deflection set at 0.2 absorbance

Column temperature: Ambient (approximately 22°C)

Guanine and adenine appear at about 11 and 15.5 min respectively. Allopurinol or caffeine can be used as internal standards. These appear at about 13.5 and 29.5 min respectively. For tannin-containing feeds, do not use caffeine since it binds with tannins, which lowers the recovery of caffeine [31].

Sample Hydrolysis

Weigh 25–100 mg sample (the lyophilized microbial fraction or the apparent undegraded residue) in 25 mL screw-cap tubes and add 2.5 mL of perchloric acid (0.6 M) and 0.5 mL of an internal standard (3 mM allopurinol or 8 mM caffeine). Incubate the mixture in a water bath at 90–95°C for 1 h. After cooling, add 7.0 mL of Buffer A of the HPLC system, adjust the pH between 6.6 and 6.9 using concentrated KOH (approximately 8 M) and centrifuge (3,000*g*) to remove the precipitate

formed. Filter through 0.45 μm filter and inject appropriate volume (15–50 μl) into the HPLC.

Express results based on adenine plus guanine.

The value for purine bases (adenine plus guanine) obtained from an amount (say 25 mg of the apparent undegraded residue) is used for calculating purine basis in the total amount of the apparent undegraded residue (in *y*; see section "Preparation of apparent undegraded residue") obtained from the syringe (purine bases after 24 h in the syringe).

Similarly, purine bases are calculated in the total apparent residue obtained by centrifuging (20,000*g*, 20 min, 4°C) 40 mL of the 0 h medium containing rumen liquor (used for filling the syringes for initiating the fermentation), washing with distilled water, followed by recentrifugation (20,000*g*, 20 min, 4°C) and lyophilisation (purine bases at 0 h in the syringe).

Microbial protein produced in 24 h (as purine basis) = (purine bases in the apparently undegraded residue after 24 h – purine bases in the apparent residue at 0 h). It is assumed that microbial lysis is negligible during this period.

Microbial protein produced in 24 h (as purine basis) *divided by* net gas produced (or short chain fatty acids produced) in 24 h is a measure of efficiency of microbial protein synthesis.

Purine amount can be converted to microbial mass microbial-N by taking rumen fluid sample, centrifuging it at approximately 20,000*g* to obtain microbial pellet, washing it once with distilled water and recentrifuging, and lyophilising it [32]. In a weighed lyophilised pellet, purines can be determined by HPLC or using a spectrophotometeric method after precipitation of purine with silver nitrate. Using this purine to microbial mass ratio, the purine can be converted into microbial mass. If there is a need to convert purine to microbial-N, a portion of the same lyophilised pellet can be subjected to the determination of N using micro-Kjeldahl method.

Microbial mass (or microbial-N) produced in 24 h *divided by* net gas produced (or short chain fatty acids produced) in 24 h is also a measure of efficiency of microbial protein synthesis.

Some Data on Purine Bases [31]

Purine base determination using HPLC method

	Adenine (μmol)		Guanine (μmol)	
	Mean	SD (n=3)	Mean	SD (n=3)
50 mg LRM	2.35	0.03	2.95	0.02

LRM, lyophilized rumen microbes

Note: It may be noted that workers can optimize their own system for a particular material being studied once equipped with a sound grasp of the rationale behind these procedures.

Table 7.4 Effects of saponins on gas production, purine content (index of microbial protein), truly degraded substrate (TDS) and efficiency of microbial protein synthesis after 24 h of incubation

	TDS (mg)	Gas (mL)	Purines (μmol)	Efficiency of microbial protein synthesis	
				μmol purine/mL gas	μmol purine/mg TDS
Control	300.0 ± 10.3^{a}	95.3 ± 0.9^{a}	6.94 ± 0.36^{a}	0.0728	0.0231
Yucca saponins	320.7 ± 7.1^{b}	91.2 ± 0.9^{b}	9.11 ± 0.34^{b}	0.0998	0.0284
Quillaja saponins	323.0 ± 1.0^{b}	94.5 ± 1.9^{a}	7.99 ± 0.34^{c}	0.0845	0.0247
Acacia saponins	297.6 ± 6.0^{a}	83.5 ± 0.3^{c}	8.38 ± 0.23^{c}	0.1004	0.0282

Data are from Makkar et al. [39], 500 mg hay (475 mg DM) was incubated in the syringes. The means with different superscripts differ ($P < 0.05$).
Data on, and implications of, purines in apparently undegraded residues and efficiency of microbial protein synthesis as a ratio of purines to short chain fatty acids on incubation of tannin-rich feeds alone or with polyethylene glycol are available in Getachew et al. [22–24].

Table 7.4 presents a unique set of data on gas production, true dry matter degradability, microbial mass and efficiency of microbial protein synthesis on addition of potential additives (0.6 mg/mL of various saponins) in the gas method. These parameters were affected to different extents by saponins. For example, addition of Quillaja saponins did not affect gas production, but increased purine content and truly degraded substrate by about 7%. Since truly degraded substrate in an in vitro system can only lead to production of gas and microbial mass, implying that all of the increase in truly degraded substrate by Quillaja saponins resulted in higher microbial protein mass (i.e., saponins increased efficiency of microbial protein synthesis). Had only gas production been measured, the conclusion could have been that Quillaja saponins had no effect on fermentation. In contrast, Acacia saponins decreased gas production, but increased microbial protein synthesis without affecting true degradability. Thus saponins affected partitioning of degraded nutrients such that more microbial mass was produced at the cost of gas, and/or short chain fatty acid production; again reflecting higher microbial efficiency. This higher microbial efficiency would not have been detected had only gas production been measured. The effect of Yucca saponins differed from those of Quillaja or Acacia saponins. Yucca saponins decreased gas, increased microbial protein synthesis and increased true degradability [39], suggesting that measurement of gas only is not sufficient to describe the "true" response of saponins (or of any additive). This highlights the importance of measuring microbial protein (and efficiency of microbial protein synthesis) along with the gas in an in vitro gas method. Similar conclusions have been arrived at by using partitioning factor as a measure of efficiency of microbial protein synthesis [20, 30]. A holistic view of the effects of an additive on rumen fermentation could only be obtained by incorporating analysis of partitioning factor or of another indicator of efficiency of microbial protein synthesis.

Determination of Microbial Protein Production and Efficiency of Microbial Protein Synthesis Using ^{15}N Incorporation

Reagents

Same as in the "reagents" section under "Method for measuring Partitioning Factor", except for the following:

Bicarbonate buffer solution: Dissolve 35 g sodium bicarbonate ($NaHCO_3$) and 4 g ammonium carbonate (NH_4HCO_3) (consisting of 5% ^{15}N-enriched N) in approximately 500 mL distilled water and then make up the volume to 1 L with distilled water.

Notes:

1. *If 50% ^{15}N-enriched NH_4HCO_3 is available, add 0.4 g of this ^{15}N-enriched NH_4HCO_3 and 3.6 g of NH_4HCO_3 in 1 L solution.*
2. *If 96.5% ^{15}N-enriched NH_4HCO_3 is available, add 0.207 g of this ^{15}N-enriched NH_4HCO_3 and 3.793 g of NH_4HCO_3 in 1 L solution.*
3. *Using 5% ^{15}N-enriched bicarbonate buffer, the expected enrichment in the apparently undegraded residue would be approximately 1%. The enrichment will also depend on the nitrogen content of the feed incubated and the rumen liquor added to the incubation medium.*

Procedure

Other procedures such as "Weighing of samples and preparation of syringes", "Preparation of in vitro rumen fermentation buffer solution and incubation", and "Net gas production" were as described in sections "Weighing of samples and preparation of syringes", "Preparation of in vitro rumen fermentation buffer solution and incubation" and "Net gas production" respectively.

After recording the gas volume after 24 h, the syringe contents were subjected to the procedure given in "Preparation of apparent undegraded residue" to obtain apparent undegraded residues (undegraded feed plus microbes after 24 h of incubation). This residue should be quantitatively collected and ground to fine powder preferably using ball mill. This residue (1–3 mg containing approximately 100 μg N; could be lower depending on the N content of the residue and sensitivity of the mass spectrometer) is weighed into tarred tin foil cups using a microbalance, and subjected to Mass Spectroscopy to measure ^{15}N enrichment. Ammonium sulphate (or ammonium chloride) solution containing 50 μg N/μl of known ^{15}N enrichment (standard), covering the observed enrichment range, is included in the measurements. An amount of ^{15}N in excess of 0.366% is considered as enrichment.

The values obtained from the Mass Spectroscopy gives the proportion of the total nitrogen as ^{15}N (percent enrichment). If the mass spectrometer is combined with an elemental analyser, total N content can also be obtained. The N content can also

be determined separately using an elemental analyser or micro-Kjeldahl method (digestion followed by ammonia determination by steam distillation, absorption in boric acid and titration with sulphuric acid or by reaction with hypochlorite-nitroprusside and measurement of the blue colour spectrophotometrically [16]. Using these values, total ^{15}N enrichment in the total amount of apparently undegraded residue can be calculated:

^{15}N in apparently undegraded residue (mg) = ((weight of apparently undegraded residue in mg × % N)/100) × (% 15 N in apparently undegraded residue/100)

As described above, the apparently undegraded residue is composed of undegraded feed and microbes produced during the incubation. However, ^{15}N incorporation from ^{15}N-labelled ammonium bicarbonate added in the buffer is in the microbes, and hence it is an index of microbial protein (or microbial mass) synthesis. Higher is the ^{15}N incorporation in the residue, higher the microbial protein or microbial mass produced.

^{15}N incorporation in the microbes in 24 h (mg) = (mg ^{15}N in the apparently undegraded residue of the test syringe after 24 h – mg ^{15}N in the apparently undegraded residue of the corresponding blank after 24 h). It is assumed that microbial lysis is negligible during this period.

Efficiency of microbial protein synthesis = ^{15}N incorporation in the microbes in 24 h/net gas (or short chain fatty acids) produced in 24 h.

Do's and Don'ts for the Gas Method

1. The plunger should be properly lubricated using white Vaseline.
2. Collect rumen liquor from both the liquid and the solid phase and handle it properly (use of warm containers, flushing the containers with carbon dioxide, always keeping the rumen liquor under carbon dioxide).
3. Reducing solution should be prepared fresh on the same day of conducting the experiment.
4. Start flushing the medium with carbon dioxide well before (approximately 10 min) adding the reducing solution. Also, flush the medium for at least 10 min after adding the rumen liquor and before starting filling the syringes. Keep flushing the medium with carbon dioxide while filling the syringes (the flow could be reduced at this stage).
5. While filling the syringes with the medium, keep an eye on the medium (carbon dioxide gas should be flushing into the medium and the medium should be stirring).
6. After dispensing 40 mL of the medium into the syringe, create a light vacuum by pushing back the plunger and then open the clip, for removing air from the syringe. This procedure will bring the medium lying in the nozzle back into the syringe. Otherwise there could be a loss of the medium and/or sample.

7. After filling of the syringes has been completed (might take 30–40 min), shake the syringes. Shake them again after every 30 min until first 2 h of the incubation, and then after every two h till the first 10 or 12 h of the incubation. Make sure that all feed particles are taken into the medium while stirring (swirling shaking action might help).
8. Wash the dispenser with distilled water immediately after finishing filling the syringes, otherwise the dispenser could get stuck up and might not then be usable.
9. Check temperature and level of water in the water bath at least twice a day.
10. In the evening before going home, if the plunger is above 80 mL level, push it back; record the readings (before and after) pushing back the plunger.
11. When you push back the syringe in the evening, give a shake after approx. 30 min in order to prevent taking up the sample along with the bottom portion of the plunger and out from the incubation medium.
12. Use carbon dioxide gas cylinder with caution. Ask someone if you do not know its operation. Misuse could cause an accident.
13. While taking the gas volume readings, use the brown ring marked on the plunger and not the bottom end of the plunger. Keep the syringe in inverted position and in parallel with eye while recording the gas reading. Immediately transfer the syringe into the water bath after taking the reading.
14. For cleaning the syringes, the syringe should be emptied (preferably pulling back the plunger and removing contents from the back and not from the nozzle). Clips should be removed. The plunger and the outer graduated part of the syringe (barrel) should be separated. Excess Vaseline on the plunger should be cleared with a tissue paper or a piece of soft cloth, and then transfer both the parts in hot water containing detergent (soap) solution. Rub the plunger with hand and inside of the barrel with a soft brush to clean these. Wash thoroughly both the portions with hot water and finally rinse them with distilled water. Dry them well before weighing sample into the syringe.
15. Fix the clip in such a manner (by keeping the portion, where pressure is applied to open or close it, facing the syringe) that it does not open by striking on the edges of the lid of the water bath while taking out the syringe for taking reading.
16. Mark the crucibles well (preferably with a diamond pencil). Keep them in increasing or decreasing order; this might help you in identifying the crucibles, which have not been marked well, especially after these have been placed in the Muffle furnace.

Note: There are a number of in vivo methods for determination of net microbial protein synthesis in the rumen (and hence of the efficiency of microbial protein synthesis) based on the use of microbial markers. They require the use of post-ruminally cannulated animals to determine flow of digesta. The cannulation approach is tedious and has several limitations [17] to its applicability under most research conditions in developing countries. A simpler technique for determination of microbial protein supply to the intestine is based on the determination of total urinary

purine derivatives [28]. Although the method is based on the collection of urine for determination of purine derivatives (allantoin and uric acid for cattle, and allantoin, uric acid, xanthine and hypoxanthine for sheep), the approach has been further simplified using spot urine samples [32]. This technique does not require cannulated animals, but involves feeding of the diets under investigation to animals, and therefore is not suitable for screening a large numbers of samples or for developing feed supplementation strategies using various feed constituents.

Conclusions

The methods reported here in which gas production and microbial mass production are concomitantly measured have several major applications:

i) study of rumen modulators or in the screening plants or plant extracts for increasing efficiency of microbial protein synthesis and decreasing emission of methane and carbon dioxide,
ii) potential for screening a large number of feed resources, for example in breeding programmes for the development of varieties and cultivars of good nutritional value, and
iii) development of supplementation strategies using locally available conventional and unconventional feed constituents to achieve maximum microbial efficiency in the rumen; and
iv) study of roles of various nutrients (by changing the composition of the incubation medium) with respect to production of fermentative gases, short chain fatty acids and microbial mass.

The choice of methods for determination of microbial mass, and estimation of efficiency of microbial protein mass synthesis, and for investigation on the partitioning of nutrients to various products depends on the facilities available and objective of the experiment. The determination of partitioning factor as the ratio of organic matter degraded to gas production is simple and can be used for evaluation of conventional feeds or for studying the effects of plant extracts, plant compounds or any other additives provided these are not rich in tannins. The use of this partitioning factor-based approach should be used with caution for feeds rich in plant secondary metabolites. The determination of microbial mass using the nitrogen balance approaches, by measuring purine or ^{15}N incorporation in the microbes could be used for all type of feeds.

References

1. Aiple, K.P., H. Steingass, and W. Drochner. 1996. Prediction of net energy content of raw materials and compound feeds for ruminants by different laboratory methods. Arch. Anim. Nutr. **49**:213–220.

2. Balcells, J., J.A. Guada, and J.M. Peiró. 1992. Simultaneous determination of allantoin and oxypurines in biological fluids by high-performance liquid chromatography. J. Chromatography. **575**:153–157.
3. Ben Salem, H., A. Nefzaoui, L. Ben Salem, and J.L. Tisserand. 1999. Different means of administering polyethylene glycol to sheep: effect on the nutritive value of *Acacia cyanophylla* Lindl. Foliage. Anim. Sci. **68**:809–818.
4. Blümmel, M., and E.R. Ørskov. 1993. Comparison of gas production and nylon bag degradability of roughages in predicting feed intake in cattle. Anim. Feed Sci. Technol. **40**:109–119.
5. Blümmel, M., and K. Becker. 1997. The degradability characteristics of fifty-four roughages and roughage neutral-detergent fibres as described by in vitro gas production and their relationship to voluntary feed intake. Brit. J. Nutr. **77**:757–768.
6. Blümmel, M., H. Steingass, K. Becker, and H. Soller. 1993. Production of SCFA, CO_2, CH_4 and microbial cells in vitro. Proc. Soc. Nutr. Physiol. 1:9.
7. Blümmel, M., H.P.S. Makkar, and K. Becker. 1997. In vitro gas production: a technique revisited. J. Anim. Physiol. Anim. Nutr. **77**:24–34.
8. Blümmel, M., H. Steingas, and K. Becker. 1997. The relationship between in vitro gas production, in vitro microbial biomass yield and ^{15}N incorporation and its implications for the prediction of voluntary feed intake of roughages. Br. J. Nutr. **77**:911–921.
9. Blümmel, M., H.P.S. Makkar, G. Chisanga, J. Mtimuni, and K. Becker. 1997. The prediction of dry matter intake of temperate and tropical roughages from in vitro digestibility/gas-production data, and the dry matter intake and in vitro digestibility of African roughages in relation to ruminant live weight gain. Anim. Feed Sci. Technol. **69**:131–141.
10. Blümmel, M., H.P.S. Makkar, and K. Becker. 1998. The partitioning kinetics of in vitro rumen fermentation products in a gas production test. J. Dairy Sci. (Suppl. 1):309.
11. Blümmel, M., K.P. Aiple, H. Steingass, and K. Becker. 1999. A note on the stoichiometrical relationship of short chain fatty acid production and gas evolution in vitro in feedstuffs of widely differing quality. J. Anim. Physiol. Anim. Nutr. **81**:157–167.
12. Blümmel, M., R. Mgomezulu, X.B. Chen, H.P.S. Makkar, K. Becker, and E.R. Ørskov. 1999. The modification of in in vitro gas production test to detect roughage related differences in in vivo microbial protein synthesis as estimated by the excretion of purine derivates. J. Agric. Sci. (Camb.) **133**:335–340.
13. Blümmel, M., A. Karsli, and J.R. Russell. 2003. Influence of diet on growth yields of rumen micro-organisms in vitro and in vivo: influence on growth yield of variable carbon fluxes to fermentation products. Brit. J. Nutr. **90**:625–634.
14. Blümmel, M., J.W. Cone, A.H. Van Gelder, I. Nshalai, N.N. Umunna, H.P.S. Makkar, and K. Becker. 2005. Prediction of forage intake using in vitro gas production methods: Comparison of multiphase fermentation kinetics measured in an automated gas test, and combined gas volume and substrate degradability measurements in a manual syringe system. Anim. Feed Sci. Technol. **123–124**:517–526.
15. Blümmel, M., D.I. Givens, and A.R. Moss. 2005. Comparison of methane produced by straw fed sheep in open-circuit respiration with methane predicted by fermentation characteristics measured by an in vitro gas procedure. Anim. Feed Sci. Technol. **123–124**:379–390.
16. Cataldo, D.A., L.E. Schrader, and V.L. Youngs. 1974. Analysis by digestion and colourimetric assay of total N in plant tissue high in nitrates. Crop Sci. **14**:854–856.
17. Chen, X.B., F.D.D. Hovell, and E.R. Ørskov. 1990. Excretion of purine derivatives by ruminants: endogenous excretion differences between cattle and sheep. Br. J. Nutr. **63**: 121–129.
18. Cone, J.W., A.H. van Gelder, G.J.W. Visscher, and L. Oudshoorn. 1996. Influence of rumen fluid and substrate concentration on fermentation kinetics measured with a fully automated time related gas production apparatus. Anim. Feed Sci. Technol. **61**:113–128.
19. Dhanoa, M.S. 1988. On the analysis of dacron bag data for low degradability feeds. Grass Forage Sci. **43**:441–444.

20. Getachew, G., M. Blümmel, H.P.S. Makkar, and K. Becker. 1998. In vitro gas measuring techniques for assessment of nutritional quality of feeds: a review. Anim. Feed Sci. Technol. **72**:261–281.
21. Getachew, G., H.P.S. Makkar, and K. Becker. 1998. The in vitro gas coupled with ammonia nitrogen measurement for evaluation of nitrogen degradability in low quality roughages using incubation medium of different buffering capacity. J. Sci. Food Agric. **77**:87–95.
22. Getachew, G., H.P.S. Makkar, and K. Becker. 2000. Tannins in tropical browses: effects of tannins on in vitro microbial fermentation and microbial protein synthesis in medium containing different amounts of nitrogen. J. Agric. Food Chem. **48**:3581–3588.
23. Getachew, G., H.P.S. Makkar, and K. Becker. 2000. Effect of polyethylene glycol on in vitro degradability of nitrogen and microbial protein synthesis from tannin-rich browse and herbaceous legumes. Brit. J. Nutr. **84**:73–83.
24. Getachew, G., H.P.S. Makkar, and K. Becker. 2001. Method of polyethylene glycol application to tannin-containing browses to improve microbial fermentation and efficiency of microbial protein synthesis from tannin-containing browses. Anim. Feed Sci. Technol. **92**: 51–57.
25. Getachew, G., H.P.S. Makkar, and K. Becker. 2002. Tropical browses. Contants of phenolic compounds, in vitro gas production and stoichiometric relationship between short chain fatty acid and in vitro gas production. J. Agric. Sci. (Camb) **139**:341–352.
26. Goering, H.K., and P.J. Van Soest. 1970. Forage fibre analyses (Apparatus, Reagents, Procedures, and Some Applications). Agric. Handbook No. 379, ARS-USDA, Washington, DC.
27. Hoeltershinken, M., U. Pitt, F.C. Tammen, P. Hoffman, and H. Scholz. 1997. Influence of mouldy grass on fermentation and thiamine metabolism in bovine rumen fluid (in vitro). Deutsche Tierartliche Wochenschrift. **104**:17–22.
28. IAEA 1997. Estimation of rumen microbial protein production from purine derivatives in urine. IAEA-TECDOC-945.
29. Makkar, H.P.S. 2003. Tannin assays, effects and fate of tannins, and strategies to overcome detrimental effects of feeding tannin-rich tree and shrub foliage. Small Ruminant Res. **49**: 241–256.
30. Makkar, H.P.S. 2005. In vitro gas method for evaluation of feeds containing phytochemicals–a review. Anim. Feed Sci. Technol. **123–124**:291–302.
31. Makkar, H.P.S., and K. Becker. 1999. Purine quantification in digesta from ruminants by spectrophotometic and HPLC methods. Br. J. Nutr. **81**:107–112.
32. Makkar, H.P.S., and X.B. Chen. 2004. Estimation of microbial protein supply in ruminant livestock through quantification of urinary purine derivatives, p. 212. FAO/IAEA Publication, Kluwer Academic Press, Dordrecht, The Netherlands.
33. Makkar, H.P.S., M. Blümmel, N.K. Borowy, and K. Becker. 1993. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. J. Sci. Food Agric. **61**:161–165.
34. Makkar, H.P.S., Blümmel, M. and K. Becker. 1995. In vitro effects of and interactions between tannins and saponins and fate of tannins in the rumen. J. Sci. Food Agric. **69**:481–493.
35. Makkar, H.P.S., M. Blümmel, and K. Becker. 1995. Formation of complexes between polyvinyl pyrrolidone and polyethylene glycol with tannins and their implications in gas production and true digestibility in in vitro techniques. Brit. J. Nutr. **73**: 897–913.
36. Makkar, H.P.S., N.K. Borowy, K. Becker, and A. Degen. 1995. Some problems in fiber determination of a tannin-rich forage (*Acacia saligna* leaves) and their implications in in vivo studies. Anim. Feed Sci. Technol. **55**:67–76.
37. Makkar, H.P.S., M. Blümmel, and K. Becker. 1997. In vitro rumen apparent and true digestibility of tannin-rich forages. Anim. Feed Sci. Technol. **67**:245–251.
38. Makkar, H.P.S., K. Becker, and M. Younan. 1997. Nutritional implications of bound proanthocyanidins. Proc. XVIII International Grassland Congress, June 8–12, Winnipeg, Canada.
39. Makkar, H.P.S., S. Sen, M. Blümmel, and K. Becker. 1998. Effects of fractions containing saponins from *Yucca schidigera, Quillaja saponaria* and *Acacia auriculoformis* on rumen fermentation. J. Agric. Food Chem. **46**: 4324–4328.

40. McDonald, I. 1981. A revised model for the estimation of protein degradability in the rumen. J. Agric. Sci. (Camb.) **96**:251–252.
41. Mehrez, A.Z., and E.R. Ørskov. 1977. A study of artificial fibre bag technique for determining the digestibility of feeds in the rumen. J. Agric. Sci. (Camb.) **88**:645–650.
42. 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 in vitro. J. Agric. Sci. (Camb.) **92**:217–222.
43. Ørskov, E.R. 1982. pp. 19–40. In E.R. Orskov (ed.), Protein Nutrition in Ruminants. Academic Press, London.
44. Pell, A.N., and P. Schofield. 1993. Computerised monitoring of gas production to measure forage digestion. J. Dairy Sci. **76**:1063–1073
45. Raab, L., B. Cafantaris, T. Jilg, and K.H. Menke. 1983. Rumen protein degradation and biosynthesis. 1. A new method for determination of protein degradation in rumen fluid in vitro. Brit. J. Nutr. **50**:569–582.
46. Russell, J.B., J.D. O'Connor, D.G. Fox, P.J. Van Soest, and C.J. Sniffen. 1992. A net carbohydrate and protein system for evaluating cattle diets. I Ruminal fermentation. J. Anim. Sci. **70**:3551–3561.
47. Rymer, C., and D.I. Givens. 1999. The use of the in vitro gas production technique to investigate the effect of substrate on the partitioning between microbial biomass production and the yield of fermentation products. Proc. Br. Soc. Anim. Sci. 36.
48. Theodorou, M.K., B.A. Williams, M.S. Dhanoa, A.B. McAllan, and J. France. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Technol. **48**:185–197.
49. Tilley, J.M., and R.A. Terry. 1963. A two stage technique for the in vitro digestion of forage crops. J. Brit. Grassl. Soc. **18**:104–111.
50. Van Soest, P.J. 1994. Nutritional ecology of ruminants, 2nd edition. Cornell University Press, New York.
51. Wolin, M.J. 1960. A theoretical rumen fermentation balance. J. Dairy Sci. **43**:1452–1459.
52. Yang C.M.J., and J.B. Russell. 1992. Resistance of proline-containing peptides to ruminal degradation in vitro. Appl. Environ. Microbiol. **58**:3954–3958.
53. Zinn R.A., and F.N. Owens. 1986. A rapid procedure for purine measurement and its use for estimating net ruminal protein synthesis. Can. J. Anim. Sci. **66**:157–166.

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

Greg W. Kemp and Chris S. McSweeney

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].

C.S. McSweeney (✉)
CSIRO Livestock Industries, St. Lucia, Queensland 4067, Australia
e-mail: chris.mcsweeney@csiro.au

P.E. Vercoe et al. (eds.), *In Vitro Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies*,
DOI 10.1007/978-90-481-3297-3_8,

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).

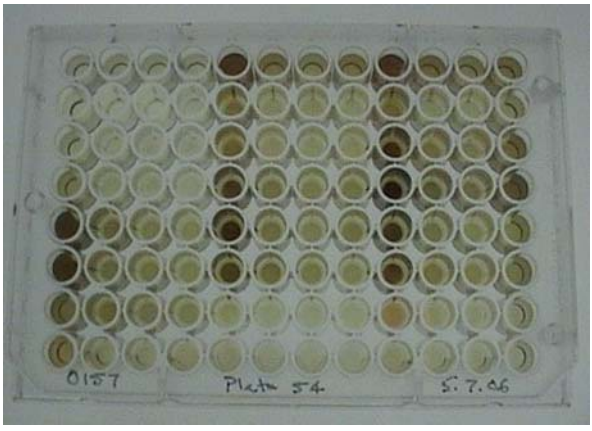

Fig. 8.1 A typical 96-well micro-titre test plate showing the masking effect (i.e. darker wells) of the test compounds

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

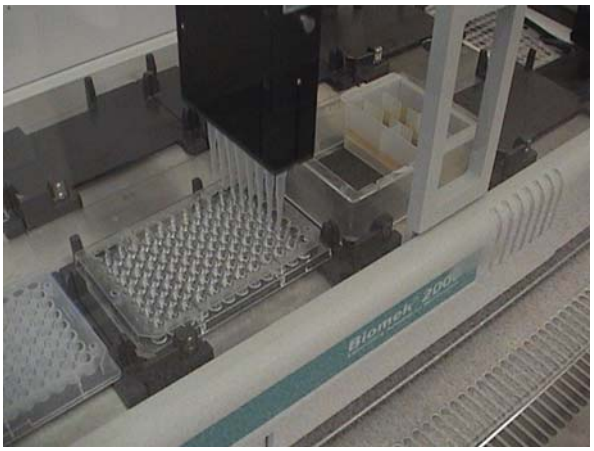

Fig. 8.2 Plates being loaded with media on the Beckman Biomek 2000^{TM} robot

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).

