METHODS IN GUT MICROBIAL ECOLOGY FOR RUMINANTS

Methods in Gut Microbial Ecology for Ruminants

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Contents

Foreword

As a result of various human activities, such as increase in human population, decrease in arable land due to soil degradation, urbanization, industrialization and associated increase in the demand for livestock products, dramatic changes are occurring in the global ruminant livestock sector. These changes include shift in the size of regional livestock populations and in the types of management and feeding systems under which ruminant livestock are held, and increased demand of a wider range of quality attributes from animal agriculture, not just of the products themselves but also of the methods used in their production. The livestock sector will need to respond to new challenges of increasing livestock productivity while protecting environment and human health and conserving biodiversity and natural resources.

The micro-organisms in the digestive tracts of ruminant livestock have a profound influence on the conversion of feed into end products, which can impact on the animal and the environment. As the livestock sector grows particularly in developing countries, there will be an increasing need to understand these processes for better management and use of both feed and other natural resources that underpin the development of sustainable feeding systems.

Until recently, knowledge of ruminant gut microbiology was primarily obtained using classical culture-based techniques, such as isolation, enumeration and nutritional characterization, which probably only account for 10–20% of the rumen microbial population. New gene-based technologies can now be employed to examine microbial diversity through the use of small sub-unit ribosomal DNA analysis (e.g. 16S rDNA) and to understand the function of complex microbial ecosystems in the rumen through metagenomic analysis. These technologies have the potential to revolutionize the understanding of rumen function and will overcome the limitations of classical-based techniques, including isolation and taxonomic identification of strains important to efficient rumen function and better understanding of the roles of micro-organisms in relation to achieving high productivity and decreasing environmental pollutants.

This book has been produced by the Joint FAO/IAEA Division of Nuclear Technique in Food and Agriculture, IAEA Vienna, Austria in collaboration with the CSIRO Livestock Industries, Brisbane, Australia. It gives a comprehensive up-to-date account of the methodologies and the protocols for conventional and modern molecular

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techniques that are currently in use for studying the gut microbial ecology of ruminants. Each chapter has been contributed by experts in the field. The techniques and procedures described are also relevant and adaptable to other gastrointestinal ecosystems and the microbiology of anaerobic environments in general. The future of ruminant gut microbiology research is dependent upon the adoption of these molecular-based research technologies, and the challenge at present is the use of these technologies to improve ruminant production and decrease environment pollutants through a better understanding of microbial function and ecology. It is hoped that this book will equip the readers better in order to meet this unprecedented challenge.

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Introduction

Current approaches to the evaluation of digestibility and the nutritive value of feed resources using conventional *in vitro* feed evaluation and animal studies have resulted in a large body of information about nutrient composition, digestion kinetics and digestibility. However, these techniques are unable to describe the microbial mechanisms involved in digestion by ruminants and other herbivores, and are unlikely to result in the development of new feeding strategies. Conventional culture-based methods of enumerating and identifying rumen bacteria are being rapidly replaced by the development of nucleic acid-based techniques that can be used to characterise complex microbial communities. Ruminant nutritionists and microbiologists have recognized the importance of molecular microbial ecology, but many have found it difficult to employ the most appropriate techniques because they are not familiar with the methods. In addition, this field is developing very rapidly and even researchers with experience in molecular microbial ecology find it difficult to keep abreast with the increasing number of techniques and alternatives.

This manual is written by an expert group of scientists interested in ruminant digestion and gut microbiology. The most recent and up-to-date methods in molecular microbial ecology with special emphasis on ruminants are collated and interpreted in this book. The methods will provide the readers an easy access to molecular techniques that are most relevant and useful to their area of interest. The authors have attempted to write in a recipe-like format designed for direct practical use in the laboratory and also to provide insight into the most appropriate techniques, their applications and the type of information that could be expected. These aspects have been supported by inclusion of the relevant literature.