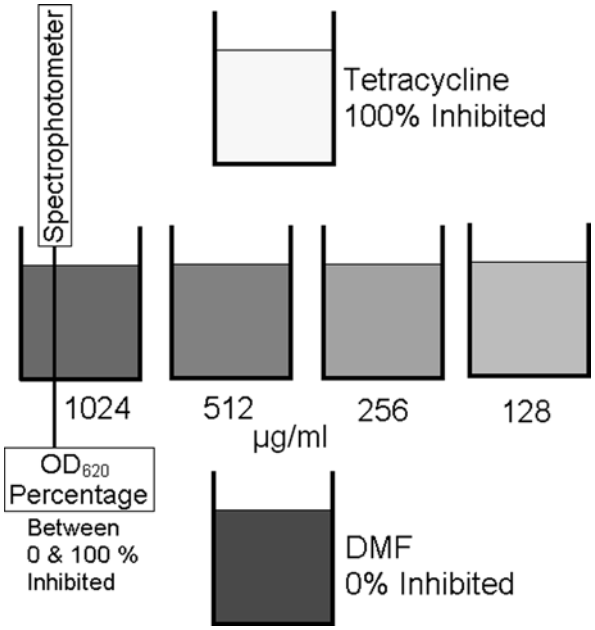

Fig. 8.3 Calculation of percentage inhibition from OD_{620} values compared between the 0 and 100% inhibition standards

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*

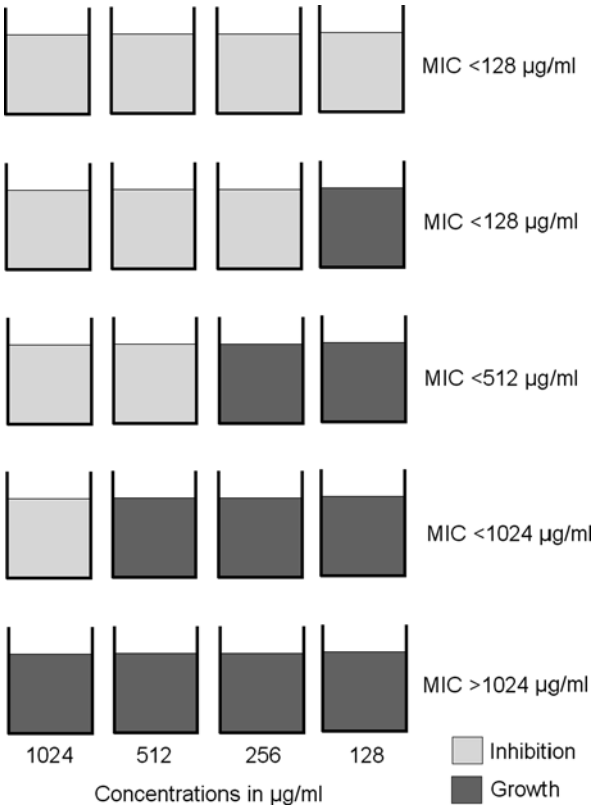

Fig. 8.4 Identification of the concentration where growth is inhibited for a particular target organism and calculation of minimum inhibitory concentration

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

Acknowledgements Our thanks to Dr. B. Lowry and Mr. L. Conlan for useful discussions regarding plant extractions and to Drs. P. Jennings and G. Wijffels for support during the early stages of assay development.

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.

Chapter 9
Screening Plants for the Antimicrobial Control of Lactic Acidosis in Ruminant Livestock

Peter G. Hutton, T.G. Nagaraja, Colin L. White, and Philip E. Vercoe

Introduction

Lactic acidosis, characterized by excessive accumulation of lactic acid in the rumen, is a major ruminal disorder that reduces productivity and the welfare of animals, particularly in dairy and beef feedlot systems. Lactate in the rumen is an intermediate microbial product that is metabolized further to volatile fatty acids (VFA). Normal concentrations of lactate in the rumen are maintained by the microbial balance between the major lactate-producers, *Streptococcus bovis* and *Lactobacillus* spp., and major lactate-utilisers, *Megasphaera elsdenii* and *Selenomonas ruminantium* [18]. However, large increases of rapidly-fermentable carbohydrate in the diet can cause a major microbial imbalance in the rumen [29]. The imbalance is due mainly to the initial rapid increase in *S. bovis* that utilises freely available and highly fermentable carbohydrate and produces lactic acid as an end product. The microbial balance then shifts to dominant populations of *Lactobacilli* that are acid-tolerant and produce more lactic acid. As the ruminal pH drops there is a simultaneous decrease in lactate-utilisers, such as *M. elsdenii* and *S. ruminantium* resulting in microbes that produce lactic acid outnumbering those that utilise it [18]. Subsequently, normal rumen fermentation is inhibited and there is a decline in the production of gas and VFA by the rumen microbes [13, 38]. A spiraling effect is initiated and at ruminal pH of below 5.2 the animal develops acute acidosis [29, 31]. During acute acidosis, lactobacilli become the predominant species and can form almost a monoculture within 24 h [8].

The addition to the feed of sub-therapeutic doses of antibiotics, such as virginiamycin inhibits the growth of lactate-producing bacteria without affecting lactic acid-utilizing bacteria or normal rumen fermentation [1, 17, 25, 39]. These

P.G. Hutton (✉)
School of Animal Biology, Faculty of Natural and Agricultural Sciences, University of Western Australia, Perth, WA, Australia; Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Palmerston North, NZ
e-mail: P.G.Hutton@massey.ac.nz

P.E. Vercoe et al. (eds.), *In Vitro Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies*,
DOI 10.1007/978-90-481-3297-3_9,

antibiotics are effective at preventing acidosis but growing concern of antimicrobial resistance that can carry over to human pathogens has led to bans or limitations on the feeding antibiotics to livestock [40]. It is therefore necessary to find alternatives that are safe for the consumer, but maintain animal production. Many plants are known to produce compounds with antimicrobial activity for defence against predators [3, 32]. Some of these compounds have broad-spectrum antimicrobial activity, while others seem to target specific bacterial species [6, 14, 22]. However, the antimicrobial activity of many of these plants has not been tested on rumen bacteria, including those responsible for lactic acidosis in ruminants.

We have developed an in vitro screening protocol to select plants that may protect against ruminal acidosis. Through this procedure we were able to identify plants with potential to control acidosis without inhibiting normal rumen function.

Overview of the Protocol

In this publication we describe a three-step protocol to screen large numbers of plants. Step one, the initial screening, provides a reliable indication of the protection that each plant may have against ruminal acidosis. Step two, the agar dilution method, provides information on the selective antimicrobial activity of screened plants on rumen bacteria. It allows us to interpret the effects of plant extracts on the microbial pathways responsible for both acidosis and normal rumen fermentation. Step three, allows more detailed analysis of the likely extent of protection against acute and sub-acute acidosis by the most promising plants as well as their likely effect on normal rumen fermentation.

The data that is generated from these methods provides reliable and meaningful information on the effect of selected plant species on rumen kinetics.

Methodology

This protocol is divided into 3 sections: (Step 1) Initial screening; (Step 2) Agar dilution method; (Step 3) Extent of acidosis protection. We provide detailed protocols for the step-wise screening of plants for their antimicrobial control of lactic acidosis in ruminant animals. The use of this protocol allows a simple, cost effective, analysis of large numbers of plants in order to identify plants that are most likely to demonstrate protection against acidosis in vivo. This protocol is intended for use at the pre-clinical stage of product development. It cannot be used to screen plants for their direct commercial application because it does not include analysis of plants for toxins or the direct effects of plants on lactic acidosis in animals.

Fig. 9.1 We used an anaerobic chamber in all three steps of the protocol to maintain an anaerobic environment. However, an anaerobic chamber is not essential to maintain an oxygen-reduced environment. An anaerobic environment in Steps 1 and 3 could be achieved by continuous flushing of containers with nitrogen. An anaerobic environment in Step 2, the agar dilution method, could be achieved by incubating the agar plates in anaerobic jars or other airtight containers with added anaerobic system envelopes containing palladium catalyst. However, bacterial species that are very sensitive to the presence of oxygen such as *Butyrivibrio fibrisolvens,* may not grow under these conditions

Step 1 Initial Screening: In Vitro Batch Fermentations

Background

Selected plants are collected, ground and tested in an in vitro system designed to mimic an acidotic rumen environment. The difference between plant treatments in pH change, during incubation, is used as an indicator of the effect of plant compounds on lactic acid production. The gas produced by microbial fermentation is used as a marker for indicating the level of rumen fermentation. The in vitro gas production method using a pressure transducer was devised in 1974 by Theodorou [37] to provide detailed information on the fermentation kinetics of ruminant feed. It is a simple yet sensitive procedure that is used as an indirect measure of the rate and extent of degradation of ruminant feeds in vitro.

Although this technique was designed to determine the nutritive value of ruminant feeds, it can be modified to suit specific requirements [23]. For the method described in this chapter it can be adapted to indirectly measure the general effect of plant compounds on microbial fermentation [19]. When accumulated gas pressure in the headspace of the treatment flasks falls below the level that is produced during a normal fermentation, it is an indication that total microbial activity has been inhibited.

The parameters of pH and gas production can be used to screen plants for their protection against acidosis. The desirable effect of plant candidates is to inhibit declining pH without significantly inhibiting total gas production relative to the positive control (antibiotic containing fermentation vessel).

Note: If a pressure transducer is not available, cumulative gas can be measured using the syringe technique of Menke et al. [21].

Material Required

Plant processing

- Insulated containers or 12 volt fridge for transporting plant material
- Crushed ice
- Balance (0.001–510 g)
- Zip lock freezer bags
- Freezer
- Freeze-drier
- Grinding mill
- 4 mm and 1 mm grinding screens
- Airtight plastic containers

Rumen fluid sampling

- Rumen fistulated wethers housed and maintained on a diet that is primarily forage-based with approximately 20% cereal grain inclusion.
- Thermos(s)
- Electric rumen pump fitted with a 1 mm screening filter, an overflow vessel and a rubber stopper to fit the thermos (Figs. 9.2, 9.3 and 9.4)
- Water and a small brush to clean the suction filter
- Extension cord and circuit breaker

Fig. 9.2 Rumen fluid collection apparatus

Fig. 9.3 Pump fitted to the overflow vessel

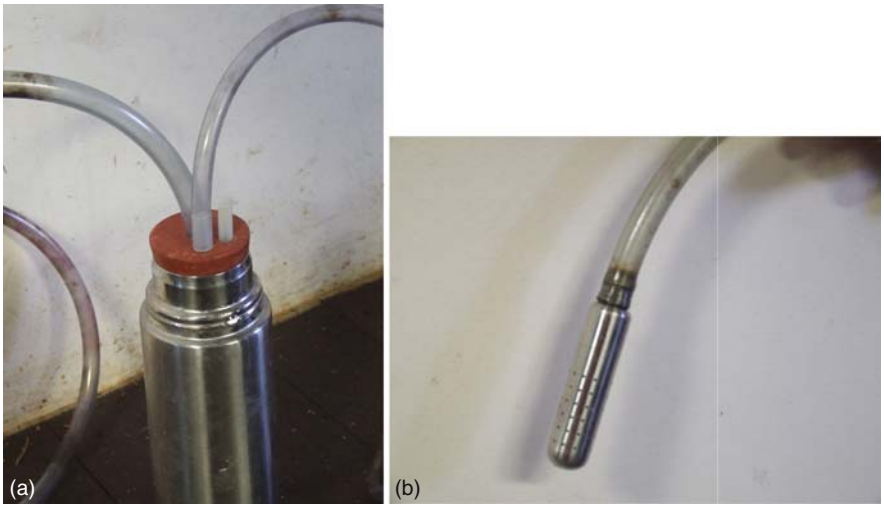

Fig. 9.4 (**a** and **b**) Custom fit a rubber stopper to the mouth of the thermos. Drill three holes in the stopper as follows; one for rumen fluid inflow line, one for the return airflow to the rumen pump and one small hole for on/off control of the vacuum. This allows direct drawing of rumen fluid from the animal to the thermos. Fit a 1 mm screening filter to the vacuum inlet

Treatments

- Rumen fluid
- Plant material
- Essential oils
- AR grade methanol
- Virginiamycin (Eskalin500®)
- D (+) Glucose
- Oaten chaff

Inside the anaerobic chamber (Fig. 9.1)

- Belco anaerobic culture tubes (18 × 150 mm) and stoppers containing treatments
- Aluminium standard seals (20 mm) with centre hole (10 mm)
- Standard seal applicator
- Magnetic stirrer
- Thermos containing rumen fluid
- Calibrated glass pipette (10 mL) and pipette controller
- Large beaker (1 L) and a stirring bar for rumen fluid
- pH meter
- 23-guage needles
- P20 and P100 micro pipette and tips

Outside the anaerobic chamber

- Graduated cylinder (50 mL)
- P200 micro pipette and tips
- Balance (0.001–510 g)
- Temperature controlled orbital shaker
- Water bath
- pH meter
- Pressure transducer (Fig. 9.5)
- 23-gauge needle, 1 mm syringe and cotton wool

Note: Insert a small wad of cotton wool into the 1 mm syringe and fit it to the pressure transducer (Fig. 9.5). The cotton wool prevents any rumen fluid that is forced through the needle during pressure readings from entering the transducer inlet. This dramatically extends the life of the transducer. Make sure that the cotton wool is replaced regularly to avoid restriction of gas flow through the syringe.

Method

Plant collection and processing

1. Chill harvested plant material during transportation from the field to the laboratory.

Fig. 9.5 Pressure transducer (Greisinger GMH 3110)

Note: Compressed, fresh plant material may begin to ferment if not chilled. Using ice or refrigerating reduces the risk of plant material fermentation. The risk of the plant material undergoing fermentation during transport or storage increases with higher moisture content of the plant.

2. Divide plants into leaf, stem and flower.

Note: It may be easier to separate the plant parts after freeze-drying.

3. Place material into zip lock bags, weigh and freeze at –20°C.

Note: Avoid repeatedly defrosting and freezing the material because this can lead to the disruption of the plant cell walls and the subsequent leakage of cell contents.

4. Freeze-dry the material and reweigh (Fig. 9.6).
5. Grind the material to a coarse powder (include the oaten chaff). We used a cyclone grinder (CYCLOTECH 1093 Sample Mill, Tecator, Hoganas, Sweden) fitted with a 4-mm screen followed by a 1-mm screen.

Note: The plant material is ground to mimic animal mastication and this increases the surface area available for microbial attachment.

6. Store the ground material at room temperature in sealed plastic containers.

Preparation of treatments

7. Measure the volumes of Belco tubes by filling them with water and pouring into a graduated cylinder. When using this non-vented system the volume of each Belco tube must be the same [37]. If volumes are not equal then the standard error will be high [34].
8. Prepare a stock solution of virginiamycin using Eskalin500® (containing 50% active Virginiamycin) as follows;

Fig. 9.6 Freeze drying of plant material is preferred over open air, drying rooms or ovens because at temperatures above 40°C the volatile components of the plant may be lost by volatilisation

- Resuspend 0.0096 g of Eskalin500® in 4 mL of methanol = 0.0024 g/mL = 0.0012 g of virginiamycin/mL
- When 100 μL of stock solution is added to 10 mL of rumen fluid, the final volume now contains 12 μg/mL of virginiamycin.

Note: Virginiamycin (Eskalin500®) was used as the antibiotic control because of its potent and selective inhibition of lactate producing bacteria. Based on the work by Dennis et al. [10] and our own in vitro experiments we determined that the ideal concentration of virginiamycin needed to protect against acidosis was 12 μg/mL of rumen fluid.

9. Prepare 3 replicates of each treatment into Belco tubes minus the rumen fluid (Fig. 9.7) according to the regime in Table 9.1. Repeat the experiment on a different day.

Notes:

1. *We determined the optimum dose of oaten chaff substrate based on the work by Rymer et al. [36] and France et al. [12] combined with results from our own testing of varying concentrations of oaten chaff substrate in vitro. The optimum concentration of glucose substrate needed to simulate acidosis was based on the work by Nagaraja et al. [25].*
2. *Essential oils are selected for screening based on their reputation as antimicrobial agents when used for human therapy.*
3. *The blank treatment is used to correct for changes in atmospheric pressure [34].*

Table 9.1 Treatment regime for induced acidosis in rumen fluid batch cultures in vitro

	Rumen fluid (mL)	Oaten chaff (g)	D-Glucose (g)	Treatment
Control	10	0.1	0	0
Uncontrolled acidosis	10	0.1	1	0
Positive control	10	0.1	1	100 μL of virginiamycin stock solution
Essential oil	10	0.1	1	6.25–25 μL of essential oil
Plant	10	0	1	0.1 g ground plant sample
Blank	0	0	0	0

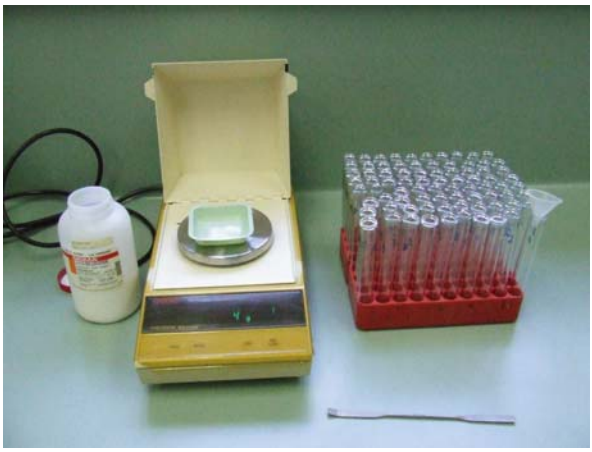

Fig. 9.7 Preparation of treatments into Belco tubes

10. Place tubes in the anaerobic chamber overnight.

Preparation of microbial inoculum

11. Fill the thermos(s) with warm water prior to taking rumen fluid.
12. Take rumen fluid samples from fistulated wethers 3 h post-feeding.
13. Remove the cannulae bung from the sheep and insert the suction hose with screening filter into the rumen of the sheep.
14. Empty the warm water from the thermos immediately prior to rumen fluid collection and fit the rubber stopper of the rumen pump assembly into the mouth of the thermos to ensure a good seal.
15. Switch the pump on and draw the rumen fluid into the thermos by placing a thumb over the hole in the thermos stopper to complete the vacuum circuit (Fig. 9.8). Ensure that the thermos remains anaerobic by filling it to the top and then sealing. Collect an equal amount of rumen fliud from at least two sheep.

Fig. 9.8 (**a** and **b**) It is also possible to take rumen fluid samples from fistulates prior to feeding the sheep to reduce the level of material that can potentially clog the suction filter. Total gas production and variability in gas production in vitro will be reduced as the period between sheep feeding and rumen sampling increases [16, 24]. However, sampling prior to feeding reduces the level of endogenous substrate in the microbial inoculum. Whatever sampling time is used, it is important to standardise the procedure for all experiments

Note: Using a screening filter is similar to straining the rumen fluid in the laboratory. However, eliminating straining of rumen fluid in the laboratory reduces oxygen contamination of the rumen fluid. Therefore more oxygen sensitive species are likely to survive the process.

16. Transport the rumen fluid immediately to the laboratory and take the thermos(s) into the anaerobic chamber.
    Inside the anaerobic chamber
17. Pool equal portions of rumen fluid from the sheep into a large beaker (Fig. 9.9).
18. Add a stirring bar to the beaker and place on the magnetic stirrer to mix.
19. Measure the pH of the pooled rumen fluid.
20. Using the dispensing syringe fitted with the dispensing tube aliquot 10 mL of rumen fluid into each prepared Belco tube (Fig. 9.9).
21. Seal the Belco tubes with a blue stopper and aluminium cap (Fig. 9.10).
22. Adjust the pressure in the gas phase of the tubes to zero by inserting a 23-gauge needle through the stopper. Gently shake the tubes and start time recording.

Note: If not using the anaerobic chamber, place the Belco tubes in a holder and immerse them in a water bath set at 39°C. Flush each Belco tube briefly with CO_2 before adding 10 mL of rumen fluid and sealing.

Outside the anaerobic chamber

23. Transfer the treatments to the orbital shaking incubator and shake constantly at 50 rpm at 39°C [36] (Fig. 9.11).

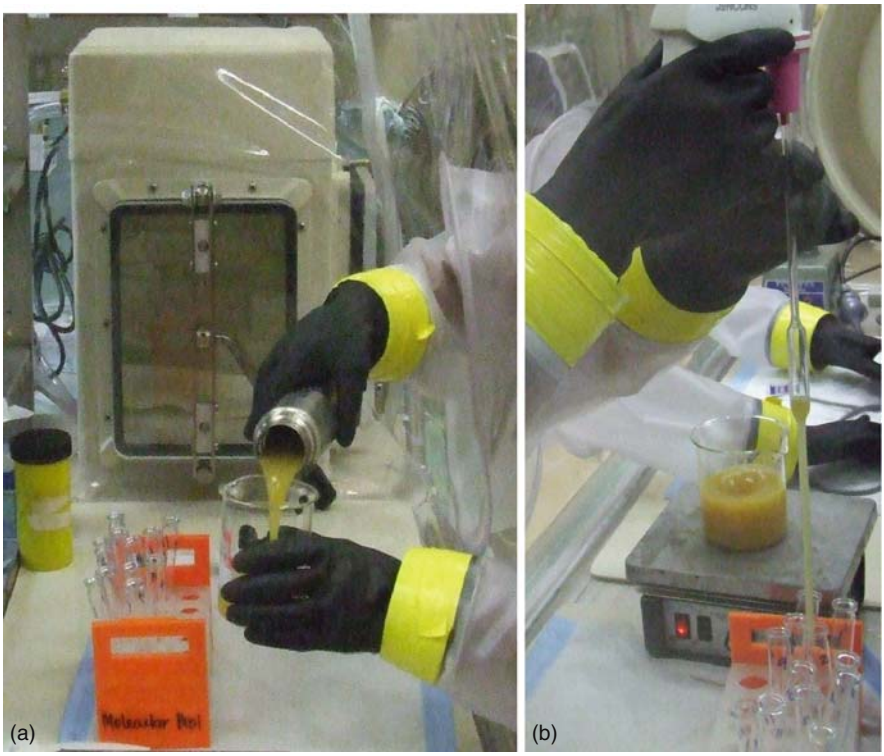

Fig. 9.9 (**a** and **b**) Pooling, stirring and dispensing rumen fluid inside the anaerobic chamber

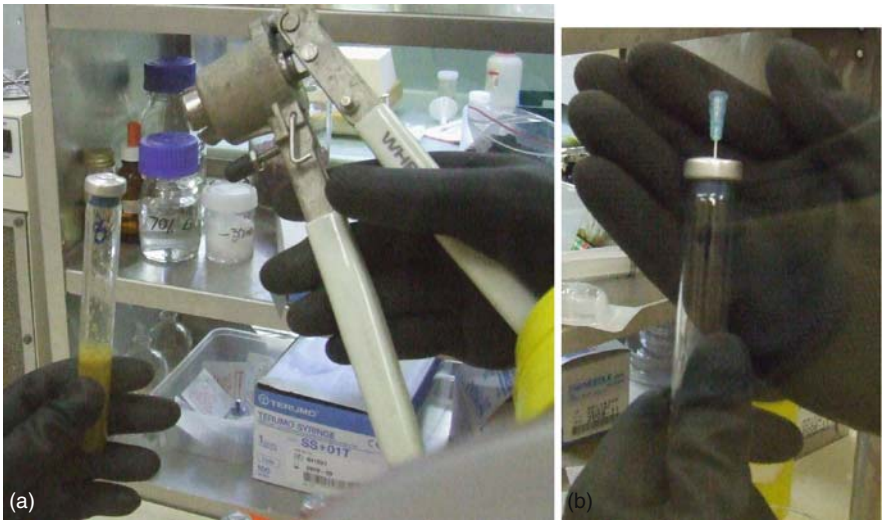

Fig. 9.10 (**a** and **b**) Sealing and venting Belco tubes

Fig. 9.11 Orbital shaking incubator with Belco tubes placed horizontally

24. Incubate for 6 h.

Note: Pell and Schofield [35] found that shaking the samples slowly, reduced the variation in gas production between replicates.

Fermentation period

25. Remove treatments from the shaking incubator at 2-h intervals and place them in a 39°C water bath. Measure cumulative gas pressures in the gas phase using the pressure transducer. Fit the 23-gauge needle and 1 mm syringe to the transducer. Set the digital display units to kPa and tare the instrument. Insert the needle through the rubber stopper of each Belco tube and record pressure readings.

Note: It is important to maintain the tubes at 39°C when taking pressure readings because, according to Gay-Lussac's law, when temperature decreases the pressure of the gas in the headspace also decreases, thus introducing error into the pressure readings [2].

26. Insert a 23-gauge needle through each Belco stopper and release the pressure.

Note: It is important when using this non-vented system that the pressure in the gas phase does not exceed 48.3 kPa. At higher pressures microbial growth becomes restricted and growth curves become non-linear [34].

27. After the final gas pressure reading, remove the stoppers and measure the pH in the liquid phase of each treatment.
28. Adjust for changes in atmospheric pressure by adding or subtracting the average pressure readings from the blank tubes at each corresponding reading. Calculate cumulative gas pressure.

Analysis

Plot treatment means as the relationship between pH and cumulative gas pressure (9.12). Compare means on Genstat 7.2 using Oneway ANOVA. When means differ by >0.05 use a Tukey's Pairwise comparison to compare individual plant treatments against the controls. Values for selection criteria are set based on the means for uncontrolled and antibiotic controlled acidosis treatments (Fig. 9.1).

Sample Results

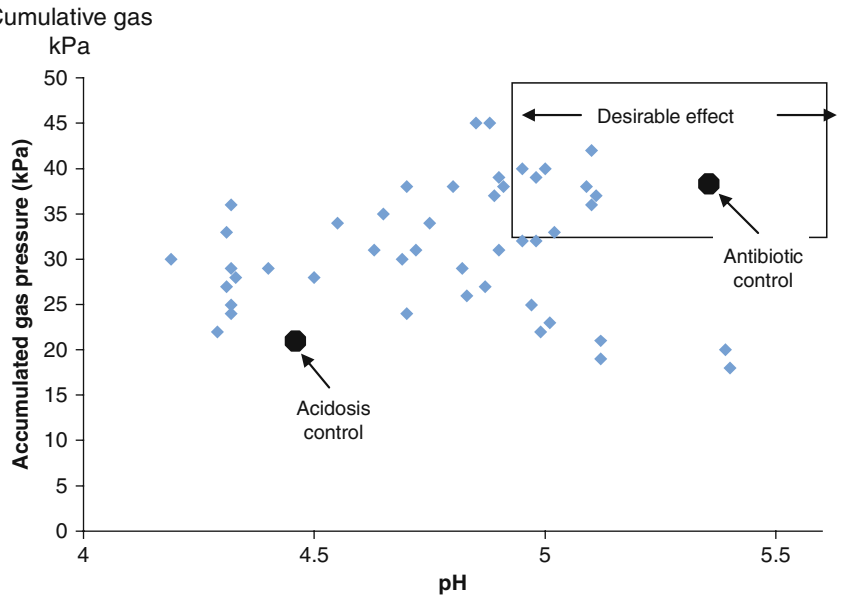

Fig. 9.12 Plant candidates were compared for their likely prevention of acidosis using gas production and pH values after a 6-h incubation and screening criteria were set based on the values for the antibiotic control and the acidosis control

Note:

1. *We did not add buffer to rumen fluid during the initial screening step. Buffer is normally used in in vitro fermentations to provide supplementary nutrients and to maintain medium pH so that substrate degradation is not compromised [23].*

However, our objective was to exaggerate differences in the degree of protection against acidosis between treatments rather than provide a quantitative assessment. We decided that adding buffer to the rumen fluid might mask any drop in pH and make it difficult to distinguish between treatments.

2. *In addition, we use a short, 6-h incubation period to synchronise measurements with the peak gas release kinetics from glucose substrate. Glucose is normally an intermediate product in the rumen resulting from the microbial enzymatic hydrolysis of carbohydrate polymers [7]. Thus, maximum gas release from glucose fermentation is between 6 and 7 h post-inoculation whereas the peak gas release from fermentable carbohydrate is 14–16 h post-inoculation [23]. In our in vitro system we eliminate the first phase of fermentation by directly introducing the intermediate glucose substrate to the medium in order to simulate acidosis.*

Step 2 Agar Dilution Method

Background

Our objective for using the agar dilution method (Step 2) was to determine the antimicrobial activity of the plants and essential oils against the major lactate-producing bacteria, *S. bovis* and *Lactobacillus* spp. and the major lactic acid-utiliser, *M. elsdenii* (Table 9.2). In particular, we were interested in the plant compounds or essential oils that inhibited *S. bovis* and *Lactobacillus* spp. but not lactate-utilising bacteria.

The agar dilution method enables the minimum inhibitory concentration (MIC) of the plant extracts on a range of rumen bacteria to be determined. The MIC is the lowest concentration of plant extract needed to inhibit the visible growth of each organism in a culture medium.

Table 9.2 Major bacterial species associated with acidosis and/ or normal rumen function [30, 15, 9, 29]

Bacterial species	Morphology/physiology	Minimum pH tolerance	Significance
Streptococcus bovis	Facultative anaerobic, Gram +ve cocci	5.1	Homofermentative: Carbohydrate to lactic acid
Lactobacillus acidophilus	Anaerobic, Gram +ve rods	4.8–5.0	Homofermentative: Carbohydrate to lactic acid
Megasphaera elsdenii	Gram –ve cocci	5.5	Ferment lactate to acetate, propionate, and butyrate

Materials Required

Ethanolic extraction

- Freeze-dried and ground plant material (1 mm screen)
- 70: 30; ethanol: water, v/v
- 500 mL conical flask
- Aluminium foil
- Stocking (or gauze bandage)
- 50 mL beaker
- Buchner funnel and Whatman filter paper (# 42)
- 500 mL filtration flask
- Temperature controlled shaker
- Vacuum pump
- Temperature controlled water bath
- Rotory evaporator
- Screw capped vials (125 mL)
- Mortar and pestle
- 20–30 mL container
- Sterile screw capped vials (12 mL)

Note: Consideration should be given to the choice of solvent for the extraction process. Although we use ethanol as the extracting solvent other extractants may be preferred. The biologically active components of plants in this screening protocol are generally unknown. Therefore, the larger the variety of compounds that are extracted by the extractant, the better the chance of recovering the active component. Eloff [11] found that acetone extracted higher numbers of different components and inhibitors from plant material than most solvents. He suggested that this is because acetone extracts both polar and non-polar components whereas ethanol, for example, extracts mainly polar components. Acetone is also highly volatile and this makes it simple to separate from the extracted plant components by evaporation. In addition, acetone has demonstrated relatively low toxicity to test bacteria [11]. It is good practice to, at least initially; use a mixture of water and alcohol or organic solvents such as acetone. Ethanol may not extract the water-soluble compounds.

Antibiotic control

- Virginiamycin (Eskalin500®)
- Technical grade methanol
- 9 x 2 mL sterile eppendorf tubes
- P100 pippette and sterile tips

Culture medium

- Rumen fluid-CHO (Carbohydrate) medium for growth of rumen bacteria (CHO broth) adapted from Bryant and Robinson Medium [4].

(Per 100 mL CHO)

Distilled water	50 mL
Glucose	0.05 g
Cellobiose	0.05 g
Soluble starch	0.05 g
Bacto-Tryptone	0.05 g
Yeast Extract	0.2 g
VFA stock	0.1 mL
Mineral Solution I	7.5 mL
Mineral Solution II	15 mL
Clarified rumen fluid	20 mL
Resazurin (0.1% w/v)	0.1 mL
Distilled water to bring to final volume	

Solutions

Volatile Fatty Acids stock (mg per 100 mL)

Acetic	170
Propionic	60
Butyric	30
Valeric	10
Isovaleric	10
Isobutyric	10
2 Methyl butyric	10

Mineral solution I (g per 100 mL)

KH_2PO_4	0.15
$(NH_4)2SO_4$	0.3
NaCl	0.3
$MgSO_4$	0.03
$CaCl_2$*	0.03
Distilled water to make volume up to	100 mL
Mineral Solution II (g per 100 mL) K_2HPO_4	0.3

Distilled water to make volume up to 100 mL

- Anaerobic chamber
- Bacto-Agar powder
- Microwave
- Schott bottle (250 mL)
- Water bath

Inside the anaerobic chamber

- $NaHCO_3$ 0.4 g/100 mL CHO medium
- Cysteine-HCl 0.05 g/100 mL CHO medium
- Media dispenser
- 100 mL serum bottles with ruber stoppers and aluminium seals
- Seal crimper and seal remover
- L-Cysteine hydrochloride
- Sodium hydrogen carbonate
- P100, P200, P1000, P5000 micro pipettes and sterilised tips
- Petri dishes

Inoculum

- Lamina flow cabinet
- Incubator
- Hungate tubes containing 5 mL of sterile rumen fluid-CHO broth
- Cysteine sulphide 1.25% (sterile)
- 1 mL syringes and 23-guage needles
- 70: 30; ethanol: water, v/v in a spray bottle

Inside the anaerobic chamber

- McFarland standards
- Steers replicator (sterile)
- Spare tubes of CHO agar

Note: Sterilise the Steers replicator prior to inoculation in a 1:8 dilution of sodium-hypochloride for about 20 min before rinsing with autoclaved, deionised water. Place in the laminar flow cabinet to dry with the ultra violet light on. Take the sterile replicator in to the anaerobic chamber at least several hours prior to inoculation to remove oxygen from the surface.

Method

Procedure for crude ethanolic extraction from plant material

1. Place 50 g of plant material (Fig. 9.13) in a conical flask and add 200 mL of 70: 30; ethanol: water (primary solvent). Place an aluminium cap on the flask and macerate for 3 h with shaking at 200 rpm at 22°C.

Note: It is important to cap the flask before maceration to prevent volatilisation of the volatile components.

2. Squeeze the material through stocking (or gauze bandage) into the beaker. Label the beaker and set aside.

3. Return the remaining fibrous material to the filtration flask and add 100 mL of 70% ethanol. Macerate the fibrous portion again for 1 h with shaking at 200 rpm at 22°C.
4. Squeeze the material through stocking (or gauze bandage) into the labelled beaker. Discard the fibrous portion.
5. Filter the combined filtrate using the Buchner funnel and filter paper into a conical flask with a vacuum outlet. Transfer the filtrate to a round-bottom Buchi flask.

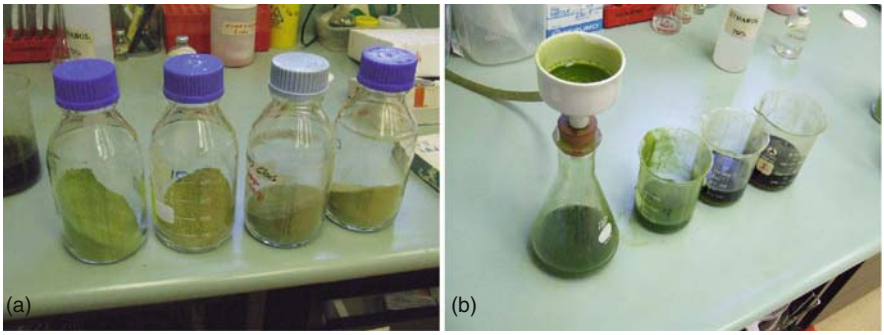

Fig. 9.13 (**a** and **b**) Weigh out 50 g of ground plant material. Applying a vacuum to the filtration flask will increase the filtration rate

6. Attach the round-bottom Buchi flask to the rotovap. Submerge the bowl of the round-bottom Buchi flask in a water bath at 40°C (Fig. 9.14). Rotate the Buchi flask and evaporate ethanol from the extract by using vacuum evaporator (Rotavap). Stop when the ethanol streak in the side bottle stops.
7. Resuspend concentrate in approximately 10 mL of water and transfer to a 125 mL plastic container and seal with a cap.

Note: Resuspending the concentrate in water will allow easier transfer from the Buchi flask and help freezing.

8. Freeze extract at –20°C. If the extract does not freeze then more of the ethanol needs to be evaporated.
9. Puncture small holes through the cap of the 125 mL screw capped vial and freeze dry overnight to give a crude dried extract.
10. Weigh the extract and calculate the proportion of extract obtained from the ground plant material.
11. Grind the extract to a coarse powder using the mortar and pestle and then transfer to the 20–30 mL container and refrigerate.

Dilution of the extract (inside the anaerobic chamber)

12. Prior to use, reconstitute 1.2 g of the extract into 12 mL of 70:30; ethanol: water (secondary solvent) for a final concentration of 100 mg/mL.

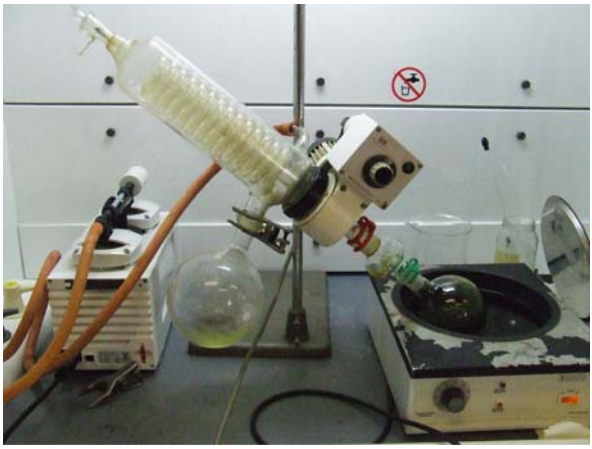

Fig. 9.14 The ethanolic filtrate may be unstable initially under vacuum. To avoid excessive bubbling begin by slowly rotating the Buchi flask while slowly sealing off the air inlet cock on the rotovap. Once the filtrate is stable, increase the rotation speed of the Buchi flask to increase the ethanol evaporation. Ethanolic extraction is complete when ethanol ceases to condense into the collection flask

13. Once resuspended, use a 0.2 μm syringe filter to sterilise the extract into a sterile screw capped vial (12 mL).

Note: For harsher solvents use suitably resistant filters.

Antibiotic control (prepare in the laminar flow cabinet). We use virginiamycin (Eskalin500®) as a positive control because of its selective inhibition of lactate producing bacteria.

14. Prepare a stock solution containing 24 mg of active virginiamycin by resuspending 48 mg of Eskalin500® in 10 mL of technical grade methanol.
15. Sterilise the stock solution by passing through a 0.2 μm syringe filter into a sterile screw capped vial (12 mL).
16. Prepare serial two-fold dilutions by adding 1 mL of methanol to each of 9 eppendorf tubes.
17. Repeat the dilutions for all eppendorf tubes to obtain the following concentrations of virginiamycin: 1200, 600, 300, 150, 75, 37.5, 18.75, 9.38 and 4.69 μg/mL.

Agar dilution method

The methods used to test the MIC of ethanolic extracts, essential oils and antibiotics on rumen cultures were modified from those of Hammer et al. [14] and Palombo and Semple [33].

Culture medium

18. Prepare bacterial growth medium by combining all the culture medium ingredients except for the Cysteine- HCl and the $NaHCO_3$ in a Schott bottle in order and with constant mixing
19. Add 1.5 g of agar powder per 100 mL of CHO broth.
20. Bring the CHO agar to the boil in the microwave and take it into the anaerobic chamber.
21. Remove the cap and allow to cool slightly before adding the L-Cysteine $NaHCO_3$.
22. Dispense the required volume of CHO agar into 100 mL serum bottles and crimp seal with aluminium caps before autoclaving (Tables 9.3, 9.4, and 9.5).

Table 9.3 Essential oil treatment regime to determine the MIC on pure cultures of rumen bacteria

CHO agar (mL)	Essential oil (μL)	Final concentration of additive (μL/mL)
50	0	0
50	25	0.5
50	50	1
50	125	2.5
49.75	250	5
49.5	500	10
49	1000	20
45	5000	70
	70% ethanol[a]	

[a] Used as a control for ethanolic extracts.

Table 9.4 Plant extract treatment regime to determine the minimum inhibitory concentration on pure cultures of rumen bacteria

CHO agar (mL)	Volume of 100 mg/mL extract (mL)	Final extract concentration (mg/mL)
49.7	0.32	0.63
49.4	0.63	1.26
48.8	1.25	2.5
47.5	2.5	5
45	5	10

Note: To obtain an anaerobic medium take the serum bottles into the anaerobic chamber at least 24 h prior to adding the CHO agar.

Note: The dose levels for virginiamycin are based on work by Nagaraja et al. [27] and Nagaraja and Taylor [25] who tested the IC50 (the dose that is required to

Table 9.5 Virginiamycin treatment regime to determine the minimum inhibitory concentration on pure cultures of rumen bacteria

Virginiamycin (μg)	Methanol (mL)	CHO agar (mL)	Virginiamycin final concentration (μg/mL)
0.00	0	50	0.00
0.00	1	49	0.00
4.69	1	49	0.09
9.38	1	49	0.19
18.75	1	49	0.38
37.50	1	49	0.75
75.00	1	49	1.50
150.00	1	49	3.00
300.00	1	49	6.00
600.00	1	49	12.00
1200.00	1	49	24.00

inhibit lactic acidosis by 50% over the control). They found that the most effective doses of virginiamycin were between 0.38 and 12 μg/mL.

23. Before taking the sterile molten agar into the anaerobic chamber maintain it at 40-60°C in a water bath to prevent the agar from solidifying but also to prevent volatile compounds in the essential oils and extracts from volatilisation.
24. Inside the chamber, label and organise the Petri dishes. Use 3 Petri dishes per dilution.
25. Bring the molten agar serum bottles into the chamber, remove the aluminium seals and pipette the required volume of additive into each serum bottle (Tables 9.3, 9.4, and 9.5).

Note: Initially, 0.5% (v/v) of Tween-20 was added to serum bottles to enhance the solubility of the essential oils. However, due to concerns over the sterility of the Tween-20 we tested it against treatments that had no added Tween-20. We found no difference in the MIC between treatments and omitted Tween-20 from further experiments.

26. Mix the solution well and divide each 50 mL dilution into 3 Petri dishes.

Note: Mix the solution by swirling but take care not to introduce bubbles into the mixture because the bubbles will transfer onto the surface of the agar plates during pouring. Bubbles can be removed from the surface using a sterile pipette tip.

27. Allow the CHO agar plates to set and dry before inoculating.

Inoculum

Cultures of bacteria were prepared from glycerol stocks that were subcultured in Bryant and Robinson's Rumen fluid Medium (CHO broth) [4] and refrigerated in Hungate tubes (Fig. 9.15).

28. Remove the bacterial cultures from the fridge and allow them to warm to room temperature. Label the Hungate tubes containing CHO broth. Use 2 tubes per culture.
29. In the laminar flow cabinet, spray the caps on the Hungate tubes and the cysteine sulphide bottle with 70:30; ethanol: water.
30. Flush a syringe 3 times with cysteine sulphide then draw 0.3 mL into the syringe. Inject 0.15 mL of cysteine sulphide into each of 2 tubes of CHO broth to reduce oxygen contamination. Invert a culture tube several times to disperse the bacteria and draw 1 mL into the flushed syringe. Inject 0.5 mL of the culture into each tube of CHO broth. Repeat the procedure for all cultures.
31. Transfer the cultures to the incubator and incubate at 39°C.

Note: We observed that after 12 h of incubation S. bovis cultures were visibly turbid whereas other cultures including M. elsdenii and Lactobacillus spp. were not. We assumed that the turbidity was due to rapid cell division of S. bovis compared with other bacterial species [28]. Based on this information, we decided to approximate the maximum growth period for M. elsdenii and Lactobacillus spp. Cell density was determined by spectrophotometry at an absorbance of 590 nm. Using the growth curves we estimated that the optimum growth period for all bacteria other than S. bovis was approximately 24 h. Hence, all bacteria excluding S. bovis are incubated for approximately 24 h before being used in the agar dilution method. S. bovis cultures are incubated for approximately 12 h prior to use. Test cultures for purity by taking a sub sample from the fresh cultures and preparing gram stain slides for morphological examination under a light microscope. Bacteria can also be tested for oxygen tolerance by inoculating onto agar plates and incubating aerobically.

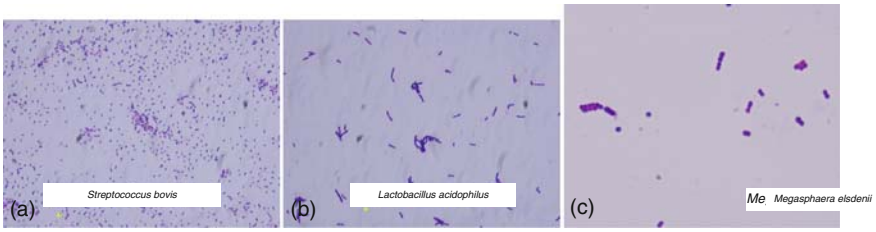

Fig. 9.15 (**a, b**, and **c**) Three of the major bacterial species involved in the production and utilisation of lactic acid in the rumen

Inoculation of CHO agar (inside the anaerobic chamber)

32. Dilute cultures by adding CHO broth to the culture to obtain a turbidity of a McFarland 1 (300×10^6 bacteria/mL).
33. Make a location plan of cultures for the replicator wells to include space for 3 wells per bacterial species and 3 wells of CHO broth control. Use a 1 mL syringe and 23-gauge needle to draw culture from the Hungate tube and dispense approximately six drops of culture broth in the corresponding wells.

34. Insert the replicator stamp into the wells, withdraw and spot approximately 10 μL of culture onto the first of the control CHO agar plates. Inoculate all bacterial cultures plus a control of uncultured CHO broth onto each CHO agar plate.
35. Repeat the inoculation for all replicates of each dilution. Inoculate the plates in the order of the lowest concentration to the highest and finishing with the solvent control (if any).

Note: If you are using more than one extract, resterilise the Steers replicator between extracts to ensure that there is no cross contamination.

36. Allow a few minutes for the CHO agar plate to absorb the culture before inverting and sealing with parafilm or placing in a sealed container. Incubate in the anaerobic chamber at 39°C for 24–48 h.
37. Score the plates for bacterial growth with a plus or minus to determine the MIC. Repeat the experiment on 2 separate days for each plant extract/antibiotic.

Note: It is good practice to take a sample of each culture from the agar plate and make gram stain slides and check the morphology under a microscope to confirm the identity and purity of each bacterial species. Confirmation is necessary due to the risk of cross contamination of bacterial species between the replicator wells.

Sample Results

There are two major outcomes from the agar dilution method; the potential of an extract to selectively inhibit rumen bacteria and the identification of the concentration needed for the inhibition (Figs. 9.16 and 9.17).

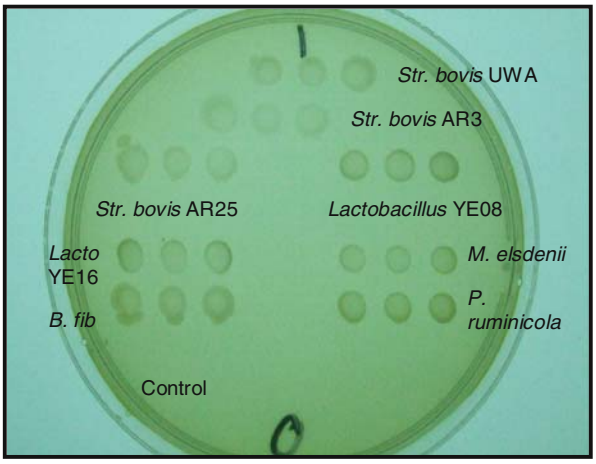

Fig. 9.16 The control agar with no extract added supported the growth of all bacterial species

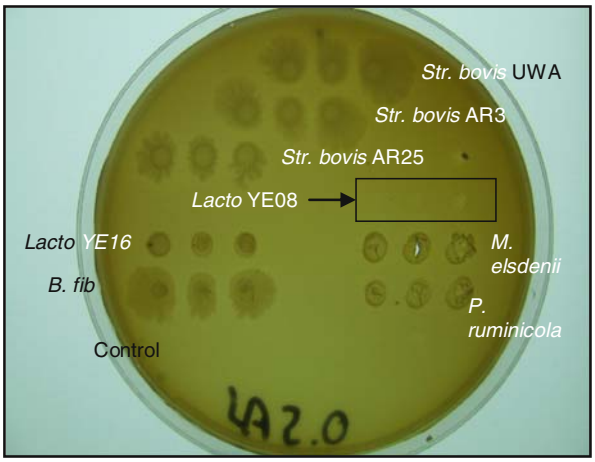

Fig. 9.17 Selective inhibition of the lactate producer, *Lactobacillus* YE08

Step 3 Extent of Acidosis Protection

Background

The previous two steps, (1) initial screening and (2) agar dilution method, enable us to identify plants with the best potential for controlling acidosis in vivo. Our objective in step (3) is to investigate these plants further and, in particular, determine the extent of the protection against acidosis in vitro by comparing them with an antibiotic-(Eskalin500®) control. We determine this by extending the fermentation period to 24 h and measuring the concentrations of accumulated lactate and VFA. Measuring these two components allows us to distinguish between sub-acute acidosis (caused by high levels of VFA) and the more serious acute acidosis (caused by high levels of lactic acid) [26]. This step allows us to determine the ability of a plant to maintain a balanced microbial environment that prevents the accumulation of lactic acid. In these types of experiments we test the hypothesis that the plants that are selected for screening will offer similar protection against in vitro acidosis as Eskalin500® and greater protection than an uncontrolled acidosis environment.

The technique that we use for the third screening is the same as that used for the initial screening (Step 1) with the following modifications;

a. We add buffer to the rumen fluid to provide some protection against declining pH and to provide supplementary nutrients so that microbial degradation is not compromised [23]. The pH is likely to decline unrealistically due to the accumulation of VFA.
b. The fermentation period is increased from 6 to 24 h
c. Fermentations are in 100 mL serum vials rather than in Belco tubes
d. Samples are taken for VFA and D-lactate analyses

Material Required

- McDougall's buffer [20]

Salt	(g/L)
Distilled H_2O	750 Ml
$NaHCO_3$	9.8
$NaHPO_4.12H_2O$	9.3
NaCl	0.47
KCl	0.57
$CaCl_2$ anhydrous	0.04
$MgCl_2$ anhydrous	0.06
Distilled H_2O	Add to make volume up to 1000 mL

- Volumetric flask (1 L)
- Large beaker (1 L)
- 3 N HCl
- 1 mL plastic squeeze pipette
- 100 mL serum bottles
- 1 mL syringes and 23 gauge needles
- 5 mL vials
- 1.5 mL micro tubes
- Concentrated H_2SO_4
- P20 micro pipette and tips

Note: 5 mL vials should be selected with a diameter that is just large enough to insert the pH probe into. It is also a good idea to select a pH probe with a small diameter. A close fit between the probe and the vial will allow 1 mL of inoculum to cover the probe.

Method

Follow the method used for (a) Initial screening: In vitro batch fermentations, with the following amendments;
Treatments

1. Prepare treatments in triplicate in 100 mL serum bottles one day prior to the collection of rumen fluid and place them in the anaerobic chamber overnight to reduce oxygen.
2. Treatments include 0.5 g of oaten chaff (uncontrolled acidosis environment), 0.5 g of oaten chaff + 12 μg/mL of virginiamycin (Eskalin500®) (antibiotic-controlled environment) or 0.5 g of plant candidate material (plant-controlled environment).

3. To create a potentially acute acidotic environment, add 5 g of D (+)-glucose to each bottle as a source of highly fermentable carbohydrate.

Note: A preliminary experiment may be necessary to determine the most effective dose of plant material needed for protection against acidosis. We use the regime in Table 9.6.

Table 9.6 Regime for dose response to plant treatment protection against acidosis in vitro

						Plant concentrations	
Serum bottle	Rumen fluid (mL)	McDougall's buffer (mL)	Glucose (g)	Oaten chaff (g)	Plant material (g)	% w/v	% of sub-strate
1–3	25	25	5	0.5	0	0	0
4–6	25	25	5	0	0.5	1	10
7–9	25	25	5	0.1	0.4	0.8	8
10–12	25	25	5	0.2	0.3	0.6	6
13–15	25	25	5	0.3	0.2	0.4	4
16–18	25	25	5	0.4	0.1	0.2	2
19–21	25	25	5	0.45	0.05	0.1	1
22–24	25	25	5	0.498	0.025	0.05	0.5
25–27	25	25	0	0.5	0	0	0

Inoculation

4. Mix the ingredients for McDougall's buffer in the volumetric flask 24 h prior to fermentations (Fig. 9.18). Place the solution in the anaerobic chamber in the large beaker to reduce the oxygen content.
5. Take the thermos containing the rumen fluid into the anaerobic chamber and mix a 1: 1 proportion of rumen fluid and McDougall's [5].
6. Bring the mixture to pH 7 while stirring by adding 3 N HCl drop wise with the 1 mL plastic squeeze pipette (Fig. 9.19).
7. Dispense 50 mL of culture fluid into 100 mL serum bottles containing treatments.

Fermentation period.

8. Measure the pH and accumulated gas pressure and take samples for VFA and lactate analysis at 5, 10, 15 and 24 h of incubation.

Note: We avoid taking inoculum samples more often than this because it requires drawing 2 mL of inoculum from each serum bottle at each measurement. This reduces the volume of inoculum for the ongoing fermentation. However, it may be necessary to take more frequent cumulative gas measurements to prevent the pressure in the headspace exceeding 48.3 kPa.

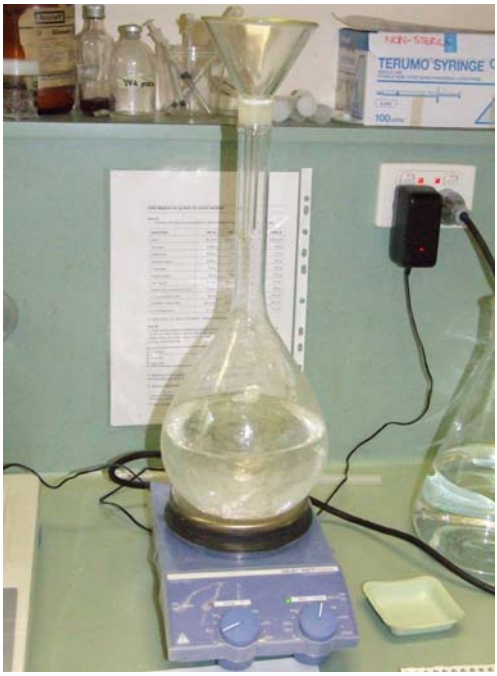

Fig. 9.18 Prepare McDougall's buffer in a volumetric flask while heating and stirring

Fig. 9.19 Bringing the pH to 7 using 3N HCl in the anaerobic chamber

9. Measure the pH by removing the serum bottle from the water and inserting the 23-gauge needle through the rubber stopper. Carefully draw 1 mL of the liquid phase into the syringe, place it in a small vial then measure the pH.

Notes:

1. *To draw the inoculum, carefully invert the serum bottle and avoid shaking the contents. Agitation of the treatments may result in blocked needles.*
2. *One needle and syringe can be used for each treatment but use a fresh needle and syringe between treatments to avoid cross-contamination.*

10. Dispense the sample into a 1.5 mL micro tube. Draw another 1 mL sample of liquid phase and dispense it into another 1.5 mL micro tube.
11. Add 5 μL of concentrated H_2SO_4 to each sample and freeze the samples at –20°C for D-lactate and VFA analysis.

Note: Samples are stable for up to 6 months.

Lactate and VFA Analysis

FA is quantified by capillary GC, equipped with a split injector and flame ionisation detection (FID), using internal standard calibration (GC Separation of VFA C2-C5 Supelco Bulletin # 749D). D (-)-lactate is determined using the Boehringer Mannheim Lactic acid kit (Product No 1112821) using a Roche Cobas Mira S auto analyser for readings.