The contents of the manual are presented in a sequence that recognizes the key elements in studying gut microbial ecology. The first chapter provides a perspective on how to design animal trials in which microbial ecology is studied. Often the power of the new molecular techniques is diminished by an inappropriate design in terms of animal number, sampling frequency, location and replication. The second chapter describes the classical culture-based methods for studying rumen microbes, as these methods are often a pre-requisite to employing molecular techniques. Chapters 3–6 provide information on the basic underpinning techniques and the protocols in

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molecular ecology, such as DNA extraction from environmental samples, the polymerase chain reaction (PCR), oligonucleotide probe and primer design and DNA fingerprinting amongst others. The application of these techniques to microbial detection and identification are discussed. Specialized techniques such as denaturing gradient gel electrophoresis (DGGE) and 16S/18S ribosomal DNA libraries for studying complex communities that contain unculturable organisms are also described. Many of these techniques are used to identify and enumerate the population of organisms that are present in a sample. However, the field is rapidly moving to a functional analysis of the microbes in an ecosystem, and some of the methods being employed to measure genes expression are described in Chapter 3. In Chapter 6, knowledge about location and spatial relationships of micro-organisms in their natural environment that are often essential for understanding the function of these organisms are discussed. The final chapter deals with metagenomic technologies, which provide the potential to capture and study the entire microbiome (the predominant genomes) from a complex microbial community, such as the rumen. The rapid high-throughput technologies developed in mapping the human genome are now being deployed to study microbial ecosystems. An explosion of knowledge in the field of microbial ecology is now expected.

The editors wish to acknowledge the contributions made by all the authors who participated in the publication of this manual. They have spent considerable time gathering information from many sources into a focussed document that enables the reader to understand how techniques have evolved and the context in which the methods should be applied to address specific issues relating to gut microbial ecology. We believe that this manual will 'demystify' the methods in molecular microbial ecology for readers, who are novice in the field but are excited by the prospects of the technology. It would also be invaluable for the experienced workers striving for giving new dimension to their research – expanding the work in other fields and initiating cross-cutting activities. This manual is seen as the first step towards understanding and manipulating gut micro-organisms as it is expected that the techniques and the methodologies associated with the study of molecular microbial ecology will continue to grow and evolve. A key challenge for the future will be the simplification of these techniques, so that these become tools of routine use in nutritional, environmental and ecological laboratories.

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PART ONE

Designing **in vivo microbial ecology studies

1.1. Experimental designs for rumen microbiology

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Introduction

Research and innovation in relation to microbiology of the rumen is based principally around functional attributes of the populations as they affect digestion and performance of the host animal. What is sought is a better understanding of the complex microbiological communities [4, 24] and identification of ways to manipulate these populations for specified objectives in ruminant production and environmental impact [27, 34]. Further objectives are to develop from that knowledge base, novel anaerobic systems for a range of purposes, such as generation of fuels, detoxification and degradation of waste materials [46].

This chapter is primarily aimed at design of experiments to describe the diversity of rumen microbial populations, identify the factors that influence the composition and nature of associations and quantify relative and absolute growth rates and functional performance of those populations. Many of the principles outlined are applicable to other types of anaerobic microbial systems.

The nature of rumen microbial populations

In research into complex microbial populations, it is well to remember that the population present at any given time is the outcome of prior successions [5]. The population is dynamic in relation to relative growth rates [15], determined by competitive advantage along with interdependencies in relation to the supply of preferred substrates and the prevailing environmental conditions [38, 40, 44, 47]. Microbes occupy microenvironments and in a system such as a compartment of the digestive tract there is always a degree of heterogeneity [1, 3, 7, 9]. Thus, microbes are distributed in broad terms between fluid phase, suspended particulate phase and the wall of compartment; in the latter two phases, they may be adherent or associated but unattached [13, 36]. The degree of heterogeneity in the environment determines which organisms are successful and what symbiotic or interdependent relationships are critical to that success. It also dictates the ease or otherwise of drawing a representative sample. These issues are raised at the outset not to deter investigation but to provide a

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conceptual framework in which hypotheses are set and experimental protocols are established.