Statistical Analysis

The data is analysed by One-way ANOVA using GenStat 8 and significant differences are reported at $p < 0.05$.

Sample Results

When the hypothesis is supported, the plant candidate will produce higher pH, VFA and cumulative gas pressure values and lower acetate to propionate ratio than the

Table 9.7 The extent of control of acidosis (pH and D-lactate) and fermentation (gas and VFA) indicators by the addition of a dried and ground plant compared with antibiotic and oaten chaff treatments after 24-h incubation in a simulated acute acidosis environment

	Antibiotic Eskalin500®	Plant candidate	Oaten chaff
pH	$5.16^a \pm 0.042$	$4.95^b \pm 0.111$	$4.31^c \pm 0.009$
D-Lactate (mmol/L)	$0.3^a \pm 0.16$	$28^b \pm 1.1$	$47^c \pm 1.3$
Accumulated gas pressure (kPa)	$171^a \pm 1.4$	$139^b \pm 0.3$	$119^c \pm 2.7$
Total VFA (mmol/L)	$153^a \pm 2.5$	$119^b \pm 0.6$	$92^c \pm 2.9$
Acetate (mmol/L)	$59^a \pm 0.3$	$52^b \pm 0.1$	$50^b \pm 0.3$
Propionate (mmol/L)	$53^a \pm 0.5$	$41^b \pm 0.6$	$26^c \pm 0.2$
Butyrate (mmol/L)	$31^a \pm 0.6$	$21^b \pm 0.3$	$14^c \pm 2.0$
Acetate: Propionate	1.23	1.28	1.90

Values are means ± SEM, n = 3. Means with different superscripts on the same row are significantly different ($P < 0.05$). VFA, volatile fatty acids.

negative control (Table 9.7). A highly successful plant candidate will produce values that are similar to the antibiotic control.

Regular sample analysis over the 24-h incubation period allows the antimicrobial effect to be plotted over time. This helps to determine the likely timing and extent of acidosis protection over a one-day interval (Figs. 9.20 and 9.21). As the pH declines the rate of gas production also declines as microbial fermentation is restricted (Figs. 9.20 and 9.21).

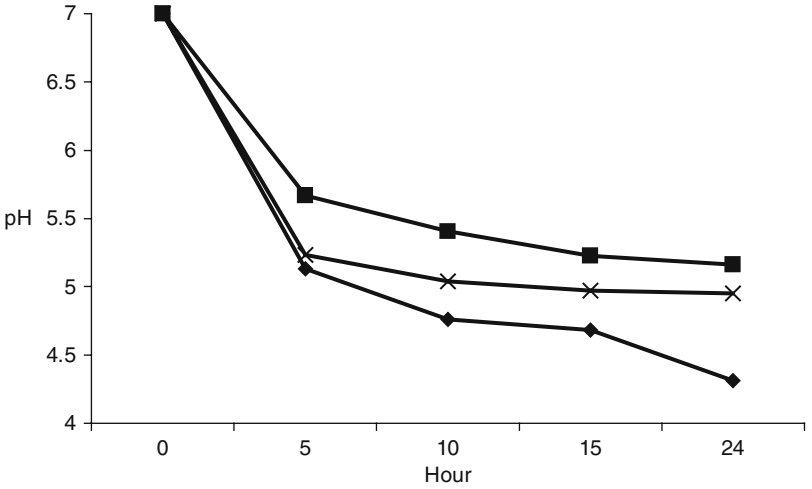

Fig. 9.20 The effects of treatments ■ Eskalin500® × Plant candidate and ♦ Oaten chaff on the pH of rumen culture fluid over a 24 h incubation period in an environment that simulated potentially acute acidosis (symbols are mean values, n = 3)

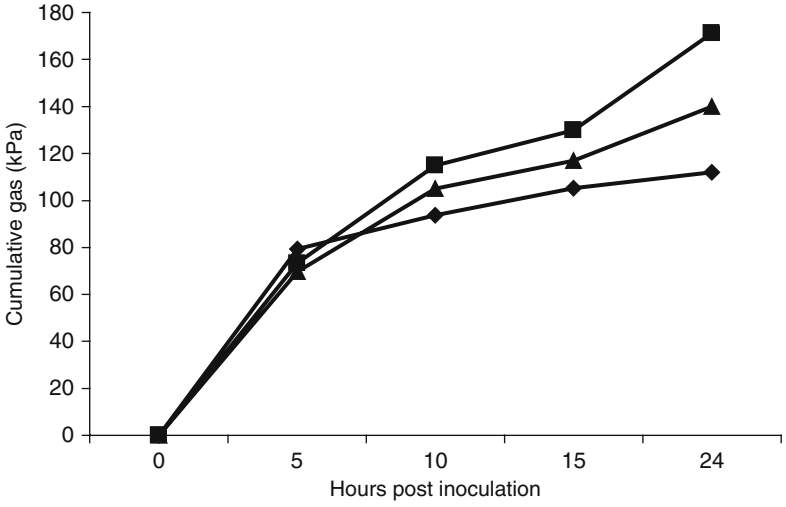

Fig. 9.21 The effects of treatments ■ Eskalin500® Plant candidate and ♦ Oaten chaff on the gas production of rumen culture fluid over a 24 h incubation period in an environment that simulated potentially acute acidosis (symbols are mean values, n = 3)

References

1. APVMA 2003. The Reconsideration of the Registration of Products Containing Virginiamycin and Their Labels: Draft Review Report. A. P. V. M. Authority, National Registration Authority for Agricultural and Veterinary Chemicals. Canberra, Australia.
2. Bettelheim, F.A. and J. March. 1998. Introduction to General, Organic & Biochemistry, Fifth Edition, Saunders College Publishing, Adelphi University.
3. Bryant, J.P., P.B Reichardt, and T.P. Clausen. 1992. Chemically mediated interactions between woody plants and browsing mammals. J. Range Manag. **45**:18–24.
4. Bryant, M.P., and I.M. Robinson. 1961. An improved non-selective culture medium for ruminal bacteria and its use in determining diurnal variation in numbers of bacteria in the rumen. J. Dairy Sci. **44**:1446–1456.
5. Busquet, M., S. Calsamiglia, A. Ferret, and C. Kamel. 2006. Plant extracts affect in vitro rumen microbial fermentation. J. Dairy Sci. **9**:761–771.
6. Carson, C.F., B.J. Mee, and T.V. Riley. 2002. Mechanism of action of Melaleuca alternifolia (tea tree) oil on Staphylococcus aureus determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. Antimicrob. Agents Chemother. **46**:1914–1920.
7. Chesson, A. and Forsberg, C.W. 1988. Polysaccharide degradation by rumen microorganisms, pp. 251–284. In P.N. Hobson (ed.). The Rumen Microbial Ecosystem, Elsevier Applied Science, London; New York.
8. Dawson, K.A., M.A. Rasmussen, and M.J. Allison. 1997. Digestive disorders and nutritional toxicity, pp. 633–660. In P.N. Hobson and C.S. Stewart (eds.). The Rumen Microbial Ecosystem, Second Edition, Blackie Academic and Professional, London.
9. Dehority, B.A. 2003. Rumen Microbiology. Nottingham University Press, Thrumpton, Nottingham.
10. Dennis, S.M., T.G. Nagaraja, and E.E. Bartley. 1981. Effects of lasalocid or monensin on lactate-producing or -using rumen bacteria. J. Anim. Sci. **52**:418–426.
11. Eloff, J.N. 1998. Which extractant should be used for the screening and isolation of antimicrobial componants from plants. J. Ethnopharm. **60**:1–8.
12. France, J., S. Lopez, J. Dijkstra, R. Sanderson, and M.S. Dhanoa. 1998. Models for interpreting in vitro gas production profiles from ruminant foods. Brit. J. Anim. Sci. **22**:79–80.
13. Gill, H.S., Q. Shu, and R.A. Leng. 2000. Immunization with Streptococcus bovis protects against lactic acidosis in sheep. Vaccine. **18**:2541–2548.
14. Hammer, K.A., C.F. Carson, and T.V. Riley. 1999. Antimicrobial activity of essential oils and other plant extracts. J. Appl. Microbiol. **88**:985–990.
15. Hungate, R.E. 1966. The Rumen and Its Microbes. Academic Press, New York.
16. Huntingdon, J.A., C. Rymert, and D.I. Givens. 1998. The effect of host diet on the gas production profile of hay and high-temperature dried grass. Anim. Sci. **67**:59–64.
17. Ives, S.E., E.C. Titgemeyer, T.G. Nagaraja, A. del Barrio, D.J. Bindel, and L. Hollis. 2002. Effects of virginiamycin and monensin plus tylosin on ruminal protein metabolism in steers fed corn-based finishing diets with or without wet corn gluten feed. J. Anim. Sci. **80**:3005–3015.
18. Klieve, A.V., G.L. Heck, M.A. Prance, and Q. Shu. 1999. Genetic homogeneity and phage susceptibility of ruminal strains of Streptococcus bovis isolated in Australia. Lett. Appl. Microbiol. **29**:108–112.
19. Lopez, S., M.S. Dhanoa, J. Dijkstra, A. Bannink, E. Kebreab, and J. France. 2007. Some methodological and analytical considerations regarding application of the gas production technique. Anim. Feed Sci. Technol. **135**:139–156.
20. McDougall, E.I. 1948. Studies on ruminant saliva 1: The composition and output of sheep's saliva. Biochem. J. **43**:99–109.

21. 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 in vitro. J. Agric. Sci. (Camb.) **92**:217–222.
22. Min, B.R., G.T. Attwood, K. Reilly, W. Sun, J.S. Peters, T.N. Barry, and W.C. McNabb. 2002. Lotus corniculatus condensed tannins decrease in vivo populations of proteolytic bacteria and affect nitrogen metabolism in the rumen of sheep. Canad. J. Microbiol. **48**:911–921.
23. Mould, F.L., R. Morgan, K.E. Kliem, and E. Krystallidou. 2005. A review and simplification of the in vitro incubation medium. Anim. Feed Sci. Technol. **123–124**:155–172.
24. Nagadi, S., M. Herrero, and N.S. Jessop. 2000. The influence of diet of the donor animal on the initial bacterial concentration of ruminal fluid and in vitro gas production degradability parameters. Anim. Feed Sci. Technol. **87**:231–239.
25. Nagaraja, T., and M. Taylor. 1987. Susceptibility and resistance of ruminal bacteria to antimicrobial feed additives. Appl. Envir. Microbiol. **53**:1620–1625.
26. Nagaraja, T.G., M.L. Galyean and N.A. Cole. 1998. Nutrition and disease. Feedlot Med. **14**:257–277.
27. Nagaraja, T.G., M.B. Taylor, D.L. Harmon, and J.E. Boyer. 1987. In-vitro lactic acid inhibition and alterations in volatile fatty acid production by antimicrobial feed additives. J. Anim. Sci. **65**:1064–1076.
28. Nagaraja, T.G., and E. Titgemeyer. 2007. Ruminal acidosis in beef cattle. Amer. Soc. Anim. Sci. **84** (Suppl. 1):424–425.
29. Nocek, J.E. 1997. Bovine Acidosis: Implications on Laminitis. J. Dairy Sci. **80**:1005–1028.
30. Ogimoto, K., and S. Imai. 1981. Atlas of Rumen Microbiology. Japan Scientific Societies Press, Tokyo.
31. Owens, F.N., D.S. Secrist, W.J. Hill, and D.R. Gill. 1998. Acidosis in cattle: A review. J. Anim. Sci. **76**:275–286.
32. Palo, R.T., and C.T. Robbins. 1991. Plant Defenses Against Mammalian Herbivory. CRC Press, Boca Raton.
33. Palombo, E.A., and S.J. Semple. 2001. Antibacterial activity of traditional Australian medicinal plants. J. Ethnopharmac. **77**:151–157.
34. Pell, A.N., R.E. Pitt, P.H. Doane, and P. Schofield. 1998. The development, use and application of the gas production technique at Cornell University, USA. Brit. Soc. Anim. Sci. **22**:45–54.
35. Pell, A.N., and P. Schofield. 1993. Computerized monitoring of gas production to measure forage digestion in vitro. J. Dairy Sci. **76**:1063–1073.
36. Rymer, C., A.R. Moss, E.R. Deaville, and D.I. Givens. 1998. Factors affecting the amount of indirect gas produced by the in vitro gas production technique. Brit. Soc. Anim. Sci. **22**:89–91.
37. Theodorou, M.K., B.A. Williams, M.S. Dhanoa, A.B. McAllan, and J. France. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Technol. **48**:185–197.
38. Wallace, R., Onodera, R., and M.A. Cotta. 1997. Metabolism of nitrogen containing compounds, pp. 683–328. In P.N. Hobson and C.S. Stewart (eds.), The Rumen Microbial Ecosystem, Second Edition, Blackie Academic & Professional, London.
39. Wallace, R.J. 1996. Ruminal microbial metabolism of peptides and amino acids. J. Nutr. **126**:1326–1334.
40. Wegener, H.C. 2003. Ending the use of antimicrobial growth promoters is making a difference. Amer. Soc. Microbiol. **69**:443–448.

Chapter 10
Screening Plants and Plant Products for Methane Inhibitors

Secundino López, Harinder P.S. Makkar, and Carla R. Soliva

Introduction

Plant secondary compounds may have important antimicrobial activity [6], and these compounds have been suggested as alternative additives for use in ruminant feeding. The plant secondary compounds may modify ruminal fermentation, enhancing the efficiency of feed utilization [16]. Manipulation of the rumen microbial ecosystem for enhancing fibrous feed digestibility, improving animal performance, and reducing methane production and nitrogen excretion by ruminants is one of the most important goals for animal nutritionists.

Methane is a potent greenhouse gas and ruminants are considered as one of the major contributors to biogenic methane emissions [20]. Methane from ruminal fermentation can be decreased by up to 25% with antibiotics, in particular ionophores, which inhibit H_2-producing species and therefore limit H_2-supply to the methanogens [32]. However, the use of ionophores as feed additives has been recently banned in the European Union. Plants and plant extracts with high concentrations of secondary compounds appear to be potential candidates for reducing ruminal methanogenesis. However, it is not well known which plant species or plant secondary compounds may be effective as anti-methanogenic agents, making the search and discovery of new plant compounds necessary to achieve this objective.

General Considerations

Screening Methodology

Research to find novel plant drugs or additives is expensive, but it is essential in the discovery of new active compounds given the high level of molecular diversity

S. López (✉)
Instituto de Ganadería de Montaña (CSIC-ULE), Dept. Producción Animal, Universidad de León, 24071 León, Spain
e-mail: s.lopez@unileon.es

P.E. Vercoe et al. (eds.), *In Vitro Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies*,
DOI 10.1007/978-90-481-3297-3_10,

that can be found in higher plants [4]. One of the main approaches for the search for new biologically active compounds is the screening of a number of candidate plants. In a first stage, a screening program consists in the collection of every readily available plant, which is subsequently tested for a particular biological activity, usually called the "target" (in our case for the decrease in methane production from ruminal fermentation). Testing of plants is usually by means of biological assays in vitro that are designed to be simple, selective, easily interpretable and low cost, with the objective to test a large number of candidates in a short period of time [4]. The objective is to spot as many candidates from the collection giving interesting results (referred to as "hits") as reasonably achievable, even though practical constraints, such as experimental errors, concentrations of active compounds or delivery mechanisms, among others, impose obvious limitations to their sustainable use. The screening also involves testing how selective the plants are for the chosen target without interfering with other related targets (in our case without non-specific inhibitory effects on ruminal fermentation). Usually, it is unlikely that a perfect candidate will emerge from an initial screening run; as more often several products may be found to have some degree of activity, and the process will require several iterative runs. Thus, depending upon the results of a first broad screening, some follow up assays can be performed with the hits to obtain further information on the narrower set, confirming and refining observations. Of particular interest is the so-called hit confirmation phase, in which compounds that are found active in the initial screening are re-tested, using the same assay conditions, and dose response curves can be generated.

A screening program includes a number of steps: target choice, assay development and validation, screening implementation, data recording and analysis and discovery of hits [26]. A specific assay method has to be developed for a given target type to ensure that a biologically relevant and robust screening is configured. An ideal assay would be simple, rapid, sensitive and precise, easy to develop and to run, stable, safe and economical, providing reliable information without compromising throughput. Initially, an assay-optimisation step is required to improve the stability and reliability of the biological system studied. This chapter will focus on the stepwise description of assays developed and validated for the specific target of decreasing methane production from ruminal fermentation.

Design of Screening Assays for Anti-Methanogenic Effects

Before the detailed description of three specific assays designed to assess effects of plant additives on methane production from ruminal fermentation is provided, some general considerations on the screening process will be outlined.

General Characteristics of the Assay

The assays designed to assess effects on methane production consist, in general, of in vitro cultures of mixed ruminal microorganisms [23]. These can be either batch or continuous cultures (Rumen Simulation Technique, RUSITEC) depending on the

objectives of the study and the number of plant additives to be tested (continuous cultures are impracticable for a large number of candidate plants). After an incubation period, a number of measurements are recorded, in particular gas and methane production.

The results from the incubation systems may be influenced by a number of factors [23]: buffer composition, pH, temperature, medium and rumen fluid proportions in the mixture, animals used as donors of rumen fluid (species, diet), incubation time, amounts of feed substrate and plant additives used, etc. The guidelines of the assays presented in this chapter are those used in our laboratories. Although guidelines can be established to ensure that the screening assay reaches an acceptable level of quality, many choices require pragmatism and the ability to compromise opposing factors. The assays presented herein can be adapted to suit to the facilities available in a laboratory. To achieve this optimally, sound knowledge of different in vitro rumen fermentation systems is required [23].

Definition of Objectives

This is a key point in guiding decision making concerning the experimental design. The objective will determine crucial features such as the size of the experiment, the parameters to be monitored and/or the number of replication.

In a large-scale screening of plant resources, the objective is to spot a few promising hits from a vast collection of plant candidates (hundreds). Only batch cultures can be used for this type of screening and it is unlikely that all the plants can be tested in a single incubation run. Thus, a series of incubations will be required to test all plants included in the collection, and each plant will be compared only with the control values observed in the same incubation run. In addition, a balance between the number of samples tested and replication is required for the assay to be feasible; if the number of samples is large, number of replications has to be small (only 3–4 replicates), otherwise the experiment becomes intractable. Results from these screening assays are subject to some limitations (small experimental replication, possible false negatives and positives). The main objective, however, is to identify new candidates that may induce noticeable changes in the ruminal fermentative pattern using simple procedures that allow to test a large number of samples in a relatively short time, rather than attaining definitive data of the effects of all the plant species, or studying all the plants in the collection in depth. Outputs from such a trial are the identification of positive hits and the information that the negatives are not likely to be effective at the dose tested.

Along with these large-scale screening trials, other experiments can be designed with a more explicit objective. For instance, after completing a large-scale initial screening, subsequent hit confirmation trials can be designed for validating the results obtained for the most promising candidates, or for testing different plant parts (leaves, flowers, fruits), different accessions (samples collected from different locations) or at different maturity stages of a given plant species (which has been identified previously as a hit). In any of these cases, as the number of plants to be studied is much lower, all the plant additives can be tested at the same time

(thus tested against the same control), and with more experimental replicates for each plant species. The whole test can be repeated in a series of incubations (using inocula from different days), resulting in a more robust statistical design.

Finally, if a small number of plant additives have to be tested (8–12) it may be of value to use continuous cultures or fermenters (such as RUSITEC) instead of batch cultures. In these continuous cultures, ruminal fermentation can be mimicked and the persistency of the effects of the plant additives (for a few weeks) can be investigated. Usually, this type of screening is restricted for detailed investigation on the hits that have been identified in previous studies using batch methods.

In this chapter, several assays designed to screen methane inhibitors are proposed. The first assay is the syringe-based in vitro gas method developed and used at the Institute of Animal Production in the Tropics and Subtropics (UH, Germany). The second assay based on measurement of gas production with a pressure transducer as set up and used at the Department of Producción Animal (ULE, Spain) will be described, and finally, a screening assay using a continuous fermentation system (RUSITEC) as routinely used in the Institute of Animal Science at the *Eidgenössische Technische Hochschule* (ETH, Zurich, Switzerland) will be outlined.

A "funnelling" approach can be programmed, starting from a large-scale screening of a vast collection of plant additives, following one or more hit confirmation assays, and then a study in continuous fermentation systems with a few candidates selected previously.

Plant Selection

The search for new feed additives in screening assays has to be based on a large and conveniently selected collection of candidates [4]. When the aim is to decrease methane production from ruminal fermentation, plant species could be selected based on their known medicinal properties, in particular those with recognized antimicrobial and/or digestive effects. Active plant compounds may decrease methane production by depressing H_2 producing bacteria (as monensin) thus limiting the substrate supply for methanogenesis, by directly inhibiting the methanogenic Archaea, or by redirecting H_2 to other products. Other criteria for plant selection can be considered, such as cost, commercial availability, or the possibility of promoting an interesting alternative crop and increasing biodiversity.

Physical Form of Plant Additives

The plants or plant products are available in a number of different forms. The form of the product to be tested as additive is important for determining its dose and the manner of its addition to the fermentation system.

Generally, plants or plant products available as raw material are in dry form and in most cases as fine powder. They may also be available as whole seeds or fruits, or as material chopped at different particle sizes. The product may be the whole plant or a part of the plant. It is suggested to use these products as a finely ground

powder (<100 mesh particle size) to facilitate the extraction of the active substances. The rate of addition of this material has to be relatively high (up to 10% of the dry matter incubated) in the fermentation system, since the concentration of secondary metabolites is generally low in most plants. Thus, the addition of some fermentable substrate with the plant material to be tested is inevitable.

The additive could also be available in the form of a plant extract. The extracts can be obtained by decoction, infusion (herbal teas), percolation, maceration, soaking or steeping in a carrier solvent (such as water, glycerine, alcohol, oil, vinegar), or by cold pressing to squeeze out the juice. Temperature, solvent and extraction time will determine the concentration and activity of functional compounds in the extracts. These products will thus be added to the batch cultures by dispensing the intended volume (with precision pipettes), or by continuous infusion to reach a steady concentration in the fermenter (RUSITEC). The dose will be lower than that of the raw plant material, as the active compounds are generally more concentrated in the extracts and, at high levels, they can become toxic to ruminal microorganisms. Caution needs to be exercised since the solvents contained in the extracts may interfere with ruminal fermentation.

Of particular interest are essential oils; plant extracts containing aromatic volatile oils usually obtained by steam distillation. The essential oils are usually more concentrated than other extracts, and their level of addition has to be relatively low. Essential oils are not water soluble, and this may cause solubility problems when added to rumen fermentation systems. In addition, essential oils have low boiling points and could vaporise while handling.

Finally, pure substances or blends of compounds isolated from natural sources, semi-synthetic or synthetic compounds can also be tested in the screening assays. The level of addition of these compounds has to be very low, which could pose problems of weighing for solids or of dispensing for liquids. Some of them may be difficult to dissolve in water or in the incubation medium used in the in vitro rumen fermentation systems. Such materials could be dissolved in water/buffer by sonication. An alternative for essential oils or pure compounds is to add them adsorbed onto an inert matrix (for example cellulose).

Plant additives need to be preserved in tightly closed plastic or glass jars and stored in a dry, dark and cool place until use.

Dose

The concentration of secondary compounds in plant material is affected by a large number of factors such as plant species, botanical variety, origin, conditions of cultivation and harvesting, climatic and atmospheric factors, phenological state of the plant, part of the plant, etc. [40]. Thus, to reach an adequate dose of active compound to express the desired response, different doses for each plant additive tested might be necessary to observe an effect. These levels can be investigated in a dose response curve assay, but it is outside of the scope of a large-scale screening test, given the large number of plant species needed to be tested. Therefore, in initial screening assays, all plants are tested at the same dose, aiming to supply adequate

quantities of active compounds. It is likely that some of the plants for which no effect was detected could have had a different response, if they had been tested at a different dose or under different experimental conditions.

The dose of plant additive can be calculated as a proportion of total DM incubated (g/g DM) or as a concentration in the fermentation medium (mg/L). The dose to be used is highly dependent on the form of presentation of the plant additive. If plant material (as powder) is used, concentration of secondary compounds is expected to be low, and a relatively high amount of material may be needed to supply an active compound in a high enough quantity to elicit a response. In batch cultures, doses of up to 0.1 g of candidate plant dry powder per g of dry matter incubated can be used. If the additive is in the form of an extract or as a pure compound, the dose must be much lower.

Blanks, Controls and Replication

Blanks are those incubation sets that contain only the buffered rumen fluid (without any feed substrate or plant additive), and are required in assays using batch cultures to calculate net gas or methane production. When liquid additives are used, cultures without feed substrate but with the plant additive may be preferred as blanks, but this approach might not be feasible when a large number of plant additives are to be tested in a large-scale screening programme (a different blank would be required for each plant).

Control sets contain feed substrate without any plant additive. These are very important, representing the reference value (baseline) against which all the other values are compared. The choice of control is not always straightforward. When the additive is in a liquid form, the amount of feed substrate should be the same in the control cultures and in those receiving the additive (test sets). The addition of plant additive in powder form means the addition of some fermentable substrate to the fermentation system. Thus, the amount of feed substrate in the control sets should be equal to the total amount of the substrate and plant additive weighed in the test sets. For instance, if 500 mg of feed substrate and 50 mg of plant additive are in the test sets, 550 mg of feed substrate will be weighed in the control sets.

Within each incubation batch, all treatments (control or test) are incubated at least in triplicate (3–5 replicates per treatment depending upon the number of plants to be tested). The number of replicates for the control sets can be higher than for the test sets, considering the importance of having a robust average value for the control. In batch cultures and continuous fermentation systems, control and test sets could be repeated with different inocula (from different animals or in different days) to have real experimental replicates, so that the residual error (variance between replicates within each treatment) can be accurately estimated. Although this would be statistically more appropriate, it is not always feasible in large-scale screening assays (hundreds of plants to be tested), and the number of samples to be tested and replication required need to be compromised.

The Hohenheim Syringe-Based In Vitro Gas Method

Two approaches, both using the syringe-based in vitro gas method [30, 31], will be outlined in this section, depending on the physical form of the plant product to be tested. The first approach ("Screening of plants") is proposed to test plant materials such as plant leaves, stems, seeds or the whole plant. In this case, the plant material (ground to powder) is directly incubated without any feed substrate, and methane production is recorded. If methane concentration in the fermentation gas is substantially reduced compared with that observed when common ruminant feedstuffs (forage, roughage) are incubated, then the plant material tested is spotted as a positive hit. These hits can be further investigated in subsequent confirmation assays in which plant material is used as a supplement (at different rates of inclusion) of a basal feedstuff used as the fermentation substrate. In the second approach ("Screening of plant extracts or additives in liquid state"), a plant extract in a liquid form is tested as an additive to a basal feed used as the fermentation substrate. Specific blanks and controls are required for each approach.

Screening of Plants

Sample Preparation

Dried sample should be milled to pass through a 1 mm sieve. Avoid fine grinding of the sample. The sample should be dried preferably using a freeze drier. If a freeze drier is not available, use a forced air oven at a temperature between 50 and 55°C.

Reagents

1. *Bicarbonate buffer solution:* Dissolve 35 g sodium bicarbonate ($NaHCO_3$) and 4 g ammonium bicarbonate (NH_4HCO_3) in approximately 500 mL distilled water and then make up the volume to 1 L with distilled water.
2. *Macromineral solution:* Dissolve 6.2 g potassium dihydrogen phosphate (KH_2PO_4), 5.7 g disodium hydrogen phosphate (Na_2HPO_4), and 0.6 g magnesium sulphate ($MgSO_4.7H_2O$) in approximately 500 mL distilled water and then make up the volume to 1 L with distilled water.
3. *Micromineral solution:* Dissolve 10 g manganese chloride ($MnCl_2.4H_2O$), 13.2 g calcium chloride ($CaCl_2.2H_2O$), 1 g cobalt chloride ($CoCl_2.6H_2O$), 8 g ferric chloride ($FeCl_3.6H_2O$) in approximately 50 mL distilled water and then make up the volume to 100 mL with distilled water.
4. *Resazurine:* Dissolve 0.1 g resazurine in 100 mL distilled water.
5. *Reducing solution:* Dissolve 996 mg sodium sulphide ($Na_2S.9H_2O$) in 94 mL distilled water and then add 6 mL of 1 N sodium hydroxide solution (dissolve 4 g sodium hydroxide in 100 mL distilled water for 1 N sodium hydroxide).

Weighing of Samples and Preparation of Syringes

Tare a specially made scoop (approximately 4 cm in length and 1 cm in depth/radius; standard sodium hydroxide-containing plastic container can be cut horizontally to half to form the scoop) on an analytical balance. Weigh 380 mg of air-dry feed sample in the scoop and then insert a 5 mL capacity pipette or a glass rod into the narrow end of the scoop and transfer the sample from the scoop into 100-mL calibrated glass syringes. The feedstuffs are incubated at least in triplicate. The blank syringes do not contain feed.

Preparation of In Vitro Rumen Fermentation Buffer Solution and Incubation

Collect the rumen fluid and particulate matter before the morning feed from 2 cattle, fed a diet of the type similar to that of the samples being analysed in vitro. Mix the contents taken from 2 cattle, homogenize, strain and filter them through 4 layers of cheesecloth. Keep all glassware at approximately 39°C and flush these with carbon dioxide before use. Carbon dioxide is heavier than air and hence it remains in the glassware for a reasonable period provided the container is not inverted up side down. The strained rumen fluid is kept at 39°C under carbon dioxide and should be prepared just before start of the incubation.

Notes:

1. *Although cattle are used as donors of rumen fluid at Hohenheim, sheep or other ruminant animals could be used.*
2. *As samples to be analysed are plants (herbs or botanicals), usually not used as feedstuffs, it is unlikely that animals can be actually fed these plants. So a diet similar to these samples could be forage-based diet.*

Medium composition

Rumen buffer solution (bicarbonate buffer)	630 mL
Macro mineral solution	315 mL
Micro mineral solution	0.16 mL
Resazurine	1.6 mL
Distilled water	945 mL
Freshly prepared reducing solution	60 mL
Rumen fluid (see above for collection and preparation)	660 mL

The above volume is sufficient for 80 syringes (30 mL/syringe) plus 7% extra.

Incubation Procedure

In a 3 or 5 L capacity glass container, mix all the above mentioned solutions (in the order given above), except the reducing solution and rumen fluid. The container is

kept immersed in a water bath adjusted at 39°C. This water bath is a plastic rectangular frame, approximately 400 × 300 × 200 cm filled with water, the temperature of which is adjusted at 39°C using a portable thermostat suspended from the top of the plastic container in water. This plastic water bath is kept on a magnetic stirrer. The contents of the container are flushed with carbon dioxide and kept stirred using a magnetic stirrer. After about 5 min, add the reducing solution and keep the mixture stirring and flushing with carbon dioxide. When the mixture has been reduced (blue colour of the dye changes to pink and then to colourless; it takes about 15–20 min for the reduction process to complete and during this time the rumen liquor and the particulate material collected from cattle are homogenized using a house-hold mixer (two 15 s bursts at maximum speed) and then strain it through 2 layers of muslin cloth or a nylon bag of 100 μm pore size to obtain approximately 700 mL of the rumen fluid), add 630 mL of the rumen fluid. Keep this mixture stirring and flushing with carbon dioxide for another 10 min. Transfer a 30-mL portion of the rumen-fluid medium into each syringe using a dispenser, and incubate in a water bath at 39°C. For filling 80 syringes, after some practice should take 40–45 min. After completion of the filling-up process, shake the syringes well and transfer them to a specially designed water bath. Shake all the syringes every hour for the first 4 h and then after every 2 h. Generally, the incubation is started at about 07.30–08.00 a.m., and after 12 h of incubation the syringes are not shaken till the termination of the incubation (24 h). If the syringes are incubated in a rotating rotor fixed in an incubator adjusted at 39°C, as suggested by Menke et al. [31], there is no need to shake the syringes.

At 24 h, the position of the piston on the calibrated syringes is recorded.

The operational aspects of the gas method are available at:
http://www.iaea.org/programmes/nafa/d3/mtc/invitro-slideshowapr01.pdf

Total and Net Gas Production

The gas volume is recorded after 24 h in test and blank syringes. The difference of the piston position at 24 h minus piston position at 0 h (generally 30 mL) gives total gas production in the test and blank syringes. The net gas production is calculated by subtracting values of the blank from that of the test syringe.

Total and Net Methane Production

In our laboratory in Hohenheim, we measure methane content of the gas in the syringe using an infrared-based methane analyser (0–30% range methane analyser from Pronova Analysentechnik GmbH & Co KG, Berlin, Germany). The methane analyser is calibrated against 10.6% or 12% standard methane. A gas chromatograph can also be used for methane measurement; however, the use of the infrared-based methane analyser is simple, convenient and takes less time and resources.

After recording the piston position at 24 h of incubation, the tubing of the syringe outlet is inserted into the inlet of the methane analyser; the piston is pushed to inject the accumulated gas into the analyser. The methane as percent of the gas is displayed on the methane analyser. This value is used for calculation of methane in the total

gas volume. When the fermentability of the feed is high (piston mark reaches above 90 mark on the syringe), the volumes of total gas and methane are measured while pushing back the piston after 8 or 10 h of incubation, and similarly volumes of gas and methane are measured after 24 h. These two values are added to obtain the total methane production in 24 h of incubation. Generally, we have observed that percent methane production in a syringe is lower for the period 0–8 or 10 h of incubation than for the period 8 or 10–24 h of incubation.

Note: Since the solubility of methane and carbon dioxide is different at different temperature (solubility of carbon dioxide in water/buffered medium is higher than methane), methane content in the gas collected in the syringe is measured without cooling the syringe to room temperature.

Calculations and Expression of Results

An Example:

Three samples (200 mg each) of Plant A and B were incubated for 24 h in the in vitro gas method.

Total gas production after 24 h (24 h piston position *minus* 0 h piston position) for:

i) Blank syringes: 5.5 mL (syringe 1); 4.5 mL (syringe 2); and 5 mL (syringe 3). Average of 3 syringes: 5 mL.
ii) Plant A: 60 mL (syringe 1); 62 mL (syringe 2); and 61 mL (syringe 3).
iii) Plant B: 58 mL (syringe 1); 57.5 mL (syringe 2); and 58 mL (syringe 3).

Net gas production:

Plant A

Syringe 1 = 60 – 5 = 55 mL
Syringe 2 = 62 – 5 = 57 mL
Syringe 3 = 61 – 5 = 56 mL

Plant B

Syringe 1 = 58 – 5 = 53 mL
Syringe 2 = 57.5 – 5 = 52.5 mL
Syringe 3 = 58 – 5 = 53 mL

Using infra-red based methane analyser, percent methane for:

i) blank syringes after 24 h of incubation: 17 (syringe 1); 17.3 (syringe 2); and 17 (syringe 3). Total methane in blank syringes (total gas × % methane)/100 = 0.935 mL (syringe 1); 0.779 mL (syringe 2); and 0.85 mL (syringe 3). Average methane in blank syringe = (0.935 + 0.779 + 0.85)/3 = 0.855 mL.
ii) Plant A syringes: 13 (syringe 1); 12.3 (syringe 2); and 12.5 (syringe 3). Total methane in syringes (total gas × % methane)/100 = 7.15 mL (syringe 1); 7.01

mL (syringe 2); and 7.06 mL (syringe 3). Net methane (total methane – 0.86) = 6.29 mL (syringe 1); 6.16 mL (syringe 2); and 6.21 mL (syringe 3).

iii) Plant B syringes: 4 (syringe 1); 4.5 (syringe 2); and 4.3 (syringe 3). Total methane in syringes (total gas × % methane)/100 = 2.12 mL (syringe 1); 2.36 mL (syringe 2); and 2.28 mL (syringe 3). Net methane (total methane – 0.86) = 3.15 mL (syringe 1); 5.36 mL (syringe 2); and 3.45 mL (syringe 3).

The results can be expressed as: % methane (as per cent of gas produced) = (Net methane produced/net gas produced)100

Plant A

Syringe 1 = 11.44%
Syringe 2 = 10.81%
Syringe 3 = 11.09%
Average: 11.1%

Plant B

Syringe 1 = 4.0%
Syringe 2 = 4.5%
Syringe 3 = 4.3%
Average: 4.3%

Expression of Results

For "usual" feeds such as hay, concentrate or a mixture of hay and concentrate the values for percent methane in the syringes range from 16 to 20%. Considering these values, and based on our experience, the methane reduction potential of plants can be arbitrarily divided in the following three categories:

i) Low potential, for % methane in gas between > 11% and ≤ 14%
ii) Moderate potential, for % methane in gas between > 6% and < 11%
iii) High potential, for % methane in gas between > 0 and < 6%

In the above example, Plant A will be categorised as having moderate methane reduction potential and Plant B as having high methane reduction potential.

Notes:

1. *Only the samples to be screened are incubated in the syringes.*
2. *Screening process can be hastened by reducing incubation time to 8 h from 24 h. From blank syringes, the gas evolved in 8 h is generally low and it is difficult to measure methane production using infrared methane analyser. Under such a situation, it is advised to transfer 60 mL rumen fluid medium (instead of 30 mL). Then half the gas and methane production to arrive at levels corresponding to 30 mL. This approach for measuring methane and gas in blank could also be useful for 24 h incubations.*

3. *Percent methane in the test syringes as displayed in the infra-red methane analyser (without calculation of the net gases i.e. without taking into consideration the methane and gas in the blank syringes) would also give a rough estimate and categorisation of plants under investigation, but the adoption of the approach (i.e. without calculation of net gases) is not advised, since it could give misleading conclusions, especially in studies illustrated in Section "Screening of plant extracts or additives in liquid state".*
4. *The plants found to be promising through investigations presented in this section could be evaluated as supplements to the basal diets of relevance to the production system by incubating different proportions of the identified plant to the basal diet. For example, by incubating 380 mg of the basal diet alone or with 19, 38 and 57 mg of the identified plant. The effectiveness of the identified plant on the basal diet could be determined by comparing the percent methane in the total gas in the sets without and with the identified plant. Decrease in percent methane on addition of the plant to the basal diet shows the possible beneficial effect of the plant on the tested basal diet.*
5. *Whether the plant identified through the approach outlined in Section "Screening of plants" has the methane reduction effect that is accountable by merely being a supplement or by acting on the rumen microbes and thus decreasing methane from the basal diet, can be studied as follows. Compare the observed and estimated values for methane (as percent of total gas or as mL methane/100 mg organic matter degraded). The observed values are for the sets in which the basal diet/substrate is incubated with the identified plant, and the expected values are the calculated values from the sets where the basal diet/substrates and the test sample/identified plant are incubated separately ([14] for details). When the observed and calculated values for methane are similar, the methane reduction is not through change of microbial fermentation but is a result of the test plant as a part of the feed; however, when the observed values of methane are lower than the calculated ones, the plant has decreased methane production from the basal diet.*

Screening of Plant Extracts or Additives in Liquid State

Preparation of Plant Extract

The dry sample is finely ground (preferably using a ball mill) and an extract is prepared in water and/or aqueous organic solvent. Solvents, which could be tried, are: 50–70% aqueous methanol, aqueous acetone and/or aqueous ethanol. There is no standard procedure for preparing the extract; however, a suggested procedure is to take 1 g of the plant material in a glass beaker and add to it 50 mL of the aqueous solvent. Sonicate the contents by placing the beaker in a sonication bath for 20 min. Centrifuge the contents at approximately 5000*g* for 10 min and collect the supernatant. Remove the organic solvent using a rotary vacuum evaporator at a temperature not greater than 40°C. Lyophilize rest of the material and dissolve

the lyophilized material in 10 mL distilled water. If the material does not dissolve in water, sonicate the contents in a sonic water bath to form a uniform emulsion. Another approach used in UH [14] is extraction by steeping 20 g of plant material in 400 mL of the solvent. The suspension was stirred overnight and centrifuged (ca. 3000*g* for 10 min). The supernatant was collected and methanol (only when aqueous methanol used) was evaporated in a rotary evaporator. The crude extract obtained was then lyophilised and recovery was calculated as weight of lyophilised extract/weight of initial test plant material. The lyophilised material was dissolved in suitable quantity of water and used for evaluation by injecting a suitable quantity. Testing of the extract in aqueous organic solvent (without removing the organic solvent) should be avoided, due to artefacts produced by the organic solvent. In some situations, where the removal of organic solvents (for example in testing essential oils) is not possible, minimum amount of organic solvent should be added and the effect of the organic solvent on rumen fermentation, and methane and gas production should be well understood.

Reagents

Reagents and their preparation are the same as above.

Weighing of Samples and Preparation of Syringes

Same as above, except that 380 mg of the feed, on which the effect of plant extract or liquid additive is to be studied, is weighed out in syringes. The feed selected should be similar to the feed being fed in the practical situations; and the rumen liquor taken for the in vitro incubation should be from animals fed similar diet.

Note: When the objective is to evaluate the effect of an additive or plant extract, a set of three syringes containing the feed, the additive/plant extract (preferably < 1 mL) and 30 mL of the incubation medium form the test set, and the corresponding blank contains a set of three syringes with the same amount of additive/plant extract as in the test and 30 mL of the incubation medium (and no feed). A set of three syringes containing only the feed and 30 mL of the incubation medium forms the control set.

Preparation of In Vitro Rumen Fermentation Buffer Solution and Incubation

Same as previously described.

Total and Net Gas Production

Same as previously described.

Total and Net Methane Production

Same as previously described.

Calculations and Expression of Results

An Example

Total gas production after 24 h (24 h piston position *minus* 0 h piston position) for:

i) Blank syringes (without additive/extract): 5.5 mL (syringe 1); 4.5 mL (syringe 2); and 5 mL (syringe 3). Average of 3 syringes: 5 mL.
ii) Blank syringes (with additive/extract): 7.5 mL (syringe 1); 7.5 mL (syringe 2); and 7 mL (syringe 3). Average of 3 syringes: 7.14 mL.
iii) Feed without additive/extract: 60 mL (syringe 1); 62 mL (syringe 2); and 61 mL (syringe 3)
iv) Feed with additive/extract: 58 mL (syringe 1); 57.5 mL (syringe 2); and 58 mL (syringe 3).

Net gas production:

Feed without additive/extract

Syringe 1 = 60 – 5 = 55 mL
Syringe 2 = 62 – 5 = 57 mL
Syringe 3 = 61 – 5 = 56 mL

Feed with additive/extract

Syringe 1 = 58 – 7.14 = 50.9 mL
Syringe 2 = 57.5 – 7.14 = 50.4 mL
Syringe 3 = 58 – 7.14 = 50.9 mL

Using infrared based methane analyser, % methane for:

i) Blank syringe (without additive/extract) after 24 h of incubation: 17 (syringe 1); 17.3 (syringe 2); and 17 (syringe 3). Total methane in these blank syringes (total gas x % methane)/100 = 0.935 mL (syringe 1); 0.779 mL (syringe 2); and 0.85 mL (syringe 3). Average methane in blank syringe = (0.935 + 0.779 + 0.85)/3 = 0.86 mL.
ii) Blank syringe (with additive/extract) after 24 h of incubation: 16 (syringe 1); 16.3 (syringe 2); and 16 (syringe 3). Total methane in these blank syringes (total gas × % methane)/100 = 1.2 mL (syringe 1); 1.22 mL (syringe 2); and 1.12 mL. Average methane in blank syringe = (1.2 + 1.22 + 1.12)/3 = 1.18 mL.
iii) Feed without additive/extract: 16 (syringe 1); 16.5 (syringe 2); and 15.5 (syringe 3). Total methane in syringes (total gas × % methane)/100 = 8.8 mL (syringe 1); 9.41 mL (syringe 2); and 8.68 mL (syringe 3). Net methane (total methane – 0.86) = 7.94 mL (syringe 1); 8.55 mL (syringe 2); and 7.69 mL (syringe 3).

iv) Feed with additive/extract: 13 (syringe 1); 12.3 (syringe 2); and 12.5 (syringe 3). Total methane in syringes (total gas × % methane)/100 = 7.15 mL (syringe 1); 7.01 mL (syringe 2); and 7.06 mL (syringe 3). Net methane (total methane – 1.18) = 5.97 mL (syringe 1); 5.83 mL (syringe 2); and 5.88 mL (syringe 3).

Average net methane:

Without additive/extract = 8.06 mL
With additive/extract = 5.89 mL

Percent methane reduction with additive/extract = (8.06 – 5.89) × 100/8.06 = 26.9%

In practical situations, the feed additive is effective in reducing methane production if the organic matter degradability of the substrate is not adversely affected by the additive/extract. Therefore, one has to determine organic matter degradability. Alternatively the effect of an additive/extract is better appreciated by comparing: "mL net methane produced/100 mg organic matter degraded" in presence and absence of additive/extract. For this, one has to determine organic matter degradability of the feed (detailed procedure is available in Chapter 7).

If on addition of additive/extract the "mL net methane produced/100 mg organic matter degraded" has decreased when compared to without its addition, the additive is effective. Different additives/extract can be ranked based on the extent of this reduction. Ideally an additive or an extract which decreases "mL net methane production/100 mg organic matter degraded" the most without affecting organic matter degradability should be the additive of the choice for further investigations.

The Pressure Transducer Method

In Vitro Incubations

This screening assay, designed to test the efficacy of a phytogenic additive to decrease methane production in vitro, consists of a series of batch cultures of mixed ruminal microorganisms [23], in which methane production is measured after the fermentation of a specific feed substrate. For this technique, a 120-mL Wheaton vial or serum bottle (Wheaton Science Products, Millville, NJ) is used as the reaction vessel where ruminal fermentation is simulated in vitro.

The procedure followed for in vitro incubations comprises below-mentioned 4 steps, executed in the sequence listed, before the measurements take place.

1. Preparation and weighing of feed substrate and plant additive to be tested
2. Preparation of fermentation medium
3. Collection of rumen fluid and dilution with the medium
4. Inoculation of cultures

Preparation and Weighing of Feed Substrate to be Fermented and Plant Additive to Be Tested

Feed Substrate

Feed substrate is the basal diet that is fermented in the batch cultures. In our screening assays, and with the aim to have a diet producing a high methane volume per g of digestible substrate, a mixture of forages and barley (500 g/kg alfalfa hay + 250 g/kg grass hay + 250 g/kg barley grain) is used. However, any other diet or feedstuff could be used depending upon the objectives of the experimental work.

The same feed substrate must be used in all the incubation runs for a particular screening programme. Preferably, a dried (air- or oven-dried at low temperature) and finely ground (mesh 1 mm in a Culatti mill) feedstuff (or mixture of feeds) should be used. Samples of feed substrate (500 mg of dry material) are weighed in a "rolled paper" and transferred into each 120-mL serum bottle.

Plant Additive

Some considerations regarding the plant additive have already been outlined in the section dealing with general design of assays for the screening of phytogenic additives. The preparation of the plant additive depends on the physical form of the product (in a liquid form if it is an extract or as a dry powder if it is the whole plant or plant parts in the solid state). When an extract or essential oil is used, a small volume will be dispensed with a precision pipette directly into each bottle. The volume dispensed depends on the additive used, its activity and its purity in the product to be tested. If the dose is too low, the response may not be observed and a potentially interesting additive may be missed. If the dose is too high, it may have non-specific inhibitory effects on fermentation. The solvent or excipient contained in the product may be important, as some compounds used with this purpose (methanol, ethanol, organic solvents) may be inhibitory to ruminal microorganisms.

If raw plant material is used, this should preferably be freeze-dried. If it is not possible, the sample can be air dried in the dark or oven dried (forced air oven) at low temperature (50–55°C). The material is then ground to pass through a screen of 1 mm for better extraction of active compounds contained in the plant matrix when it is incubated with the buffered medium containing rumen fluid.

The plant to be tested should be weighed separately from the feed substrate and then transferred directly into the fermentation bottle. In our screening trials, 50 mg of plant additive are weighed in a precision scale ($\pm$ 0.1 mg) and added directly to

the 500 mg of feed substrate into the bottom of the vial, just before dispensing the buffered rumen fluid.

The dose or rate of addition has to be decided considering the objectives and design of the experimental work.

Preparation of Fermentation Medium

The next step is to prepare the fermentation medium, consisting of a mixture of solutions that are prepared in advance following these instructions [15, 30]:

1. *Bicarbonate buffer solution:* Dissolve 70 g sodium bicarbonate ($NaHCO_3$) and 8 g ammonium bicarbonate (NH_4HCO_3) in approximately 1 L distilled water and then make up the volume to 2 L with distilled water.
2. *Macromineral solution:* Dissolve 12.4 g potassium dihydrogen phosphate (KH_2PO_4), 11.4 g disodium hydrogen phosphate (Na_2HPO_4), and 1.2 g magnesium sulphate ($MgSO_4.7H_2O$) in approximately 1 L distilled water and then make up the volume to 2 L with distilled water.
3. *Micromineral solution:* Dissolve 1.0 g manganese chloride ($MnCl_2.4H_2O$), 1.32 g calcium chloride ($CaCl_2.2H_2O$), 0.1 g cobalt chloride ($CoCl_2.6H_2O$), 0.8 g ferric chloride ($FeCl_3.6H_2O$) in approximately 50 mL distilled water and then make up the volume to 100 mL with distilled water.
4. *Resazurine:* Dissolve 0.1 g resazurine in 100 mL distilled water.
5. *Reducing solution:* Dissolve 1.875 g cysteine-HCl and 1.875 g of sodium sulphide ($Na_2S.9H_2O$) in 200 mL distilled water, then add 12 mL of 1 N sodium hydroxide solution (dissolve 4 g sodium hydroxide in 100 mL distilled water for 1 N sodium hydroxide), and finally make up the volume to 300 mL with distilled water.

Note: The reducing solution should be prepared fresh. Other reagents could be prepared in bulk and stored at room temperature for up to approximately 1 month.

All the above-mentioned solutions are mixed in a 10-L capacity glass container placed on a magnetic hot plate stirrer. A stir bar is placed at the bottom of the bottle, and CO_2 is flushed to purge all air present in the empty bottle. Then, 1282.5 mL of the bicarbonate buffer solution are poured into the bottle, and both the magnetic stirrer and the heater of the plate are switched on. From this time onwards, the medium is kept under continuous agitation (with the magnetic stirrer), temperature is adjusted at 39°C and monitored frequently using a digital thermometer, and the contents are bubbled continuously with carbon dioxide (CO_2 standard grade) to saturate the medium with this gas. Then, macromineral (1282.5 mL), micromineral (6.5 mL), and resazurin (6.5 mL) solutions, and 2565 mL distilled water are added in this order [15, 30]. Once the medium temperature is stable at 39°C, 260 mL of freshly prepared reducing solution are added, keeping the mixture stirring and flushing with CO_2. On adding the reducing solution,

the mixture starts getting reduced (blue colour of the dye changes to pink and then to colourless; it takes about 15–20 min to complete the removal of any O_2 present in the medium) and the medium thus becomes anaerobic. This will be the time to add the filtered rumen fluid (1350 mL for the volumes indicated above) to the medium.

Collection of Rumen Fluid and Dilution with the Medium

Samples of rumen contents (rumen fluid and particulate matter) are collected before the morning feed from three cannulated sheep (any other ruminant species can be used), fed a diet of the kind similar to the feed used as fermentation substrate in the vials. The rumen contents taken from three sheep are mixed and collected in thermos bottles and transported to the laboratory. Before collecting the rumen liquor, thermos should have been filled up to the brim with water at 39°C, and emptied just before collection of the ruminal samples in them. In the laboratory, rumen contents are homogenized, strained and filtered through several layers of cheesecloth. It is important to keep all glassware at approximately 39°C and flush them with carbon dioxide before use (carbon dioxide is heavier than air and hence it remains in the glassware for a reasonable period provided the container is not inverted up-side down). Generally, rumen fluid is prepared while the medium is being reduced upon the addition of the cysteine-sulphide solution. The strained rumen fluid is kept at 39°C under a continuous flux of CO_2 and prepared just before the start of the incubation. Once the medium is completely reduced (based on observed change in colour) 1350 mL of strained rumen fluid are added to the medium (5400 mL of the mixture as described above), giving a final volume of 6650 mL (rumen fluid diluted to 20% v/v; i.e. the final ratio of rumen fluid to medium is 1:4 as suggested by Goering and Van Soest [15]).

Inoculation of Cultures

The buffered rumen fluid is stirred and flushed with CO_2 for another 10 min. In the meanwhile, serum bottles containing feed substrate and plant additive are kept in an incubator adjusted at a temperature of 39°C, and flushed with CO_2 to purge any air. Transfer 50-mL of the buffered rumen-fluid into each bottle using a dispenser or an automatic peristaltic pump adjusted to dispense 50 mL of liquid (peristaltic pump is more expensive but more precise and repeatable). Include bottles with feed substrate but no plant additive (controls) and without feed substrate or plant additive (blanks) in each incubation run.

Immediately after inoculation, the bottles are closed with butyl rubber stoppers and crimp sealed with aluminium tear-off cap seals using a hand-held crimper. Then, vials are shaken vigorously, headspace gas pressure in each bottle is adjusted to ambient pressure (just by inserting a hypodermic needle through the rubber stopper to release any gas accumulated) and bottles are immediately placed in an incubator adjusted at 39°C. It is estimated that, after some practice, 30 min are required for filling 100 serum bottles.

Measurements at the End of Incubation

During the incubation, bottles are shaken regularly by hand until the end of fermentation after 24 h of incubation. This incubation time has been chosen considering the type of feed substrate fermented (high proportion of forage), the amount of gas and methane usually recorded under these conditions (based on previous experience) and the convenience of the timing for inoculation and measurement. The incubation time can be changed depending on the objectives of the assay. However, the time chosen must be the same for all the incubation runs of a given screening programme.

At the end of incubation, the volume of gas produced from substrate fermentation is measured and sampled, the gas composition is analysed, and other fermentation features are measured to characterize the fermentation pattern.

Measurement of Gas Production Using a Pressure Transducer

A pressure transducer attached to a digital readout voltmeter is used to measure raised pressure in the headspace of each serum bottle [36]. The transducer is connected to one of the female ports of a disposable Luer-lock three-way stopcock valve. The male port of the stopcock is connected to a disposable hypodermic syringe needle. The second (usually the one on the side) female port is connected to a disposable calibrated plastic syringe of 125 mL capacity (Fig. 10.1).

When incubation is finished, gas pressure in the headspace is measured after insertion of the hypodermic syringe needle through the butyl rubber stopper into the bottle headspace. The three-way stopcock is in the position where the needle male port and the transducer port are directly connected, whereas the syringe port is closed. In this position, the pressure in the headspace is measured by the transducer recording the value shown in the display unit. Most transducers measure pressure above atmospheric pressure, and in units of pounds per square inch (psi). Then, the stopcock is turned to open the link to the syringe so all the three ports of the valve are opened. In this position, the syringe plunger is gently pulled out so the gas accumulated in the bottle headspace is displaced into the syringe barrel. The value shown in the digital display unit will decrease progressively. When the headspace gas pressure is returned to ambient pressure, as indicated by a reading of zero on the display unit, it can be assumed that all fermentation gas accumulated in the headspace has been collected in the syringe. Then, the stopcock is rotated again closing the needle male port, and the pressure transducer assembly is withdrawn from the bottle closure. The volume of gas collected in the syringe can be recorded considering the position of the plunger in the scale of the calibrated syringe. It is important that the temperature of the headspace gas remains unaltered during the measuring period.