The definition of objective or statement of the hypothesis

What is it that you wish to know? Is it how much microbial protein is generated on a daily basis? Is it whether a specified species or group of species are present, something about their relative numbers or biomass, and perhaps the relationship to processes of degradation of substrates? Or is it the broad profile of all major species or functionally identifiable groups present and the changes due to a set of dietary or other treatments imposed? Is it a qualitative or quantitative question? Are the questions about general trends that can be expected in response to a given set of variable conditions, so that the experiment is conducted to derive empirical equations for incorporation into a mechanistic model? Or are they about a specific result to explain performance of animals under specific sets of dietary conditions? Or combinations of the above?

Investigations of microbial populations therefore require very clearly defined objectives or specific hypotheses in order to specify the necessary and sufficient conditions, the experimental design and the protocol for all measurements made. Thus, for example, some questions can be answered under conditions where substrate supply is continuous and the system tightly controlled to minimize variability in conditions through time – so-called *steady state*. Many experiments *in vivo* or in continuous fermenters in which attempts have been made to quantify the rate of microbial growth or flow of microbial cells from the rumen have been based on such protocols [25].

Other important questions, however, relate to the transitional and cumulative effects of changing conditions on the growth rates or population density of specific organisms or groups. Under *non-steady* state dynamics, there is potential for changes in pool size, dilution rates and relative efficiencies of growth that can dramatically affect the nature of the population present at any given sampling time [15, 25]. Most questions relating to microbial activity and the species composition of the microbial population under normal animal behavioural patterns of intake of feed and water call for protocols that allow for this, particularly those involving grazing and/or the feeding of supplements. Here, the patterns of intake may be relatively repeatable in cycles on a 24 h basis [43], but any regularity will depend on frequency of feeding and even prevailing weather conditions.

Variables

The potential sources of variability in experiments to explore the microbial population of the rumen (or any other gut compartment), its diversity and the factors affecting the structure of that population include combinations of the following:

The animals

- between species of animals
- between animals of the same species at different ages/stages of development
- between animals of the same species and age but reared under different conditions
- between like animals in a cohort from the same rearing conditions (the most common approach in selecting animals for nutritional experiments)
- between fermenters started with the same innoculum (replicate systems)
- within individual animals (replicated in time)

The diets

- between previous diets (carry-over effects)
- between current diets
- between levels of intake (*ad libitum* or controlled)
- between meal eating patterns or periodicity of feeding of components of the diet

Time of sampling

- between samples taken at a specified time relative to the feeding regime
- between bulked samples taken at several specified times in the feeding cycle
- between individual samples taken at specified times in a feeding regime

Site of sampling

- between samples drawn at a set position of the sampling device within the digesta
- between samples taken from several set positions but with samples bulked
- between several set positions of sampling with samples analysed separately

Fraction sampled

- between samples of mixed digesta
- between samples of strained fluid phase
- between samples of strained particulate phase
- between samples extracted, for example, by centrifugation methods

Experimental conditions, treatments and sampling protocols are designed to remove the influence of selected sources of potential variability in accordance with the demands of the specific objective or hypothesis. Interactions can occur between the various sources of variability, so that, for example, there may be animal by diet interactions revealed only at specific times of sampling. That may or may not be of immediate interest, depending on the objectives of the experiment, but may be of importance when separate experiments are compared and we seek to explain differences in results or interpretation. Replication at the sampling level is necessary if one is to evaluate the influence of any one of the above potential variables to the total variability.

Variability associated with unresolvable interactions plus the variance due to replicates is treated as residual 'error'.

The experimental unit

A critical element in design of experiments involves the establishment of the variables that must be isolatable in subsequent statistical analysis of the data. The experimental unit is the finest subdivision of data that can legitimately be treated as truly independent. Clearly the objective or hypothesis will determine one layer or set of such isolatable variables. However, additional variables become important if, for example, comparisons to related work of others are important and the experiment can provide some support towards being 'right for the right reason' or contribute to explanations of differences in results.