A mathematical approach has been suggested to calculate gas volume from the headspace pressure recordings [25], so that only pressure measurements are required. This approach is based on Boyle's law, which states that volume (V, mL) is inversely proportional to pressure (P, psi) at a given temperature, i.e. $V \propto 1/P$. This relationship means that pressure increases as volume decreases, and *vice versa*, and

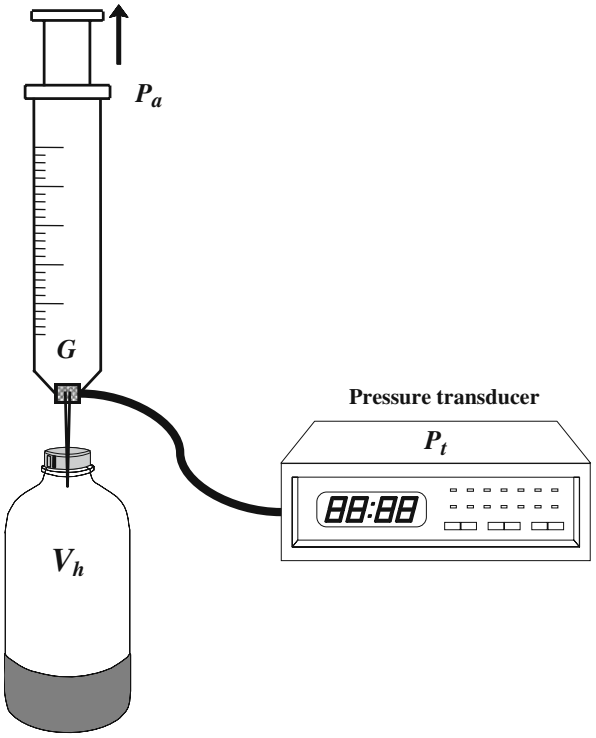

Fig. 10.1 Measurement of gas production in gas-tight culture bottles by measuring changes in pressure in the headspace using a pressure transducer (G = volume of gas produced recorded in the syringe; V_h = headspace volume; P_a = atmospheric pressure; P_t = pressure measured by the transducer above P_a)

that the product of pressure and volume at a given temperature is constant. Initial values (before withdrawing the gas) for P and V are the pressure (P_t, psi) measured by the transducer (above atmospheric pressure P_a psi) and the headspace volume (V_h, mL), respectively. After all the gas is withdrawn from the incubation bottle, the pressure is restored to atmospheric pressure (P_a, psi), the total gas volume is the sum of the headspace volume (V_h, mL) plus the volume recorded in the syringe where the gas is collected, *i.e.* the actual volume of gas produced (G, mL) during incubation. Therefore:

$$(P_t+P_a)V_h=P_a(V_h+G),$$

allowing calculation of G from P_t using the expression:

$$G = \frac{V_h}{P_a} \times P_t.$$

This expression has been evaluated using gas production data recorded at the University of León (Spain). Considering the nominal volume of the Wheaton bottles used (120 mL) and the volume of the incubation medium (10 mL rumen fluid + 40 mL buffer and mineral solution), the calculated volume of the headspace (V_h) is 70 mL. Considering that out laboratory is situated at an altitude of 850 m, average P_a> at this location is, on average, 13.1 psi. With this information, the theoretical relationship between G and P_t in our laboratory is:

$$G = \frac{70}{13.1} \times P_t = 5.34\,P_t.$$

This relationship will have to be adapted accordingly if different serum bottles are used or if the volume of incubation medium is different, and bearing in mind that atmospheric pressure will be different at a different location.

Sampling of Gas

Once total gas production has been recorded, a representative sample of the gas produced has to be taken for subsequent analysis. Sampling is conducted using special gas-tight glass syringes provided with removable needles (pressure-lock gas syringes Series A-2 with matching needles; Valco Instruments Co. Inc. (VICI) Precision Sampling; Baton Rouge, LA, USA; www.vici.com/contact.htm). These syringes are fitted with a push-button valve allowing for sample locking and storage within the syringe.

A sample can be taken directly from each bottle headspace, after inserting the needle through the septum of the stopper, taking a sample in the syringe and closing the valve. Therefore, it is assumed that gas composition will be the same in the gas released and in that remaining in the headspace. This procedure is followed provided the samples can be analysed immediately after the sample collection. If this is not the case, samples can be collected in 10-mL glass vacuum airtight tubes (Vacutainer® from Becton, Dickinson & Company, Franklin Lakes, NJ, USA; www.bd.com or Venoject® from Terumo Europe, Leuven, Belgium; www.terumo-europe.com). In this case, once the headspace gas has been collected in the calibrated syringe connected to the three-way stopcock, the syringe contents are injected into the vacuum tube for taking a gas sample. It is important to inject a volume of gas greater than the capacity of the sampling tube, so that contents in the vacuum tube are pressurized. This will avoid the entry of air from outside. It is important to take samples in duplicate, to check for any leak of gas from the airtight tube during storage. At the time of analysis, a sample of gas is taken from the sampling tube using the special gas-tight syringe by inserting its needle through the rubber septum of the vacuum tube.

Finally, the needle of the gas-tight syringe can be inserted into the injection port of the gas chromatograph, the valve opened and the sample injected.

Analysis of Methane in Fermentation Gas

Methane is measured by gas chromatography, following the methods described in detail by López and Newbold [24]. Briefly, methane content in fermentation gas is determined using a Shimadzu GC-14 B GC (Shimadzu, Japan) equipped with Carboxen™ 1000, 45/60, 2 m × 1/8" column (Supelco, USA) and flame ionization detector. Temperatures are 170, 200 and 200°C in column, injector and detector, respectively and carrying gas (He) flux is adjusted to 24 mL/min. Each gas sample (300–500 μL) is manually injected using Pressure-Lok® syringes A-2 Series of 500 μL (Supelco, USA). Methane content in samples (mmol methane/mol gas) is calculated by external calibration, using a certified gases mixture with (per L) 100 mL CH_4, 250 mL N_2, 50 mL H_2 and 600 mL CO_2 (Carburos Metálicos, Spain).

Fermentation Pattern

Although the main target of the screening assays reported herein is methane production, other features of in vitro fermentation can be measured to examine if any significant shift in the fermentation pattern may have occurred, explaining the changes in methane production.

Thus, once gas production has been measured and a sample of gas collected from the headspace, the protocol followed in our laboratory is described stepwise.

Bottles are swirled on ice to stop fermentation and opened to measure pH of the incubation medium. A sample of supernatant (0.8 mL) is taken for short chain fatty acid analysis. This sample is mixed thoroughly with 0.5 mL of a solution containing 20 g metaphosphoric acid and 4 g crotonic acid per litre of 0.5 N HCl. This is an acidifying and deproteinising solution, and crotonic acid is an internal standard to quantify the concentration of short chain fatty acid from the chromatogram peaks. This mixture is centrifuged at 20,000*g* for 10 min (4°C) and transferred to gas chromatography vials. In our laboratory, a Perkin Elmer Autosystem XL GC (Perkin Elmer Inc., USA), equipped with a semi-capillary, TR-FFAP, 30 m × 0.53 mm × 1 μm column (Supelco, USA), flame ionization detector and auto-sampler is used. Temperatures are 140°C in the column and 250°C both in the injector and the detector, and carrier gas (He) flux is adjusted at 13 mL/min. Each sample (1 μl) is injected automatically with split ratio of 1/3. Chromatograms are integrated using software Star Chromatography Workstation 6.2 (Varian Inc., USA). For calculation of short chain fatty acid production, concentration of acids in the initial mixture of buffered rumen fluid dispensed into the bottles should be known. This amount initially present in the bottles is subtracted from the amount measured at the end of incubation, to estimate the short chain fatty acid produced during the incubation.

Finally, all contents remaining in the bottle are filtered through sintered Pyrex® crucibles (pore size number 1), and the residue recovered is oven-dried (100°C for 48 h). A sample of the dry residue is collected and weighed in F57 ANKOM filter bags for extraction with neutral detergent solution (100°C for 1 h) in an ANKOM fibre analyzer (ANKOM Technology Corporation, USA). The residual neutral detergent fibre (NDF) is used for calculation of true DM and NDF in vitro degradability.

Neutral detergent fibre in the substrate, plant additives and incubation residues (in all cases expressed inclusive of residual ash) is determined using the procedure of Van Soest et al. [38], using sodium sulphite, but not α-amylase, in the neutral detergent solution.

With all the information obtained, it is possible to evaluate if decrease in methane is due to a specific effect on methane production mechanisms or could be attributed to non-specific inhibitory effects of the plant on ruminal fermentation (i.e. if methane is reduced just because ruminal fermentation has been depressed). Methane, gas and short chain fatty acid production can be expressed not only per g of DM incubated, but also per g of DM digested. The partitioning factor (mg DM digested/ mL gas), suggested by Blümmel et al. [3] as an indicator of fermentation efficiency, can also be calculated. The molar proportion profile of short chain fatty acid can be examined, in particular effects on acetate to propionate ratio and molar proportion of propionate. A decrease in methane is usually accompanied by an increase in propionate. The molecular hydrogen generated in ruminal anaerobic fermentation is disposed of mainly through methane production, and it may be interesting to evaluate H balance in the fermentation system. According to Demeyer and Van Nevel [9], H recovery is:

mol H utilized/mol H produced, where:

$$\text{H utilized} = 2\text{P} + 2\text{B} + 4\,\text{M},$$

and

$$\text{H produced} = 2\text{A} + \text{P} + 4\text{B},$$

with A, B, M and P being mol of acetate, butyrate, methane and propionate produced, respectively.

Calculations

Total and Net Production of Gas and Methane

Total gas production is the volume of gas measured from pressure recordings in the bottle headspace as described above. Net gas production is calculated by subtracting average value of the blanks from that of each test bottle.

The volume of methane (M, mL) produced at the end of the incubation can be calculated from the volume of gas and the gas composition data as:

$$M = (G + V_h)\ \ C,$$

where:

G is the volume (mL) of total gas produced at the end of the incubation,
V_h is the volume (mL) of the headspace in the serum bottle,

C is the percentage or proportion (mL methane/mL gas) of methane in the analysed sample.

It is assumed that the gas is homogeneously mixed, and thus the composition of this gas remaining in the headspace at end of the incubation is the same as that of the gas withdrawn from the bottle. Net methane production is calculated by subtracting average value of the blanks from that of each test bottle.

The approximate amount of gas or methane produced (in mmol) can be calculated applying the ideal gas law:

$$PV = nR\theta,$$

$$n = \frac{PV}{R\theta},$$

where:

n is the amount of gas or methane (in mmol),
P is the atmospheric pressure (in atm),
V is the volume of gas or methane (in mL),
R is the gas constant (0.082 L $\times$ atm/[mol $\times$ K]),
θ is the absolute temperature (K).

If $P = 1$ atm and $\theta = 273$ K, then one mol of any gas occupies 22.415 L of volume.

Expression of Results and Statistical Evaluation

The analysis of data involves the calculation of a number of parameters, starting with mean (μ) and standard deviation (σ) of values of methane production recorded in test (with a particular plant additive added) and control (with substrate but no plant additive) bottles. When several incubation runs are required to complete a screening programme (the number of plant additives to be tested is so large that it is not possible to test all of them in a single batch), each plant will have to be compared with the corresponding control of the same incubation run. From this, it is possible to assess the effects of a particular plant additive (compared with the control) using different approaches [26].

Absolute effect (also called specific signal or signal window) is just the difference (D) between test and control values:

$$\mathrm{D} = \mu_{\mathrm{test}} - \mu_{\mathrm{control}}$$

This can also be calculated for each individual replicate (i), and then the average effect calculated.

Relative (R) effect (also called signal to control) provides an indication of the comparative separation between test and control means:

$$R = \mu_{test}/\mu_{control}$$

If R equals unity, test value is identical to control, lower values indicate a decreasing production in the test treatment and values higher than one indicate that methane has been increased in response to the plant additive.

The effect of each plant additive (difference to the control) can also be expressed as the relative (percentage) increase (positive) or decrease (negative) in relation to the control average value of that incubation batch:

$$R' = (\mu_{test} - \mu_{control})/\mu_{control}$$

Expressed in percentage, this parameter provides the percentage of change (increase or decrease) observed in the test bottles compared with the control values.

Statistical evaluation of the differences between mean values of the test and control sets is compromised by the small number of replicates. Thus, the decision of whether a plant has caused a significant decrease in methane production may be a matter of personal judgment on the grounds of previous expertise. For instance, based on the experience acquired in our laboratory, we could say that a plant causing a relative decrease (R′) of 15% or higher can be considered a promising plant additive deserving further investigation.

Pharmaceutical researchers use statistics to assess the differences between plant tested and control in screening of compounds to be used as new drugs [26]. One of them is called "signal to noise" (S/N) ratio, calculated as:

$$S/N = (\mu_{test} - \mu_{control})/\sigma_{control}$$

A more powerful statistics, as a relative indication of the separation between signal (plant tested) and control, and a useful way of assessing the statistical performance of an assay is Z′ factor, calculated as:

$$Z' = 1 - \frac{3\,(\sigma_{test} + \sigma_{control})}{|\mu_{test} - \mu_{control}|}$$

Z′ factor is a dimensionless parameter, with a maximum value of one for an infinite difference, whereas both values overlap when $Z' \leq 0$. Nevertheless, the acceptance criterion (value of Z′ indicating a sample can be considered a positive hit) has to be established based on previous experience.

Some statistical approaches can be applied, from a more liberal to a more conservative perspective. A Student *t*-test can be used to compare means of test and control sets using all individual values, but it has to be taken into account that small differences may reach statistical significance using this approach. Finally, one-way analysis of variance (ANOVA) can be performed with all data recorded, with plant additive as the only source of variation. Contrast analysis allows us to test the

statistical significance of predicted specific differences in particular parts of our complex design (comparison between a specific plant and the control). Every plant additive included in the analysis can be compared with the control using the Dunnett test (designed for multiple comparisons with control). Tests for multiple comparisons of means can be used, although given the small replication it is recommended to choose the most conservative tests (Bonferroni, Scheffe's), with which a wider difference between the means is required to declare statistical significance. It is important to bear in mind that the primary goal of this sort of screening is to distinguish accurately the "hits" from the "nonhits" in a vast collection of samples, and thus only plant additives showing a remarkable effect in the screening assays are expected to produce a substantial effect of practical significance in vivo.

Example

Table 10.1 shows data from an assay in which three plant additives were screened, each in triplicate. Results are mmol methane produced (net production after subtracting blank) after 24 h of incubation per g of digested dry matter.

From the results shown in Table 10.1, it can be concluded that plants 1 and 3 cause a substantial decrease in methane production (of 15.7 and 36.1% relative to control, respectively), whereas with plant 2 a very slight increase (of 2.4 %) compared with control was observed. Parameter S/N is an indication of the magnitude of the difference between plant and control means relative to the variability among control observations. Z' parameter is even more cautious, and in this case it can be interpreted that plant 1 and 2 are not positive hits in the screening (negative values indicate that test and control values are overlapping), whereas plant 3 is slightly different from control.

Table 10.2 shows the results of a pair-wise (each plant vs. control) Student *t*-test. Differences between plant 1 or plant 3 and control were significantly different ($P < 0.05$), whereas plant 2 had no effect on methane production.

Table 10.1 Methane produced (mmol/g digested dry matter) in control and test sets of batch cultures and derived statistics (see text for details)

	Control	Plant 1	Plant 2	Plant 3
Bottle 1	1.92	1.70	2.30	1.41
Bottle 2	2.06	1.65	1.90	1.19
Bottle 3	2.15	1.82	2.08	1.32
Mean	2.04	1.72	2.09	1.31
Standard deviation	0.116	0.087	0.200	0.111
Standard error	0.067	0.050	0.116	0.064
Coefficient of variation	5.7	5.1	9.6	8.5
D		–0.32	0.05	–0.74
R		0.843	1.024	0.639
$R'(\%)$		–15.7	2.4	–36.1
S/N		–2.76	0.43	–6.36
Z'		–0.91	–17.97	0.08

Table 10.2 Comparison between control and test sets using pair-wise Student *t*-test

	Control	Plant 1	Plant 2	Plant 3
Mean	2.04	1.72	2.09	1.31
Student *t*-tests				
s.e.d.		0.084	0.134	0.092
$P =$		0.019	0.727	0.002

Table 10.3 Comparison between control and test sets from ANOVA

Test for multiple comparison of means	Minimum significant difference
Dunnett test	0.319
Bonferroni test	0.385
Scheffe's test	0.386

Results of an ANOVA performed are shown in Table 10.3. This analysis shows that effects of plant additives are highly significant ($P < 0.001$). The multiple comparison of means (s.e.d. = 0.111) shows that Bonferroni and Scheffe's tests are more rigorous to declare a difference statistically significant (a larger minimum significant difference). Using the Dunnett test, both plants 1 and 3 would be significantly different from control, whereas with Bonferroni and Scheffe's tests only plant 3 would have a significant effect on methane production.

In Vitro Continuous Fermentation System (RUSITEC)

The RUSITEC (Rumen Simulation Technique) system is a continuous fermentation system designed by Czerkawski and Breckenridge [7], ideal to screen and in depth evaluate up to 8 different plants or plant extracts for their anti-methanogenic potential in the rumen. Many such investigations have been carried out in various laboratories using the RUSITEC system [1, 11–13, 17, 18, 33–35, 39].

Preparation of Nylon Bags and Experimental Feed

In the RUSITEC, feed is supplied and incubated inside porous polyester (nylon or Dacron) bags. It is advisable to prepare new bags for each experiment as besides contamination, nylon fabric might get stretched due to washing and squeezing. This could possibly change pore size of the fabric. Nylon fabric (e.g. from Nitex 03-100/32; Sefar AG, Heiden, Switzerland) of 100-μm pore size is recommended. The nylon cloth is cut in the dimensions of 145 × 150 mm. The longer side of the fabric should be folded once and the borders on the two sides should be sewed together using polyester thread in a way to get a bag size of 70 × 140 mm with an outside border of about 5 mm. Bags can also be made by heat-sealing. Bags should be washed with cold tap water before being used in the fermenter. If bags have been

already used previously, it is necessary to wash them thoroughly to avoid contaminations with feed residues. Cable clips can be used for closing the open end of the bags after inclusion of the feed.

Experimental feed has to be chopped to simulate the chewing activity of the ruminant preceding the arrival of the feed in the rumen. A sieve size of 5 mm for dry roughage seems to be ideal for incubation in the nylon bags. For the concentrate a smaller sieve (3 mm) should be used to ensure that all the grains are broken up. Substantial moisture-containing feeds, such as silage and green forage, should be frozen and chopped to particle sizes of 5–10 mm in a food mixer equipped with cutting blades. An amount of 15 g dry matter of experimental feed was observed to be the most suitable daily portion when using dry feed. When using bulky high moisture feeds, such as silage and green forage, limitation might be imposed by their high packing density, and the daily amount of feedstuff to be incubated has to be determined by filling and weighing the full nylon bags (see Note 2).

Before the experiment starts, all experimental feed portions for daily administration into the fermentation system should have been weighed into weighing bowls using analytical balance, and then nylon bags for each experimental day can be filled up (with the help of a funnel and a brush to avoid any feed losses while transferring) and stored until incubation. This is necessary because the dry matter content of the feed might change during the experiment and therefore the amount of feed added to the fermenter can change when preparing the feed portions during the experiment. Feeds such as silage and other high-moisture feedstuffs should be kept frozen until the day of their incubation. Addition of frozen feeds to the fermenter should be avoided. Frozen feeds should be taken out from the freezer for thawing approximately 4 h before transferring them to the fermenters.

Notes:

1. *Regarding feedbag cloth pore size, it has to be considered that the size of the ruminal microbes varies between 1 and 13 μm length × 0.5–1 μm diameter to 70–120 μm length × 35–70 μm diameter for bacteria and protozoa, respectively. This means that a pore size of 100 μm is too small to allow the largest ciliate protozoa having access to the feed while all other microbes can enter and, at the same time, losses of feed particles are still tolerably low. This pore size was recommended by Carro et al.* [5].
2. *When using silage or high moisture feeds, it might be necessary to test in advance the appropriate amount which needs to be filled into the nylon bags; since stuffing in the nylon bag with feed might lead to reduced nutrient degradability due to limited access of the microbes to the diet, while a feed amount too low may result in an insufficient level of fermentation.*

Preparation of Plant Additive

The plants or plant extracts are often available as powders, extracts or oils. Plant powder can be added to and mixed with the daily feed portion and thus added to

the fermenter in the nylon bags. With this approach, however, dry matter disappearance (degradability) of experimental feed cannot be measured separately, as some residual matter in the bag will correspond to the candidate plant material. Thus, at the University of León, an alternative approach is used. Feed substrate is weighed in nylon bags and, in addition, the candidate plant (as a powder) is weighed in small synthetic cloth bags, such as F57 Ankom filter bags (5 × 5 cm, porosity 25 μm), although other filter bags can be used (with porosity up to 50 μm).

The dose of plant additive to be used in the test fermenters has to be decided considering a number of factors such as physical form of the additive (powder, extract, oil), type and concentration of active compound (if known) in the plant or extract, amount of feed incubated (dose may be calculated per g of substrate incubated), volume of the fermentation unit (dose may be calculated to reach a given concentration in the fermenter) and dilution rate in the fermenter.

The plant products in a liquid form (e.g., plant extracts) – mostly to be administered in small amounts – can be added as a pulse dose directly to the incubation fluid (rumen fluid/buffer solution mixture) during the process of the daily feed administration to avoid any losses. Oils and essential oils can be previously dispensed over the feed substrate or adsorbed onto inert carrier substances such as Isolute (Isolute HM-N; International Sorbent Technology Ltd., Hengoed Mid Glam, UK) to avoid the oil from being flushed out of the nylon bag. Once on the carrier, the substance can be added to the daily feed portion like the powders. Another mode of application of plant extracts could be via the buffer solution. The plant extracts are added to and dissolved into the buffer solution and infused into the fermenter at a continuous rate throughout the day. Using this approach, dilution of the extract as a result of incubation fluid turnover can be avoided. It has also to be considered that this approach simulates a continuous application of the additive, in contrast with a single application when the additive is added to the basal feed, and extrapolations to feed supplementation might be restricted.

Preparation of Incubation Medium

Reagents

Buffer Solution

The buffer solution commonly used in the RUSITEC is a so-called artificial saliva solution (simulating the ruminant's saliva) proposed by McDougall [29].

The final buffer solution is composed of a bicarbonate-phosphate buffer solution (Solution A) and a micromineral solution (Solution B).

- Solution A: Dissolve 49 g sodium bicarbonate ($NaHCO_3$) and 23.38 g disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) in 4950 mL distilled water.
- Solution B: Dissolve 47 g sodium chloride (NaCl), 57 g potassium chloride (KCl), 5.3 g calcium chloride dihydrate ($CaCl_2.2H_2O$), and 12.8 g magnesium

chloride hexahydrate ($MgCl_2.6H_2O$) in approximately 500 mL distilled water and then make up the volume to 1 litre with distilled water.

To 4950 mL of solution A add 50 mL of solution B drop-wise with the help of a titration pipette, otherwise the minerals in the solution might precipitate. While solution A should be prepared daily, solution B can be stored in a cool place for up to one month.

Prepare fresh buffer solution each day (daily requirement is 500–550 mL per fermentation unit).

Collection of Rumen Fluid and Solid Inoculum

Rumen fluid is collected from one or more rumen-fistulated animals. When two or more animals are used, rumen fluid should be pooled upon collection. The donor animal(s) should be fed a mixed diet consisting of forage and concentrate to ensure the presence of fibrolytic, amylolytic, and proteolytic bacteria. Rumen fluid should be kept anaerobic, out of light and at the animal's body temperature during its transportation to the laboratory. Therefore glass flasks, pre-warmed in water bath to approximately 39°C, are filled up to the brim with rumen fluid, sealed with a lid, and transported in an isolated box heated with water. An alternative can be the transport in thermo glass flasks or similar systems keeping rumen fluid at animal's body temperature and in an anaerobic atmosphere. Approximately one litre of filtered rumen fluid is needed to set up each fermentation unit of the RUSITEC system.

Solid rumen inoculum (i.e. particulate rumen contents) is taken from the rumen using a cable grabber (needed only in case of small fistulae, while with large fistulae it can be taken by hand), put in a pre-warmed glass flask (39°C) and filled up to the brim with rumen fluid to keep it anaerobic. Solid rumen inoculum helps to rapidly establish stable conditions in the fermenter system as it is a good source of microbes, which are firmly attached to the (fibrous) particles.

Notes:

1. *RUSITEC fermenters require a large amount of rumen fluid and therefore the ideal donor is cattle. Investigations using rumen fluid from small ruminants are only possible when several fistulated animals are available or when the animals are slaughtered.*
2. *In contrast to the rumen fluid used in the syringe-based short-term incubation in the gas method; rumen fluid for RUSITEC studies can be collected after morning feeding.*

Description of the RUSITEC Fermentation System

The original RUSITEC was designed and described in detail by Czerkawski and Breckenridge [7] and was modified in the meantime [27]. The system, comprising

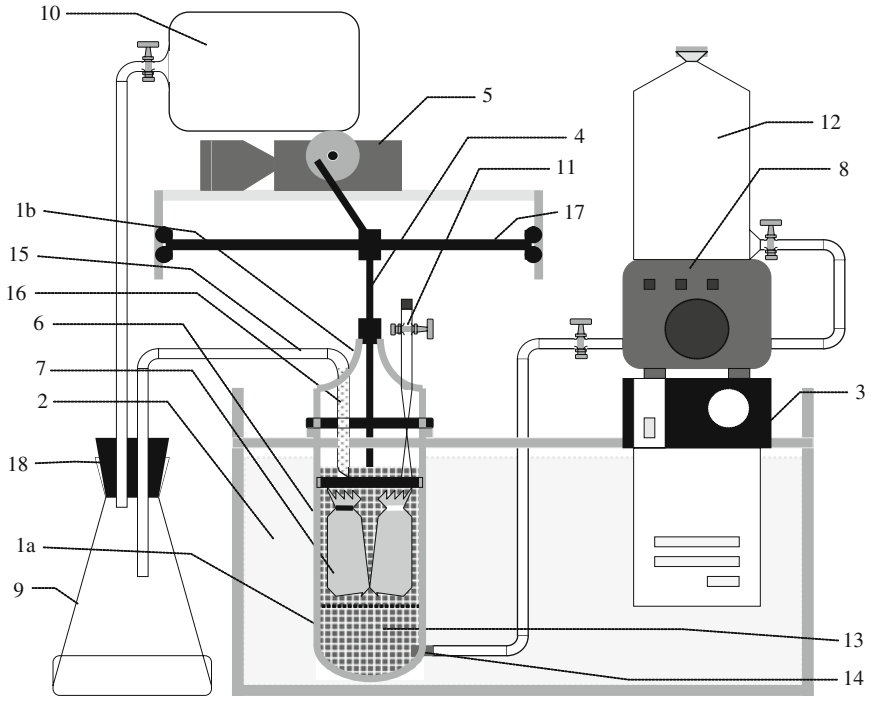

Fig. 10.2 Schematic representation of the Rumen Simulation Technique (RUSITEC)

the fermentation unit (or reaction vessel) as the main part and other required attached elements, is shown in Fig. 10.2.

The RUSITEC fermentation system consists of the following elements:

i. A container (12) for artificial saliva (5 L capacity). The artificial saliva is continuously infused into each fermentation unit (1a) using a peristaltic pump (8) for precise supply of buffer solution. Connection tubing between container and the pump and from the pump to the fermentation unit (14) are required.
ii. Fermentation unit consists of a gas-tight 1 L fermenter (or reaction vessel) (1a) closed with a fermenter top (1b) or a screw cover. This fermenter is usually made of glass, translucent plastic or resinous material (such as Perspex or methacrylate) and is placed in a water bath (2) maintaining a constant temperature of 39°C with the help of a heating system (3). Thus, incubation fluid is at an incubation temperature of 39°C. The fermenter has an inlet at the bottom of the cylinder (14) for the infusion of saliva. Artificial saliva is stored in a container (12) and continuously pumped with into the fermenter with the help of a peristaltic pump (8). A perforated feed container (6) with a screw cap and made of glass or plastic material is put inside the fermenter. Feed is incubated in nylon

bags (7) that are placed into the container. The fermenter is filled with incubation fluid (13) composed of a mixture of rumen fluid and artificial saliva in which the feed container is submerged.
The fermenter top has two outlets, one connected to a 3-way valve (11) for sampling incubation fluid, and the other (16) connected to the outflow tube (15). Out flowing incubation fluid and gas generated during fermentation leave the fermenter through this overflow outlet (16).

iii. A motor (5) is connected to an eccentric (17), and this in turn to a stainless-steel rod (4) passing through the fermenter top and linked to the feed container. The motor drives the entire device to transmit motion with the final result that feed containers are moved up and down inside the fermenters, simulating ruminal movements and causing a continuous mixture of the incubation fluid (13).

iv. Incubation fluid is flowing out of the fermenter through the overflow outlet to a flask (9) in which all the daily effluents from a fermenter are collected. Fermentative activity in the effluent collection flasks has to be stopped immediately. In the system used in ETH-Zurich, the flasks are placed in an aluminium block and cooled immediately to –20°C (not shown in the schematic illustration). Another possibility is to add acids to the flasks that act inhibiting the ruminal microbes. The effluent collection flask is tightly sealed (18). Gas generated in the fermenter also streams into the collection flask and from there through tubing to a gas-tight bag (10) of 8-L capacity (tecobag, PETP/AL/PE: 12/12/75 quality; Tesseraux Container GmbH, Bürstadt, Germany). All tubing used in the system has to be impermeable to gas to avoid any gas leakage.

Setting Up of the Fermentation Units

Before starting the incubation, the water bath of the RUSITEC system (into which the fermentation units are placed into) is to be heated to 39.5°C. McDougall buffer solution (artificial saliva) is dispensed (100 mL) into each fermenter. Fermentation units used in each RUSITEC system are set up subsequently, one at a time, to ensure that ruminal microbes are never exposed to oxygen or low temperatures for too long. Prior to inoculating each fermentation unit, rumen fluid collected from the animals is strained through 4 layers of medicinal gauze (1000 μm pore size, Type 17; MedPro Novamed AG, Flawil, Switzerland) or one layer of cheesecloth to get rid of feed particles. Afterwards, strained rumen fluid is transferred into each fermenter (900 mL rumen fluid is needed for a 1 L fermenter to get a buffer solution to rumen fluid ratio of about 1:9 when incubation is started). On the first day of the incubation 2 nylon bags, one containing the experimental feed and the other containing about 50 g fresh solid rumen inoculum, are inserted separately into the perforated feed container, which then is placed into the fermenter. Then the fermenter is immediately sealed with the fermenter top. Then the gas phase of the fermentation unit (i.e., the headspace of the fermenter top, overflow tubing and effluent collection

flask) is flushed with gaseous nitrogen to purge it from air (in particular oxygen) and establish anaerobic conditions. Therefore, a tube connected to a cylinder containing gaseous nitrogen is plugged to the tube connecting the tecobag with the effluent collection flasks. Gaseous nitrogen flushes through the incubation unit for 3 min at a gas flow rate of 3 L/min. The 3-way valve, situated at the fermenter top, is used as the gas exit port during flushing and has to be opened to maintain gas circulation with a constant flow. After this flushing, the 3-way valve is closed, and the gas tecobag is attached to the tube connected to the effluent collection flask, so all the system is hermetically sealed. The motor unit is switched on, so feed containers are continuously moved up and down inside the fermenters at 8 cycles per minute, simulating ruminal movement. McDougall buffer solution is infused continuously into the fermenter through a tube connecting the saliva container to the fermentation unit via a peristaltic pump. When using a 1 L fermenter the average buffer solution flow rate is about 500 mL/day (fractional dilution rate of 0.020–0.025/h), which is controlled with a precision peristaltic pump (e.g., ISM444; Ismatec SA, Glattbrugg-Zurich, Switzerland) and small tubing (e.g., Tygon LFL, 0.48 mm ID; Ismatec SA, Glattbrugg-Zurich, Switzerland). With buffer solution continuously flowing into the fermenters, the liquid surplus overflows through the outlet situated on the fermenter top and is transferred to the effluent collection flask. In the system set up in ETH-Zurich, all the collection flaks are placed in freezing units, insulated containers (aluminium frame wrapped with expanded polyethylene) whose contents (a coolant solution with a freezing point of –40°C) are chilled to –20°C using a freezing machine (e.g., Werner Kuster AG, Zurich, Switzerland). Collection flaks are immersed into the coolant, so effluents are immediately cooled down to stop all fermentation processes in this effluent fluid. Along with the effluent fluid, the fermentation gases released are evacuated from the incubation unit through the overflow outlet. The fermentation gas is collected in the gas-tight tecobags. If necessary, the 3-way valve can be used to take samples of the incubation fluid for various analyses.

Notes:

1. *The pH of the fermentation medium can be varied by changing the buffer type (salt concentrations) and buffer flow rate through the peristaltic pump. Some microorganisms (in particular protozoa) may be washed out from the fermentation unit with higher dilution rates. Using a higher buffering capacity buffer allows decreasing the buffer flow rate while keeping the pH of the incubation fluid constant, thus reducing the washing out of rumen protozoa.*
2. *Instead of using gaseous nitrogen to flush the incubation fermenter system, carbon dioxide can also be used depending on whether or not fermentative carbon dioxide needs to be quantified.*
3. *It is important to always use tubing with a low gas diffusion rate for carbon dioxide, to avoid losses of carbon dioxide from the fermentation system.*

Daily Feed Supply to the Fermenter

The feed supply to the fermenter system takes place at the same time every day for each of the fermenters (i.e. maintain the same filling routine). Before opening the fermenter top, the incubation unit should be flushed with gaseous nitrogen to make sure all the fermentation gases are quantitatively collected in the fermentation gas collection tecobags. For this, a nitrogen gas cylinder is connected to the fermenter top (to the three way valve that is also used for daily collection of incubation fluid to be analysed) and nitrogen (3 L/min) is flushed through the system for 30 s. After 30 s of flushing, the tecobag are closed, disconnected from the system and replaced with an empty tecobag. The total amount of gas collected in the tecobags therefore consists of the fermentation gases diluted by gaseous nitrogen from the flushing of the system. Thereafter the fermenter top can be opened and the perforated feed container with the 2 nylon bags carrying the respective feed treatment can be removed from the fermenter. From the 2 feedbags only the one already incubated for 48 h should be replaced by a nylon bag containing fresh feed. To keep the loss of incubation fluid and of ruminal microbes as low as possible, the nylon bag already incubated for 48 h should be squeezed over the open fermenter while being rinsed with 50 mL of pre-warmed (39°C) buffer solution. The nylon bags removed from the fermenters are then washed in a commercially available washing machine (using cold water, without spin cycle) to remove the microbes still attached to the non-digested feed. The bags are frozen and stored at –20°C. This removal of the microbial nitrogen is important in order to avoid underestimation of protein (and organic matter) degradation of the feed. After exchanging the feedbags, the feed containers are re-inserted into the fermenters and fermenter tops are closed. The liquid outflow flask, whose content has to be determined after thawing for its liquid volume, is replaced with an empty flask. Finally, the closed incubation system should be flushed with gaseous nitrogen again for 3 min (3 L/min) to re-establish anaerobic conditions.

Notes:

1. *It is important that during supply of the feed, the fermenter is opened only for a short time to limit the exposure of the microbes to oxygen. On average, time for feed supply should not exceed 4 min (time when the incubation fluid is exposed to air) per fermenter. If the fermenter has to be kept open for a longer time, the incubation fluid surface should be flushed with gaseous nitrogen or carbon dioxide as long as the fermenter is open.*
2. *After the first 5 days of incubation (adaptation time for the ruminal microbes to the experimental feed and RUSITEC fermentation conditions), steady-state conditions are reached. Under favourable conditions, the microbial ecosystem in the fermentation units can be kept stable for another 15 days (20 days of incubation in total). Generally a total incubation period of 10 days, with a 5-day measurement period after an adaptation period of 5 days) is adequate for testing the plant or plant extracts.*

Measurements and Laboratory Analyses

Gas and Methane Production and Composition

The fermentation gases produced during the 24 h of incubation are quantitatively collected in the tecobags by flushing the system with gaseous nitrogen, meaning that the fermentation gas collected in the tecobags is diluted with nitrogen. The total volume of gas in the tecobags is quantified by water displacement using a water-filled 5 litre-flask connected to the tecobag. The amount of water replaced in the flask by the gas is measured with a graduated cylinder or with a second flask placed on a balance. Although the solubility of carbon dioxide in water is very high (0.76 mL/mL water at 25°C) a loss of carbon dioxide due to its solubility and, consequently, the underestimation of fermentation gas volume is not to be expected when following the recommended procedure. In this procedure, the flask water is used during the whole experimental period and therefore it gets saturated with carbon dioxide after the first 3–4 days of each experimental run. The data from these 4 days are not considered in the statistical evaluation anyway since the microbes in the fermentation unit need 4–5 days of adaptation to the dietary treatment. Optionally the water in the flask can also be saturated in advance by flushing with carbon dioxide.

An entirely different approach would be measuring the gas volume with a gas flow meter. At the University of León (Spain) a RITTER® wet-test (drum-type) gas flow meter (Ritter Apparatebau GmbH & Co. KG Bochum, Germany) is used to measure and record gas volume and gas flow rate. This wet-test gas meter functions upon the principle of positive displacement. The sample gas stream rotates a measuring drum within a packing fluid (usually water or low viscous oil), and a needle-dial and counting mechanism, coupled to the rotating drum, records the volume of gas flow.

A sample of gas is taken directly from the tecobag and analyzed for the concentrations of methane, hydrogen and carbon dioxide, e.g. by a Hewlett Packard gas chromatograph (model 5890 Series II; Hewlett Packard, Avondale PA, USA) equipped with a thermal conductivity detector, a flame ionization detector, and a Carboxen-1000 column (mesh size 60/80; Fluka Chemie AG, Buchs, Switzerland).

Other Measurements

Daily, incubation fluid is analyzed for pH and redox potential (to control the anaerobic conditions), with the respective electrodes connected to a potentiometer (e.g., model 713; Metrohm, Herisau, Switzerland). Ammonia can be measured by the ammonia electrode procedure. These measurements take place at the same time before feed supply to the system. At this time, there is minimum fluctuation in the fermentative parameters, as no fresh feed has been supplied to the system for several hours. Therefore, incubation fluid should be withdrawn from the incubation unit without opening the fermenter top. This can be done through the 3-way valve situated at the fermenter top and a syringe attached with a tubing of small diameter (e.g., medical catheters were shown to be ideal) but long

enough to reach the incubation fluid surface. Subsequently, ciliate protozoa (entodiniomorphs and holotrichs) and bacteria are counted, for instance by using Bürker counting chambers (0.1 and 0.02 mm depth, respectively; Blau Brand®, Wertheim, Germany). To immobilize ruminal microbes and to dilute the incubation fluid to establish better counting conditions, Hayem solution is used (also called Mercury solution; prepared dissolving 2.5 g mercury chloride ($HgCl_2$), 25 g disodium sulphate (Na_2SO_4), and 5 g sodium chloride (NaCl) in approximately 500 mL distilled water and then making up the volume to 1 L with distilled water. This solution can be stored at room temperature for 4–6 months). The incubation fluid is diluted in a ratio of 1:20 (0.1 mL incubation fluid + 1.9 mL Hayem solution) and 9:10 (0.9 mL incubation fluid + 0.1 mL Hayem solution) for bacterial and protozoal counts, respectively. If specific rumen microbial species need to be detected or quantified by using molecular techniques such as fluorescence in situ hybridisation or real time PCR, incubation fluid samples should be collected into cryovials and frozen in liquid nitrogen. These samples should be stored at –80°C until analyses. Samples for determinations of short chain fatty acid (acetate, propionate, butyrate, valerate, and the branched-chain fatty acids iso-valerate and iso-butyrate) are prepared, e.g., according to the method of Doane et al. [10] and determined by HPLC (e.g. a LaChrom, L-7000 series; Hitachi Ltd., Tokyo, Japan). As short chain fatty acid analysis is carried out after the completion of fermentation in the RUSITEC system, daily 3 mL of incubation fluid is taken from each of the fermenters, centrifuged at 4000*g* for 5 min, and 2 mL of the liquid supernatant frozen at –20°C. After thawing, 40-µl acid solution (50 mM H_2SO_4 and 100 mM quinic acid) is added to 360 µl of incubation fluid. The samples are allowed to rest for 10 min at room temperature, centrifuged at 4000*g* for 5 min and the supernatant filled in HPLC vials. The HPLC is equipped with a guard-column (Cation-H Refill Cartridge, 4.6 mm ID × 30 mm; Bio-Rad Laboratories, Hercules, CA, USA) to protect the main column against aggressive solvents or irreversibly bound material. The main column (Aminex® HPX-87H, 7.8 mm ID × 300 mm; Bio-Rad Laboratories, Hercules, CA, US) is operated at 30°C with 5 mM H_2SO_4 at 1.0 mL/min as the mobile phase. The sample injection volume is 100 µl. The short chain fatty acid can also be determined by gas chromatography (Section "Fermentation pattern").

Feed degradability is estimated from substrate disappearance from the nylon bags. After 48 h of incubation, the nylon bags withdrawn from the fermentation units are washed in a commercially available washing machine (using cold water, without spin cycle) to remove the microbes still attached to the non-digested incubation residue. Removal of microbial matter from incubation residue is important to prevent underestimation of feed degradability. The bags are frozen, stored at –20°C and freeze dried. Dry residue represents the residual (undigested) dry matter.

Samples of experimental feed and of incubation residues are milled to pass a 0.5 mm sieve and analyzed in triplicate for dry matter (DM), total ash, ether extract (EE), and nitrogen, following standard procedures [2]. The DM content of feed and

fermentation residues is determined by drying to a constant weight at 105°C, and ash content by heating to 550°C for a period of at least 8 h. Organic matter (OM) is calculated by subtracting total ash from DM content. Crude protein (CP) is calculated as 6.25 × N.

The fibre analyses (neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL)) are performed in triplicate using either the Fibertec technology (Fibertec System M, Tecator; 1020 Hot Extraction, Flawil, Switzerland) or the Ankom system (ANKOM 200/220 Fibre Analyzer, Ankom Technology Corporation, Macedon, NY, USA). Contents of NDF should be analyzed with heat-stable α-amylase as suggested by Van Soest et al. [38] but without sodium sulphite and corrected for ash content (aNDFom [37]). When evaluating the NDF content of diets supplemented with tannin-rich plant extracts the addition of sodium sulphite might be necessary [21], although it might be difficult to get reliable values, and negative degradation values when comparing the NDF content of feed and residue might occur [18, 28]. Ash-free ADF (ADFom [37]) and ADL are determined successively as outlined in method no. 973.18 (procedures C and D) of [2]. For the ADL analysis, the samples are soaked in 12 M sulphuric acid for 3 h and thoroughly washed with boiling distilled water. Ash contents of NDF and ADL are determined by incinerating residues at 550°C in a muffle furnace for 3 h. Detailed descriptions on fibre and lignin analyses can be found in Hindrichsen et al. [19].

Calculations

The total volume of methane production can be calculated from its concentration in the total amount of gas collected in the tecobags:

Example:

Total gas measured in the tecobag = 4500 mL
Methane proportion in the gas as determined by gas chromatography = 8%
Methane production after 24 h of incubation = 4500 × 8/100 = 360 mL

It is assumed that all methane present in the gas phase of each fermentation unit is displaced to the collection tecobags upon flushing with N_2.

Feed degradability is calculated from disappearance from the feedbag after 48 h of incubation.

Example:

NDF incubated = 7.1 g
Residual NDF recovered in the feedbag after 48 h = 4.7 g
NDF disappearance = 7.1 – 4.7 = 2.36 g
DM degradability = 2.36/7.1 = 0.33

Knowing the composition of feed incubated and of incubation residue, the degradability of any feed component can be calculated (DM, OM, CP, and ADF).

Total NDF digested in 24 h of fermentation would be the NDF fermented from the first 24 h in one of the bags plus the NDF fermented in the other feedbag from 24 to 48 h of incubation. Therefore, total NDF digested can be assumed to be equal to the NDF disappearance from one feedbag after 48 h of incubation. This can be used to calculate methane production per g of NDF digested.

Example:

Total NDF digested in 24 h of fermentation = 2.36 g
Methane production after 24 h of incubation = 360 mL
Methane production after 24 h of incubation = 152.5 mL/g NDF digested

This can be important to elucidate if any decrease in methane production is due to a specific effect of the plant additive on ruminal microorganisms or to a non-specific effect depressing fibre fermentation.

Total short chain fatty acid production after 24 h of incubation can be calculated from the total volume of effluent collected and the concentration of short chain fatty acid measured in an effluent sample.

Example:

Total volume of effluent collected = 500 mL
Short chain fatty acid concentration in the effluent = 100 mmol/L
Total short chain fatty acid production after 24 h of incubation = 0.5 × 100 = 50 mmol short chain fatty acid

The same calculation is applicable to any acid (acetate, propionate, butyrate, etc.).

Hydrogen balance can be calculated using the equation propose by Demeyer [8] considering the daily production of acetate (A), propionate (P), butyrate (B), valerate (V) and iso-valerate (iV), and methane (M) as:

Hydrogen recovery (%) $= 2H_u/2H_p \times 100$,
$2H_u = 2P + 2B + 4M + V$; H_u representing hydrogen utilized
$2H_p = 2A + P + 4B + 2iV + 2V$; H_p representing hydrogen produced.

To assess effects of a plant additive on methane production, values recorded in the test fermentation units are compared with control values using statistical analyses. Main parameters to be compared are total methane production, and methane production per gram of NDF or OM digested. Other parameters (short chain fatty acid production, substrate degradability, H recovery) can be studied to investigate effects on fermentation pattern.

Statistical Analyses

For the statistical analyses, the average values e.g. of the last 5 days of each experimental run are used. Thereby the respective dietary treatments and experimental runs are included as effects in the statistical model. Data are subjected to the general linear model (GLM) procedure of the SAS program (version 8.2; SAS Institute, Inc., Cary, NC) while multiple comparisons among means are carried out e.g. by the Tukey method. This allows the identification of the plants or plant extracts with a significant anti-methanogenic potential.

To study some effects on any of the parameters examined, the MIXED procedure of the SAS program can be used with the random and repeated statements as recommended by Littell et al. [22]. In some cases, the calculation of Pearson correlation coefficients between fermentation traits or multiple regression analysis as carried out with the MAXR selection method within the REG procedure of SAS may be of interest.

References

1. Abel, H., I. Immig, and E. Harman. 2002. Effect of adding caprylic and capric acid to grass fermentation characteristics during ensiling and in the artificial rumen system RUSITEC. Anim. Feed Sci. Technol. **99**:65–72.
2. AOAC (Association of Official Analytical Chemists). 1990. Official Methods of Analysis (15th edition), Association of Official Analytical Chemists Inc., Arlington, VA, USA.
3. Blümmel, M., H. P. S. Makkar, and K. Becker. 1997. In vitro gas production: A technique revisited. J. Anim. Physiol. Anim. Nutr. **77**:24–34.
4. Borris, R. P. 1996. Natural products research: Perspectives from a major pharmaceutical company. J. Ethnopharmacol. **51**:29–38.
5. Carro, M. D., P. Lebzien, and K. Rohr. 1995. Effect of pore size of nylon bags and dilution rate on fermentation parameters in a semi-continuous artificial rumen. Small Ruminant Res. **15**:113–119.
6. Cowan, M. M. 1999. Plant products as antimicrobial agents. Clin. Microbiol. Rev. **12**:564–582.
7. Czerkawski, J. W., and G. Breckenridge. 1977. Design and development of a long-term rumen simulation technique (RUSITEC). Br. J. Nutr. **38**:371–384.
8. Demeyer, D.I. 1991. Quantitative aspects of microbial metabolism in the rumen and hindgut, pp. 217–237. In J. P. Jouany (ed.), Rumen Microbial Metabolism and Ruminant Digestion, INRA Editions, Paris, France.
9. Demeyer, D. I. and Van Nevel, C. J. 1975. Methanogenesis, an integrated part of carbohydrate fermentation, and its control, pp. 366–382. In I. W. McDonald, and A. C. I. Warner (eds.), Digestion and Metabolism in the Ruminant, The University of New England Publishing Unit, Armidale, NSW, Australia.
10. Doane, P. H., A. N. Pell, and P. Schofield. 1998. Ensiling effects on the ethanol fractions of forages using the gas production. J. Anim. Sci. **76**:888–895.
11. Dohme, F., A. Machmüller, A. Wasserfallen, and M. Kreuzer. 2000. Comparative efficiency of various fats rich in medium-chain fatty acids to suppress ruminal methanogenesis as measured with RUSITEC. Can. J. Anim. Sci. **80**:473–482.
12. Dong, Y., H. D. Bae, T. A. McAllister, G. W. Mathison, and K. J. Cheng. 1997. Lipid-induced depression of methane production and digestibility in the artificial rumen system (RUSITEC). Can. J. Anim. Sci. **77**:269–278.

13. García-González, R., S. López, M. Fernández, and J. S. González. 2006. Effects of the addition of some medicinal plants on methane production in a rumen simulating fermenter (RUSITEC). International Congress Series **1293**:172–175.
14. Goel, G., H. P. S. Makkar, and K. Becker. 2008. Effects of *Sesbania sesban* and *Carduus pycnocephalus* leaves and Fenugreek (*Trigonella foenum-graecum* L.) seeds and their extracts on partitioning of nutrients from roughage- and concentrate-based feeds to methane. Anim. Feed Sci. Technol. **147**:72–89.
15. Goering, M. K., and P. J. Van Soest. 1970. Forage Fiber Analysis (Apparatus, Reagents, Procedures and Some Applications). Agricultural Handbook No 379, Agricultural Research Service, USDA, Washington DC, USA.
16. Greathead, H. 2003. Plants and plant extracts for improving animal productivity. Proc. Nutr. Soc. **62**:279–290.
17. Hess, H. D., M. Kreuzer, T. E. Dìaz, C. E. Lascano, C. E. Carulla, C. R. Soliva, and A. Machmüller. 2003. Saponin rich tropical fruits affect fermentation and methanogenesis in faunated and defaunated rumen fluid. Anim. Feed Sci. Technol. **109**:79–94.
18. Hess, H. D., L. M. Monsalve, C. E. Lascano, C. E. Carulla, T. E. Díaz, and M. Kreuzer. 2003. Supplementation of a tropical grass diet with forage legumes and *Sapindus saponaria* fruits: effects on in vitro ruminal nitrogen turnover and methanogenesis. Austr. J. Agric. Res. **54**:703–713.
19. Hindrichsen, I. K., H.-R. Wettstein, A. Machmüller, K. E. Bach Knudsen, J. Madsen, and M. Kreuzer. 2006. Digestive and metabolic utilisation of dairy cows supplemented with concentrates characterised by different carbohydrates. Anim. Feed Sci. Technol. **126**:41–61.
20. Johnson, K. A., and D. E. Johnson. 1995. Methane emissions from cattle. J. Anim. Sci. **73**:2483–2492.
21. Krueger, C. G., K. A. Albrecht, J. D. Reed, E. J. Bures, and V. N. Owens. 1999. Sodium sulphite effects on recovery and composition of detergent fibre and lignin from forage legumes varying in levels of proanthocyanidins. J. Sci. Food Agric. **79**:1351–1356.
22. Littell, R. C., P. R. Henry, and C. B. Ammerman. 1998. Statistical analysis of repeated measures data using SAS procedures. J. Anim. Sci. **76**:1216–1231.
23. López, S. 2005. In vitro and in situ techniques for estimating digestibility, pp. 87–121. In J. Dijkstra, J. M. Forbes, and J. France (eds.), Quantitative Aspects of Ruminant Digestion and Metabolism (2nd edition), CAB International, Wallingford, UK.
24. López, S., and C. J. Newbold. 2007. Analysis of methane, pp. 1–13. In H. P. S. Makkar, and P. E. Vercoe (eds.), Measuring Methane Production from Ruminants, Kluwer Academic Publishers, Dordrecht, The Netherlands.
25. López, S., M. S. Dhanoa, J. Dijkstra, A. Bannink, E. Kebreab, and J. France. 2007. Some methodological and analytical considerations regarding application of the gas production technique. Anim. Feed Sci. Technol. **135**:139–156.
26. Macarrón, R., and R. P. Hertzberg. 2002. Design and implementation of high throughput screening assays, pp. 1–29. In W. P. Janzen (ed.), High Throughput Screening: Methods and Protocols (Methods in Molecular Biology, vol. 190), Humana Press, Totowa, NJ, USA.
27. Machmüller, A., C. R. Soliva, and M. Kreuzer. 2002. In vitro ruminal methane suppression by lauric acid as influenced by dietary calcium. Can. J. Anim. Sci. **82**:233–239.
28. Makkar, H. P. S., N. Borowy, K. Becker, and R. Degen. 1995. Some problems in fiber determination in tannin-rich forages. Anim. Feed Sci. Technol. **55**:67–76.
29. McDougall, E. I. 1948. Studies on ruminant saliva 1. The composition and output of sheep's saliva. Biochem. J. **43**:99–109.
30. Menke, K. H., and H. Steingaβ. 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim. Res. Develop. **28**:7–55.
31. Menke, K. H., L. Raab, A. Salewski, H. Steingaβ, D. Fritz, and W. Schneider. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor. J. Agric. Sci. **93**:217–222.

32. Nagaraja, T. G., C. J. Newbold, C. J. Van Nevel, and D. I. Demeyer. 1997. Manipulation of ruminal fermentation, pp. 523–632. In P. N. Hobson, and C. S. Stewart (eds.), The Rumen Microbial Ecosystem, Blackie Academic and Professional, London, UK.
33. Sliwinski, B. J., C. R. Soliva, A. Machmüller, and M. Kreuzer. 2002. Efficacy of plant extracts rich in secondary constituents to modify rumen fermentation. Anim. Feed Sci. Technol. **101**:101–114.
34. Soliva, C. R., M. Kreuzer, N. Foidl, G. Foidl, A. Machmüller, and H.D. Hess. 2005. Feeding value of whole and extracted *Moringa oleifera* leaves for ruminants and their effects on ruminal fermentation in vitro. Anim. Feed Sci. Technol. **118**:47–62.
35. Soliva, C. R., L. Meile, A. Cieslak, M. Kreuzer, and A. Machmüller. 2004. Rumen simulation technique study on the interactions of dietary lauric and myristic acid supplementation in suppressing ruminal methanogenesis. Br. J. Nutr. **92**:689–700.
36. Theodorou, M. K., B. A. Williams, M. S. Dhanoa, A. B. McAllan, and J. France. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Technol. **48**:185–197.
37. Udén, P., P. H. Robinson, and J. Wiseman. 2005. Use of detergent system terminology and criteria for submission of manuscripts on new, or revised, analytical methods as well as descriptive information on feed analysis and/or variability. Anim. Feed Sci. Technol. **118**:181–186.
38. Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods of dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. **74**:3583–3597.
39. Wang, Y., T. A. McAllister, C. J. Newbold, L. M. Rode, P. R. Cheeke, and K. J. Cheng. 1998. Effects of *Yucca schidigera* extract on fermentation and degradation of steroidal saponins in the rumen simulation technique (RUSITEC). Anim. Feed. Sci. Technol. **74**:143–153.
40. Wenk, C. 2003. Herbs and botanicals as feed additives in monogastric animals. Asian-Aust. J. Anim. Sci. **16**:282–289.

Chapter 11
Challenges in Extrapolating In Vitro Findings to In Vivo Evaluation of Plant Resources

Juan J. Villalba and Frederick D. Provenza

Introduction

Conflicting evidence exists regarding findings on the biological activity of natural products when extrapolated from the laboratory to the animal. The first approach to testing for the biological activity of a chemical – i.e., against parasites, or to favour rumen function – is to conduct screening procedures in vitro. This is because of the substantial advantages of this methodology such as low cost and rapid turnover, which allows for screening of a large number of compounds in a relatively short period of time. Nevertheless, the advantage of bioprospecting through in vitro testing comes with a cost: A positive in an in vitro test does not necessarily warrant biological activity when the chemical or product is subsequently tested in the animal. Using the same logic, it is also possible that lack of activity by a natural product in an in vitro test does not strictly mean lack of biological activity in vivo.

There are several reasons for the conflicting evidence found frequently between in vitro and in vivo testing in natural plant products or plant secondary metabolites. The main point to keep in mind is that there is a reason for laboratory bioprospecting tests to be called "in vitro:" There are several assumptions and deviations from processes typically occurring in the animal; from the physiology and kinetics of digestive processes, to the pharmacokinetics of natural plant products to the ingestive behaviour of herbivores. The present chapter is an attempt to explain how these assumptions and deviations from processes occurring in the animal influence and bias in vitro testing. Using this framework we attempt to understand discrepancies between in vitro and in vivo testing and to propose some potential solutions to the problem. We hope that this effort will aid in the development of more reliable techniques for testing the biological activity of plant resources.