The degree to which a researcher can add complications either of additional experimental treatments or sampling schedules to cover suspected sources of variability obviously depends on cost and time constraints. The question resolves to the importance placed on getting a result that represents a good 'general case' or getting a result that defines the magnitude and impact of the various sources of variability. For example, samples taken at different times of the day and from different sites in the rumen can be bulked to 'average out' the broad picture of differences due to diet. The experimental unit is clearly the bulked sample. However, if it is desirable to get a more intimate picture of the changes going on or to ensure that the chosen sampling and bulking schedule (e.g. equal volumes only before and 6 h after feeding) does not grossly bias the results derived through the bulking process, the individual samples should be analysed separately and become the experimental unit.

Individual animals differ in the microbial populations established, which may reflect the source of the inoculum, but importantly also anatomical and physiological variables [18]. These include factors, such as digesta pool size, effectiveness of rumination, the kinetics of fluid and solid particle entry and exit rates [20], overlain by the individual animal response to diet composition expressed in selection and/or meal patterns where these are not constrained in the management system applied. While the broad outcomes in terms of digestion rates for dietary constituents may be similar, the organisms occupying the various microenvironmental and particularly substrate niches can differ. Likewise, the patterns of production of fermentation products, rates and energetic efficiencies of microbial growth and the net microbial cell yields presented for subsequent digestion vary. Such diversity in the solutions of microbial success under the prevailing conditions in each animal constitutes the so-called biological variability and will have an influence on the numbers of animals required for robust statistical analysis and interpretation, and the appropriate source, selection and preparatory treatment of those animals. From this, it is also clear that in any experimental program that is undertaken *in vitro* (e.g. continuous flow fermenters) the source and constitution of rumen digesta inoculum should be well described. Guidelines can be established in order that more secure comparisons between experiments can be made. However, guidelines are often aimed at reducing variability and so may

constrain the circumstances to which the results can be extrapolated. While we may justify the simplifications inherent in over-riding the complicated realities of microbial dynamics *in vivo*, we need to be alert to those matters where such simplification could lead to incorrect interpretations.

All that said, the following discussion has the aim of assisting in establishing robust, purpose-specific experimental designs and protocols for investigations of microbial populations in the rumen and their contribution to processes of digestion and the supply of nutrients to the host animal. Because of the diverse objectives of individual experiments in such research what is presented is in the form of principles and processes in arriving at best solutions for specific cases.

Design, conditions, sampling and measurements

In many studies of the rumen microbes, the studies have drawn on samples obtained from digesta of free-ranging ruminants or from animals in experiments designed to investigate wider aspects of animal performance. Samples taken have been used to establish in the laboratory libraries of readily culturable anaerobic genotypes. Once isolated, the organism can be characterized on the basis of substrate range and specificities and the nature of the end products of fermentation. The challenge has been to increase the array of culturable organisms by finding the conditions under which each can be maintained. This has allowed development since the early 1940s of knowledge of substrate range, cofactor requirements and end products for many rumen anaerobes.

While these objectives remain, new opportunities have arisen through advances in molecular genetics permitting, for example, description of hereto uncultured organisms using metagenomic approaches and the application of biotechnological approaches to manipulation of organisms.

For all experiments, there are several guiding principles.

1. A *full description of the experimental conditions* is mandatory, to provide key information in terms of the type and sources of animals, where and under what environmental conditions they are held, the diet composition and feeding regime. This is necessary but rarely sufficient.
2. The *specific objective and hypothesis* to be tested must be explicit, because it determines the constraints to be set on the design and protocol to be followed.
   Many experiments are designed on the basis of constraining sources of variability other than the primary (treatment) variables or to obviate spatial and time-sensitive differences. Thus, many experiments and much of the data used in construction of mechanistic models are based on experiments using total mixed rations (TMR) (dietary mixtures aimed at delivery of all feed components synchronously) and short-interval feeding regimes (e.g. 2 h feeding in equal-sized meals). While such conditions produce relatively stable and therefore more easily measured digestion parameters, they do not provide an understanding of the effects of the fluctuating conditions established during many natural feeding and particularly grazing cycles.
3. When setting the *experimental design*, decisions are required not only on what *treatments* are to be imposed, but also on the nature of the baseline conditions.