J.J. Villalba (✉)
Department of Wildland Resources, Utah State University, Logan, UT 84322-5230, USA
e-mail: juan.villalba@usu.edu

P.E. Vercoe et al. (eds.), *In Vitro Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies*,
DOI 10.1007/978-90-481-3297-3_11,

Simple vs. Complex Environments: Interactions of Bioactives with Other Chemicals

Testing purified compounds allows their activity to be quantified reliably without the interference of other plant components or nutrients [1]. Nevertheless, such a controlled environment comes with a cost when attempting to extrapolate results to the whole animal: in vitro studies normally do not deal with the interference of the complex environment present in the realm of the gastrointestinal tract.

In vitro assays designed to test for antiparasitic activity typically involve the extraction and purification of the natural compound (e.g., tannins) and subsequently the preparation of test solutions with the compound and the parasite larvae (e.g., [31, 32]). Such solutions are devoid of the chemical and biological complexity of the gastrointestinal tract. Chemicals that normally occur in the rumen fluid, digesta and faeces such as nutrients and other plant secondary metabolites have the potential to interact with bioactives creating complexes that may either depress or enhance activity significantly.

Screening for natural plant products that influence rumen function, generally use rumen fluid in the testing medium, which offers a chemical and biological dimension closer to an in vivo situation, particularly when the diets fed to the donor animals are similar to those that will be tested in vivo [6, 7]. Likewise, parasite viability in response to antiparasitic bioactives has been tested in faecal samples [18], and thus biological and chemical complexity of the media is kept during in vitro testing.

The different effects of plant actives when tested in vitro using a simple chemical and biological environment, versus when tested in vivo – under a complex chemical and biological environment – is illustrated by the strong activity of condensed tannins against abomasal nematodes of sheep in vitro [2], but not in vivo [2]. Condensed tannins are polyphenolic compounds with high affinity for proteins. The majority of condensed tannins present in the abomasums of sheep fed tannin-rich diets have been found in complexes with proteins and consequently are unavailable for action against abomasal parasites [1].

Tannins not only complex with proteins; these chemicals are highly reactive compounds which form complexes with saponins [15], alkaloids [36], and terpenes [34]. Tannins, terpenes and alkaloids can co-occur in the digesta of animals consuming diverse diets and thus the biological activity of plant secondary metabolites has the potential to be reduced significantly after the formation of such complexes.

Incubation of Plant Bioactives with Rumen Fluid: Static vs. Dynamic Fermentation Systems

The low cost and high numbers of samples that can be screened per unit of time makes the in vitro systems – similar to those originally developed by Tilley and Terry [43] – the first choice for investigating the potential impact of natural products on rumen function. Nevertheless, the in vitro system is a surrogate for the "reference" method, which involves the in vivo measurement of food digestibility.

The problem, however, with the in vitro system is that it assumes that the conditions in the incubators remain similar to rumen content for 48 h (or more) in spite of the accumulation of end products. On the contrary, end products, and even substrates such as natural plant products typically cross the rumen wall or they flow with the digesta and thus processes in the rumen fluid do not remain static but are inherently dynamic. As an example, terpenes – an array of natural plant products found in many shrubs and trees- are small, fat-soluble molecules that are absorbed quickly through the rumen epithelium. The average terminal elimination of plasma terpene concentrations is in the order of minutes after terpenes are infused into the rumen [11]. Consequently, during in vitro testing the ruminal activity of terpenes could be overestimated because under static fermentation techniques terpenes remain in the medium during the whole incubation process, which increases the likelihood for interactions. In contrast, when animals ingest terpenes these compounds are rapidly absorbed and metabolized and thus the residence time of terpenes in the rumen is much shorter.

Due to the dynamism of ruminal processes and the static nature of in vitro testing, discrepancies have been found between in vivo and in vitro digestibility studies. Terpenes in *Artemisia tridentata* have marked antibacterial effects in the rumen and in vitro studies they have been reported to inhibit digestibility [35]. However, in a recent in vivo study we found that terpenes from *A. tridentata* caused increases rather than decreases in dry matter and fibre digestibility [48]. Digestibility depression is a function of the competition between rates of digestion and passage [44], variables that are not taken into account in static systems. Terpenes apparently increase rumen retention times, which in turn increase the extent of digestion [48].

Because of the dynamic nature of digestion processes, in vitro continuous systems are a step closer to "reality" than standard in vitro techniques because they mimic the constant digesta turnover that occurs in the animal [44]. In vitro continuous systems have been used successfully to determine the effects of natural plant extracts on ruminal fermentation [6].

A proposed approach to in vitro studies is to initiate the screening process in a traditional non-dynamic in vitro system and then test the most promising bioactives in a continuous system before the development of in vivo trials.

Concentration of Bioactives in Biological and Artificial Media

Crude or purified extracts from plants are used for in vitro testing. This procedure may not give results that are always relevant to the in vivo situation [1]. For instance, purified condensed tannins in *Cichorium intybus* have been shown in vitro to inhibit deer nematodes [33]. However, the concentration of condensed tannins in *C. intybus* is very low and thus it is highly unlikely that the concentrations found to be successful in vitro could be attained in vivo [1]. A constraint on using plant chemicals as biological active agents is the potentially large quantities of plants required to achieve meaningful doses in the herbivore [49]. Concentrations yielding promising results in vitro may never be reached in vivo.

Another constraint on using purified chemical compounds during in vitro testing is that it may be unpractical, economically unfeasible or even impossible to feed purified compounds or plant extracts to animals. When feeding plants with bioactives – instead of purified compounds – a point to consider is that the concentration of a chemical in the plant's tissue may not represent the amount, which is actually available to the animal.

A new approach for testing biological activity of plants in vitro against gastrointestinal nematodes is the use of rumen fluid taken from animals grazing pure stands of plant secondary metabolites-rich plants [1]. With this methodology researchers can test for activity in a medium that contains concentrations of chemicals in the rumen fluid that are realistic instead of theoretical. By using rumen fluid, the technique also adds chemical and biological complexity to the medium (see above), which is also a step closer to the in vivo scenario. Nevertheless, when testing for activity against intestinal nematodes, the possible lack of plant secondary metabolites activity in the rumen fluid does not necessarily mean lack of activity in the duodenum or large intestine, as plant secondary metabolites might become active in the lower parts of the gastrointestinal tract [1].

Ruminal Adaptation: Short vs. Long Term Effects

The diverse microbial populations in the foregut can perform many reactions that modify plant products and thus influence their biological activity [13]. For instance, Cardozo et al. [6] found that although some natural plant extracts have a short-term effect on ruminal microbial fermentation, ruminal microbes were adapted after 6 days and differences from controls (without plant extracts) disappeared. This led Cardozo et al. [6] to suggest that data from short-term in vitro fermentation studies may lead to erroneous conclusions.

There is evidence suggesting rumen microbes have a direct impact on transforming and inactivating natural plant products. Gradual exposure to increasing levels of oxalic acid to ruminants leads to a change in the composition of the rumen microbial population, which results in the breakdown of oxalic acid [10]. Chronic exposure to terpenes in sheep increases their ability to consume terpenes in *A. tridentata* [45]. Rumen microbes adapt to monoterpenes [11, 21], and ruminal microbes in goats modify diterpene diesters present in *Euphorbia esula* [23]. Deconjugation of phytoestrogens [4] and metabolism of mycotoxins [26] by the gut microflora has been reported in livestock.

Ingestive Behavior

Food Aversions and Willingness to Consume Bioactives

A problem with validating in vitro results for biological activity is the assumption that animals will be willing to consume concentrations of natural plant

products, which parallel the amounts that yield biological activity in artificial systems. However, many of the chemicals in plants with biological activity are secondary compounds, which at certain doses can adversely affect mammals through their negative actions on cellular and metabolic processes [8, 9]. Herbivores feed to avoid exceeding a threshold dose of particular plant secondary metabolites [28, 40, 41] such that ingestion of plant bioactives would not exceed their capacity to biotransform and eliminate these compounds.

A mechanism used by herbivores to prevent toxicosis is the stimulation of the emetic system and the development of food aversions [40]. Food aversion learning is the process by which after eating or drinking a specific food, a physiological event or physiochemical agent causes nausea [17]. Thus, aversions result from the stimulation of the emetic system of the midbrain and brain stem [30]. This system can be stimulated by toxins in the cardiovascular system and cerebrospinal fluid and through vagal and splanchnic afferents from the gastrointestinal tract [5]. After ingesting a food containing a plant secondary metabolite, afferent impulses to the central nervous system cause malaise, which in turn causes the animal to decrease intake of food. In turn, efferent impulses from the central nervous system to the gut cause a decrease in motility and a decrease in absorption of the plant secondary metabolites [42]. Consistent with this mechanism, antiemetic drugs attenuated food aversions in sheep caused by the toxicant LiCl [39]. Likewise, administration of a selective antiemetic (ondansetron), an antagonist of 5-hydroxy tryptamine (5HT3) serotonin receptors, increased intake by marsupials of diets containing secondary metabolites present in *Eucalyptus* [25]. Collectively, the information presented suggests that there is a limit on how much plant bioactive an herbivore is willing to ingest. In certain circumstances, small amounts of plant secondary metabolites will stimulate the emetic system causing strong food aversions that will prevent animals from consuming the therapeutic or active doses found during in vitro studies.

A possible course of action to enhance the animal's willingness to consume plants with bioactives is to provide an adequate level of nutrition with the basal diet. When animals ingest adequate amounts of energy and protein, they can eat more foods that contain plant secondary metabolites. This is because rates of detoxification are influenced by the nutrient status of an animal. The general mechanism of detoxification involves converting more toxic lipophilic compounds to less toxic water-soluble compounds that can be excreted in the urine [8, 9]. Biotransformation of toxins is carried out largely in the liver and usually occurs in two steps. The first step (Phase I) introduces a reactive group – such as OH, NH_2, COOH, or SH – into the structure of the toxin; those interactions typically produce a less toxic compound. During the second step (Phase II), the newly formed compound is conjugated with a small molecule such as glucuronic acid, amino acids (e.g., glycine), sulphates, acetates, or methyl groups [37]. Importantly, these transformations require nutrients such as protein and energy in the form of glucose [19, 20]. Thus, detoxification processes reduce the protein and energy that otherwise would be available for maintenance and production [14, 20]. Lambs can ingest more of the toxin LiCl as the energy content of their diet increases [50]. Likewise, sheep offered terpene-containing diets with increasing concentrations of energy or protein

consume terpenes in a graded fashion with a direct relationship between energy or protein available and terpene intake [47]. Supplemental energy and protein increase the ability of sheep and goats to eat foods that contain plant secondary metabolites such as terpenes [45], tannins [46], and saponins [27]. Conversely, herbivores restrict ingestion of higher amounts of plant secondary metabolites when levels of nutrients such as sodium are low [16].

Pulse-Delivery of Bioactives to the Digestive Tract

Even if we assume animals are willing to consume concentrations of plant secondary metabolites that yield biological activity, the rate at which those compounds enter the digestive tract is not continuous but in pulses. Ingestion of plant bioactives in nature is not continuous because, as mentioned before, plant secondary metabolites at certain concentrations have negative impacts on cells and metabolic processes. Thus, animals consume plant secondary metabolites in small amounts during discrete feeding bouts distributed throughout the day. At critical thresholds, toxins satiate the detoxification capabilities of herbivores [13]. At these levels, animals quit feeding, and they resume eating only after plant secondary metabolites concentrations in the body decline due to detoxification and elimination [11, 12, 38]. These processes cause cyclic patterns of intakes of particular foods with peak intakes at the lowest concentration of plant secondary metabolites in the body [13, 38]. During these cycles, pulses of substrate are sent down the gastrointestinal tract. Pulsative feeding could create cyclic perturbations of the microbial populations through shifts in the relative proportions of such populations [44]. Consequently, pulse-like feeding behaviour could create conditions in vivo that deviate substantially from the continuous or static conditions normally present in artificial fermentation or incubation systems. Experience from the administration of antibiotics to patients has clearly demonstrated the advantages of continuous dosage over intermittent administration of antibiotics [22].

Voluntary Intake and Sequence of Feeding Patterns

The sequence in which “medicinal” and other components of the diet are ingested may be another reason findings in vitro diverge from those obtained in vivo. The temporal order at which foods enter the rumen may influence the likelihood of interactions among different dietary chemicals. For instance, lambs offered plant secondary metabolites in the sequence of tannins followed by terpenes consume twice as much food as lambs offered a meal of terpenes followed by a meal of tannins [34]. Tannins are large molecules that interact with other compounds as they move slowly through the gastrointestinal tract [24, 29]. Consumption of tannins first increases the likelihood of interaction, and possible deactivation, of terpenes fed subsequently in a meal. In contrast, terpenes are small non-polar molecules, highly

Table 11.1 Some causes of discrepancy between in vitro and in vivo studies and a proposed course of action to reduce such differences

Cause of discrepancy	Action
• In Vitro: Chemically simple testing media In Vivo: Chemically and biologically complex media with more likelihood for chemical interactions and inactivation	Testing medium should be as close as possible to the chemical and biological conditions where bioactives are expected to act
• In Vitro: Static systems (e.g., in vitro digestibility) In Vivo: Dynamic system	Use static systems as a first screening approach, followed by a continuous system such as artificial rumen, Rusitec, etc.
• In Vitro: Purified extracts, concentrations may never reach the amounts used in vivo. Reduced bioavailability	Use lyophilized plant material. Use rumen fluid taken from animals grazing pure stands of plant secondary metabolites-rich-plants. It might not be always possible
• Ruminal adaptation due to changes in microbial populations	Conduct screening studies for periods longer than 1 week
• Animals may develop aversions to the bioactives and thus they will not consume the doses of bioactives tested in vitro	Offer bioactives along with a nutritious food such that the likelihood of a food aversion declines
• Pulsative feeding on bioactives creates gastrointestinal disturbances	Deliver bioactives in pulses in continuous in vitro systems
• Sequential feeding patterns may influence the likelihood of interactions among bioactives and other chemicals in the digesta	In a continuous system try to mimic the feeding patterns which occurs in vivo

soluble in membranes; they are absorbed readily through the gastro-intestinal tract walls [11]; if they are fed first in the sequence the likelihood for interaction with tannins decreases. Likewise, the sequential supply of a tanniferous shrub (*Acacia cyanophylla*) followed by protein rich feed substantially increases the chances of protein interacting with tannins, which in turn reduces ammonia formation and increases protein retention in sheep and goats [3]. Collectively, the information presented suggests that the biological activity of plant secondary metabolites will depend on the sequence at which plant secondary metabolites-containing plants are fed relative to the remaining components of the diet (Table 11.1).

References

1. Athanasiadou, S., and I. Kyriazakis. 2004. Plant secondary metabolites: antiparasitic effects and their role in ruminant production systems. Proc. Nutr. Soc. **63**:631–639.
2. Athanasiadou S, I. Kyriazakis, K. Jackson, and R.L. Coop. 2001. The effects of condensed tannins supplementation of foods with different protein content on parasitism, food intake and performance of sheep infected with *Trichostrongylus colubriformis*. Br. J. Nutr. **86**: 697–706.
3. Ben Salem, H., H.P.S. Makkar, A. Nefzaoui, L. Hassayoun, and S. Abidi. 2005. Benefit from the association of small amounts of tannin-rich shrub foliage (*Acacia cyanophylla* Lindl.) with

soya bean meal given as supplements to Barbarine sheep fed on oaten hay. Anim. Feed. Sci. Technol. **122**:173–186.
4. Bennetau-Pelissero, C., K. Latonnelle, A. Sequeira, and V. Lamothe. 2000. Phytoestrogens, endocrine disrupters from food. ANALUSIS. **28**:763–775.
5. Borison, H.L. 1986. Anatomy and physiology of the chemoreceptor trigger zone and area postrema, pp. 10–17. In C.J. Davis, G.V. Lake-Bakaar, and D.G. Grahame-Smith (eds.), Nausea and Vomiting: Mechanisms and Treatment, Springer-Verlag, NY.
6. Cardozo, P.W., S. Calsamiglia, A. Ferret, and C. Kamel. 2004. Effects of natural plant extracts on ruminal protein degradation and fermentation profiles in continuous culture. J. Anim. Sci. **82**:3230–3236.
7. Cardozo, P.W., S. Calsamiglia, A. Ferret, and C. Kamel. 2005. Screening for the effects of natural plant extracts at different pH on in vitro rumen microbial fermentation of a high-concentrate diet for beef cattle. J. Anim. Sci. **83**:2572–2579.
8. Cheeke, P., and L.R. Shull. 1985. Natural Toxicants in Feeds and Poisonous Plants. Avi Publishing, Westport, CT.
9. Cheeke, P.R. 1998. Natural Toxicants in Feeds, Forages, and Poisonous Plants. Interstate Publ. Inc., Danville, IL.
10. Duncan, A.J., P. Frutos, and S.A. Young. 2000. The effect of rumen adaptation to oxalic acid on selection of oxalic-acid-rich plants by goats. Br. J. Nutr. **83**:59–65.
11. Dziba, L. E., J.O. Hall, and F.D. Provenza. 2006. Feeding behavior of the lambs in relation to kinetics of 1.8-ceneole dosed intravenously or into the rumen. J. Chem. Ecol. **32**(2): 391–408.
12. Dziba, L.E. and F.D. Provenza. 2008. Dietary monoterpene concentrations influence feeding patterns of lambs. Appl. Anim. Behav. Sci. 109: 49–57.
13. Foley, W.J., G.R. Iason, and C. McArthur. 1999. Role of plant secondary metabolites in the nutritional ecology of mammalian herbivores—how far have we come in 25 years? pp. 130–209. In H.J.G. Jung and G.C. Fahey Jr (eds.), Nutritional Ecology of Herbivores, 5th Int. Symp. Nutr. Herbivores, Savoy, IL: Am. Soc. Anim. Sci.
14. Freeland, W.J., and D.H. Janzen. 1974. Strategies in herbivory by mammals: The role of plant secondary compounds. Am. Nat. **108**:269–286.
15. Freeland, W.J., P.H. Calcott, and L.R. Anderson. 1985. Tannins and saponin: interaction in herbivore diets. Biochem. Syst. Ecol. **13**:189–193.
16. Freeland, W.J., and D. Choquenot. 1990. Determinants of herbivore carrying capacity: Plants, nutrients, and *Equus asinus* in northern Australia. Ecology **71**:589–597.
17. Garcia, J. 1989. Food for Tolman: cognition and cathexis in concert, pp. 45–85. In T. Archer and L.Nilsson (eds.), Aversion, Avoidance and Anxiety, Earlbaum, Hillsdale, NJ.
18. Helle, O., W. Velle, and J. Tharaldsen. 1989. Effect of ovine urine and some of its components on viability of nematode eggs and larvae in sheep faeces. Vet. Parasitol. **32**:349–354.
19. Illius, A.W., and N.S. Jessop. 1995. Modeling metabolic costs of allelochemical ingestion by foraging herbivores. J. Chem. Ecol. **21**:693–719.
20. Illius, A.W., and N.S. Jessop. 1996. Metabolic constraints on voluntary intake in ruminants. J. Anim. Sci. **74**:3052–3062.
21. Johnson, A.E., L.F. James, and J. Spillet. 1976. The abortifacient and toxic effects of big sagebrush (*Artemisia tridentata*) and juniper (*Juniperus osteosperma*) on domestic sheep. J. Range Manage. **29**:278–280.
22. Kasiakou, S.K., K.R. Lawrence, N. Choulis, and M.E. Falagas. 2005. Continuous versus intermittent intravenous administration of antibiotics with time-dependent action – A systematic review of pharmacokinetic and pharmacodynamic parameters. Drugs **65**:2499–2511.
23. Kronberg, S.L., and J.W. Walker. 1993. Ruminal metabolism of leafy spurge in sheep and goats: A potential explanation for differential foraging on spurge by sheep, goats, and cattle. J. Chem. Ecol. **19**:2007–2017.
24. Kumar, R., and M. Singh. 1984. Tannins: their adverse role in ruminant nutrition. J. Agric. Food Chem. **32**: 447–453.

25. Lawler, I.R., W.J. Foley, G.J. Pass, and B.M. Eschler. 1998. Administration of a 5HT3 receptor antagonist increases the intake of diets containing *Eucalyptus* secondary metabolites by marsupials. J. Comp. Physiol. B **168**:611–618.
26. Mackie, R.I., and White, B.A. 1990. Recent advances in rumen microbial ecology and metabolism: Potential impact on nutrient output. J. Dairy Sci. **73**:2971–2995.
27. Martinez, J.H., T.T. Ross, K.A. Becker, and G.S. Smith. 1993. Ingested dry snakeweed foliage did not impair reproduction in ewes and heifers during late gestation. Proc. Western Sect. Am. Soc. Anim. Sci. **44**:32–35.
28. Meyer, M., and W.H. Karasov. 1989. Antiherbivore chemistry of *Larrea tridentata:* effects on desert rats (*Neotoma leipida*) feeding and nutrition. Ecology **70**:953–961.
29. Min, B.R., and S.P. Hart. 2003. Tannins for suppression of internal parasites. J. Anim. Sci. **81**(E. Suppl. 2):E102–E109.
30. Mitchelson, F. 1992. Pharmacological agents affecting emesis: A review (Part I). Drugs **43**:295–315.
31. Molan, A.L., S.O. Hoskin, T.N. Barry, and W.C. McNabb. 2000. Effect of condensed tannins extracted from four forages on the viability of the larvae of deer lungworms and gastrointestinal nematodes. Vet. Record. **147**:44–48.
32. Molan, A.L., G.C. Waghorn, and McNabb, W.C. 2002. Effect of condensed tannin on egg hatching and larval development of *Trichostrongylus colubriformis* in vitro. Vet. Record. **150**: 65–69.
33. Molan, A.P., A. Duncan, T.N. Barry, and W.C. McNabb. 2003. Effects of condensed tannins and sesquiterpene lactones extracted from chicory on the viability of deer lungworm larvae. Proc. N.Z. Soc. Anim. Prod. **60**:26–29.
34. Mote, T.E., J.J. Villalba, and F.D. Provenza. 2008. Sequence of food presentation influences intake of foods containing tannins and terpenes. Appl. Anim. Behav. Sci. **113**: 57–68.
35. Nagy, J.G., H.W. Steinhoff, and G.M. Ward. 1964. Effects of essential oils of sagebrush on deer rumen microbial function. J. Wildl. Manage. **28**:785–790.
36. Okuda, T., K. Mori, and M. Shiota. 1982. Effects of interaction of tannins and coexisting substances. III Formation and solubilization of precipitates with alkaloids. J. Pharm. Soc. Japan. **102**:854–858.
37. Osweiler, G.D., T.L. Carson, W.B. Buck, and G.A. Van Gelder. 1985. Clinical and Diagnostic Veterinary Toxicology. Kendall/Hunt, Dubuque, IA.
38. Pfister, J.A., F.D. Provenza, G.D. Manners, D.R. Gardner, and M.H. Ralphs. 1997. Tall larkspur ingestion: Can cattle regulate intake below toxic levels? J. Chem. Ecol. **23**: 759–777.
39. Provenza, F.D., L. Ortega-Reyes, C.B. Scott, J.J. Lynch, and E.A. Burritt. 1994. Antiemetic drugs attenuate food aversions in sheep. J. Anim. Sci. **72**:1989–1994.
40. Provenza, F.D. 1995. Potingestive feedback as an elementary determinant of food preference and intake in ruminants. J. Range Manage. **48**:2–17.
41. Provenza, F.D. 1996. Acquired aversions as the basis for varied diets of ruminants foraging on rangelands. J. Anim. Sci. **74**:2010–2020.
42. Stricker, E.M., and J.G. Verbalis. 1990. Control of appetite and satiety: Insights from biologic and behavioral studies. Nutr. Rev. **48**:49.
43. Tilley, J.M.A. and R.A. Terry. 1963. A two-stage technique for the in vitro digestion of forage crops. J. Brit. Grassland Soc. **18**:104.
44. Van Soest, P.J. 1994. Nutritional Ecology of the Ruminant, 2nd edition, Cornell University Press, Ithaca and London.
45. Villalba, J.J., F.D. Provenza, and R.E. Banner. 2002. Influence of macronutrients and activated charcoal on utilization of sagebrush by sheep and goats. J. Anim. Sci. **80**:2099–2109.
46. Villalba, J.J., F.D. Provenza, and R.E. Banner. 2002. Influence of macronutrients and polyethylene glycol on intake of a quebracho tannin diet by sheep and goats. J. Anim. Sci. **80**:3154–3164.

47. Villalba, J.J., and F.D. Provenza. 2005. Foraging in chemically diverse environments: Energy, protein and alternative foods influence ingestion of plant secondary metabolites by lambs. J. Chem. Ecol. **31**:123–138.
48. Villalba, J.J., F.D. Provenza, and K.C. Olson. 2006. Terpenes and carbohydrate source influence rumen fermentation, digestibility, intake, and preference in sheep. J. Anim. Sci. 84:2463–2473.
49. Waghorn, G.C., and McNabb, W.C. 2003. Consequences of plant phenolics compounds for productivity and health of ruminants. Proc. Nutr. Soc. **62**:383–392.
50. Wang, J., and F.D. Provenza. 1997. Food deprivation affects preference of sheep for foods varying in nutrients and a toxin. J. Chem. Ecol. **22**:2011–2021.

List of Participants

Joint FAO/IAEA Division and Writtle College Meeting on
"Alternative Feed Resources: A Key to Livestock Intensification in Developing Countries"
Writtle College, United Kingdom
12–13th September 2006

1	Dr. Philip Edward Vercoe Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Department of Nuclear Sciences and Applications, International Atomic Energy Agency Wagramerstrasse 5, P.O. Box 100 A-1400 Vienna Austria	Tel.: +43 1 2600-26057 Fax: +43 1 2600-26007 E-mail: p.vercoe@iaea.org
2	Dr. Devki Kamra Centre of Advanced Studies in Animal Nutrition, Indian Veterinary Research Institute, Izatnagar – 243 122 India	Tel.: +91 581 230 1318 Fax: +91 581 230 1318 E-mail: dnkamra@rediffmail.com
3	Dr. Harinder Makkar Institut für Tierproduktion in den Tropen und Subtropen Universität Hohenheim Postach 91 70593 Stuttgart Germany	Tel.: +49 711 459 3640 E-mail: makkar@uni-hohenheim.de
4	Dr. Juan Villalba Department of Wildland Resources, Utah State University, Logan, UT 84322-5230 USA	Tel.: +1 435 797 2539 E-mail: juan.villalba@usu.edu

P.E. Vercoe et al. (eds.), *In Vitro Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies*,
DOI 10.1007/978-90-481-3297-3,

5	Dr. John Wallace Microbial Biochemistry Group Rowett Research Institute Bucksburn, Aberdeen AB21 9SB UK	Tel.: +44 1224 716656 Fax.: +44 1224 716687 E-mail: john.wallace@rowett.ac.uk
6	Dr. Dean Revell Senior Research Scientist CSIRO Livestock Industries Private Bag 5 Wembley WA 6913 Australia	Tel.: +61 8 9333 6492 Fax: +61 8 9383 7688 E-mail: dean.revell@csiro.au
7	Dr. Chris McSweeney CSIRO Livestock Industries Queensland BioScience Precinct 306 Carmody Road, St. Lucia Australia	Tel.: +61 7 3214 2665 Fax: +61 7 3214 2900 Email: chris.mcsweeney@csiro.au
8	Dr. Frank Jackson Moredun Research Institute Pentlands Science Park Bush Loan, Penicuik, Midlothian EH26 0PZ UK	Tel.: +44 131 445 5111 Fax: +44 131 445 6111 E-mail: info@moredun.ac.uk
9	Dr. Evelyn Mathias League for Pastoral Peoples and Endogenous Livestock Development Germany Müllenberg 5a, 51515 Kürten, Germany	Tel.: +49 2268 801 691 Fax: +49 2268 801 692 E-mail: evelyn@mamud.com
10	Dr. Zoey Durmic Lefroy Research Fellow School of Animal Biology, The University of Western Australia 35 Stirling Highway Crawley WA 6009 Perth, Australia	Tel.: +61 8 6488 1475 Fax: +61 8 6488 2243 E-mail: zdurmic@cyllene.uwa.edu.au

Index