Often there needs to be a control treatment that will allow inter-experiment comparisons through some consistent baseline condition and perhaps provide data on between-animal variability. There are some traditions about the length of any preliminary treatment or feeding period, the numbers of animals required for robust statistical analysis, the use of Factorial, Latin Square or Cross-over designs and the benefits of a covariate period. However, as we move into an exploration of the functional diversity of rumen organisms and the potential for reliable manipulation for production purposes, longitudinal studies involving dietary changes in individual animals in the treatment cohort may prove more illuminating.

4. *Individual animals differ*, for example, protozoa or anaerobic fungi may be abundant in some but not all animals particularly on some but not all diets studied [14, 15]; the reasons for this again call for further experimental work. This imposes a degree of statistical heterogeneity in data obtained with any type of design, and designs are selected either to explore the differences by keeping individual animal as the experimental unit or to gain a 'coarser' view by bulking samples or combining data obtained over groups of animals as the experimental unit.

   In all cases, the animals are randomly assigned to groups to receive the respective experimental treatments *except* where the class of animal is to be an experimental variable. If the animals are deemed to be of a single class, unbiased allocation to treatments is by simple random number drafting. Where the animals are clearly differing in some respect and there is no immediate interest in the variance due to such differences, randomization should be on a stratified basis. *Stratified randomization* requires animals to first be assigned to a defined class such as breed, sex, age and/or weight and members of each class are assigned in rotation to the respective treatments randomly.

   Animals for which results appear to be 'outliers' in relation to any measurement made contribute to the overall variability and create a greater level of heterogeneity in the cell into which their data are assigned. Their unusual status may make them a target for closer examination. In terms of data relating to microbial populations, such animals may have special significance.
5. In any given design, *the measurements* to be made are selected on two bases. They are the measurements that are essential in testing the primary hypothesis. Additional measurements to be considered are those that characterize more thoroughly the conditions of the experiment, inform the interpretation and support efforts to compare and contrast results with those of other apparently similar experiments.
6. The numbers of *samples* and the times and the sites of sampling need close attention. The decisions revolve around the nature and magnitude of differences due to time, to any stratification or imperfection in digesta mixing and to interactions between these factors.

   Simply adopting the protocols of others in the field is not always best practice. Always the capacity for analysis of samples depends on time and funds available, but the compromise arrived at needs to acknowledge that the reason for spending any time or money is to take a robust step towards reliable additional knowledge.

Pragmatic solutions such as sampling cows only at milking times or choosing a single 'best time' of the day for sampling need to be challenged and strong biological reasons advanced that this is sufficient to the objective. In terms of sample size and sampling site, in some cases extreme efforts to take a 'representative sample' may be unwarranted; in other cases samples taken at the same time from different sites may need to be viewed as describing the basic heterogeneity rather than to be pooled to provide an aggregate result.

Key questions

The following considerations, expressed as questions to be addressed, form an important step in planning for most experiments and have general application here. They cannot all be answered once and for all in a stepwise fashion but have to be revisited as provisional decisions are reached.

- To what degree do I have control over each of the variables?
- Which of the potential variables am I interested in, in terms of main effects and possible interactions?
- Which of the potential variables must be 'removed' to address the objective or test the hypothesis?
- Which of the potential variables cannot be removed given the constraints on the experiment and the conditions under which it will be conducted *and* how then do I provide sufficient information to ensure that others can see the results in that context?
- How many treatments are necessary and sufficient to the objective?
- Over what ranges do I seek to set the levels for treatment variables?
- What samples are to be taken from all animals, in relation to time, site and fractionation of the sample?
- What replication is required in order to establish a sufficient basis for robust statistical analysis at the level of the experimental unit?
- What are the samples to be analysed for in terms both of data essential to the objective and data desirable for more effective description of the conditions achieved in the experiment?
- How many samples can be analysed (level of precision, time, cost) and what is the compromise on issues such as bulking of samples?

Strengths and weaknesses of experimental designs and protocols for evaluation of microbial populations

In the following section, several common designs are reviewed and comments made on the issues that arise in their application. All readers are advised to discuss fully with their statistical adviser the design that they consider most appropriate to their objectives and ensure at the outset that they have a clear view of the way the data will be treated in subsequent statistical analysis.

Samples take at one time from individual animals

The results are a snapshot of the microbial population present. Samples may be taken from one or more animals, from different sites, fractionated and replicated to allow analysis for variance due to animals, sites and fractions [8, 26, 33, 41]. Such studies may provide the initial basis for a hypothesis or yield unusual data of microbiological importance setting the scene for further experimental work. Results cannot reveal what factors influenced the arrival at that population; any relationships to diet, season and digestive physiology of the animal are by inference.

Longitudinal studies on individual animals

Each animal is its own control and data obtained through time relate to the sequence of changes in conditions over that time course and the consequent patterns of microbial successions [10, 12, 18, 21]. Samples may be taken at successive intervals at times within a day or over an extended period, relating to events or time elapsing since imposition of a treatment. Samples may be taken from different sites, fractionated and replicated, to allow analysis for variance due to animals, times, sites and fractions. Relationships to season, diets and physiological changes over the period can be inferred, but because of confounding of these influences, direct evidence of the influence of any critical variable can only be derived by further testing of hypotheses under more controlled experimental conditions. However, longitudinal studies can be established within more complex designs described below.

Studies on animals subjected to different treatments within the same time period

These types of experiments provide opportunity to investigate the influence of a limited array of selected variables such as species, age, diet, environmental conditions, physiological state or physiological intervention where these are imposed as 'treatments' [5, 30]. Animals are usually, but not always, drawn from groups with a known common history and are assigned to treatments by randomization or by stratified randomization. Any differences in recent dietary or drug treatment or in familiarity with the conditions for the experiment are to be reported and are usually dealt with by including a preparatory or preliminary period under a common management system. Replication is needed and individual animals can be treated as replicates if they correctly define the *experimental unit*.

Block design
Individual animals or groups of animals (in each case replicated) are subjected to several treatments to compare effects of, say, Treatment A vs. Treatment B etc. in a single experimental period [11, 23, 28]. The variance due to animals within a treatment may be significant but such interactions can result in high residual variances (error term). Samples may be taken at successive intervals at times within a day or over an extended period, relating to events or time elapsing since imposition of a treatment [32]. Samples may be taken from different sites, fractionated and

replicated, to allow analysis for variance due to treatments, animals, times, sites and fractions.

Factorial design

Replicate animals or groups of animals are necessary. The way that animals are managed (e.g. individually fed vs. group fed) and the way the samples taken are treated for analysis determine the experimental unit.

Under these types of design, it is possible to investigate interactions between treatments by imposing several treatments separately and in selected combinations on randomized groups of animals [29, 35, 37]. For example, a basic treatment might be pasture or roughage diet (R), and the further treatments imposed may be added, for example, type of supplement (R + A, R + B), level of supplementation (R + A, R + 2A) or various combinations of supplements (R + A + B). Samples may be taken at successive intervals at times within the experimental period relating to events or time elapsing since imposition of the respective treatment. Samples may be taken from different sites, fractionated and replicated, to allow analysis for variance due to treatment, times, sites and fractions. However, even when the experimental unit is set correctly, differences due specifically to individual animals within groups cannot be separated from other residual variability (error term).

Studies on animals subjected to different treatments in a sequence over time

These designs are aimed at increasing the database and ensuring that all animals receive all treatments, but they increase the length of time and hence the opportunity for time-related factors to influence the results. There are advantages particularly where infrastructure and equipment are limiting.

Cross-over design experiments allow for each animal or group of animals as a set to receive one of a number of treatments in one period of time and other treatments in following periods in a balanced design [19]. Often this design is used to make simple comparisons between two treatments; Group 1 receives Treatment A in period 1 and Treatment B in period 2, while a second group receives the same two treatments but in the reverse order. Usually the analysis is most robust when the experimental unit is an individual animal (i.e. each animal is managed on a truly independent basis). Replication is needed. Samples may be taken at successive intervals at times within the experimental period relating to events or time elapsing since imposition of the respective treatment. Samples may be taken from different sites, fractionated and replicated, to allow analysis for variance due to treatment, times, sites and fractions.

Latin Square design experiments provide a basis for investigation of variance due to individual animals. It can help uncover a consistent bias in data due to some peculiarity of the individual. In its basic form, there are as many animals as there are treatments, and each animal receives each treatment in a randomized sequence over successive periods of time (Table 1). In any period, no two animals receive the same treatment [42]. The data can be analysed for variance due to treatment, period and animal; any interactions are treated as residual variability (error). In this case, the animal is managed as an individual and is the experimental unit. Interactions

Table 1. A Latin Square design

	Period 1	Period 2	Period 3	Period 4
Treatment 1	Animal (Group) 2	Animal (Group) 4	Animal (Group) 1	Animal (Group) 3
Treatment 2	Animal (Group) 4	Animal (Group) 1	Animal (Group) 3	Animal (Group) 2
Treatment 3	Animal (Group) 3	Animal (Group) 2	Animal (Group) 4	Animal (Group) 1
Treatment 4	Animal (Group) 1	Animal (Group) 3	Animal (Group) 2	Animal (Group) 4

between animal, treatment and time period are embedded in the residual variability (error term).

A Latin Square design can also be based on a group of animals managed together as the experimental unit, so that a more aggregated view of effects of treatment and period is achieved. Samples may be taken at successive intervals at times within the experimental period relating to events or time elapsing since imposition of the respective treatment. Samples may be taken from different sites, fractionated and replicated, to allow analysis for variance due to treatment, animals, periods, times within periods, sites and fractions. The samples can be physically bulked across animals within a given treatment group for each time, site and fraction of sample. However, this means that individual animal variability cannot be isolated. If the samples are analysed separately, individual data can be viewed (any outliers?), but the data will still be analysed on the basis of the treatment group; the individual variability within groups becomes part of the residual variability (error term).

The issue of treatment sequence and its effects on the microbial population also becomes important in the Cross-over and Latin Square designs, because no two animals receive the same sequence of test treatments. In analysis of the data, this has the effect of lumping together the different carry-over effects. If there are any carry-over effects of a preceding treatment on the microbial succession under the new treatment, this will increase the heterogeneity of the data attributed to the current treatment, and in analysis this will appear in the error term. Therefore, there is a need to reduce any influence of carry-over effects. This can be achieved by including longer periods for adaptation to the new set of treatments. Another approach is to return all animals to a common set of conditions during an interval before imposing the new treatment. All these strategies are expensive in use of resources including time and in some cases are not warranted.

Time and site of sampling

The following section relates particularly to the study of the microbiology of the rumen, though some considerations may help in choice of sampling procedures in other compartments of the digestive tract.

Sampling time can be a most critical decision depending on the objectives of the experiment. Since the current potential to track the dynamics of microbial population change by repeated sampling is strongly constrained by cost and time, most researchers

will have to arrive at a restricted sampling schedule based on their knowledge of time patterns in the changing environment in the compartment of the digestive tract under investigation. The major environmental factors implicated are rates of entry of new substrates, their individual rates of fermentation and the concentrations or rates of accumulation of end products (rate of production minus rate of removal). A review of existing mechanistic models that predict rumen function can help gain some overview of the important factors involved, but most of these aggregate to a daily average level for predicted variables [6, 16, 22, 39]. For shorter-term fluctuations during a day, the reader should refer to individual published papers such as Dixon et al. [17] and Williams et al. [45]. In broadest terms, the chemical composition and physical form of the dietary ingredients and time patterns of ingestion set the substrate entry rates and changes in rates of their fermentation. For dietary carbohydrates, the rate of accumulation of fermentation end products is very broadly associated with pH of the digesta and for dietary N compounds, with digesta ammonia concentration. Both of these variables can reflect important changes in the conditions affecting the relative competitive success or fitness of various functional classes of micro-organisms, though evidence has mostly been indirect through measured changes in rates of digestion of, for example, dietary fibre. In Figs. 1 and 2, taken from Williams et al. [45] a few times for sampling are proposed in order to detect the most likely times at which important changes in numbers, growth rates or species composition of the microbial population will be apparent.

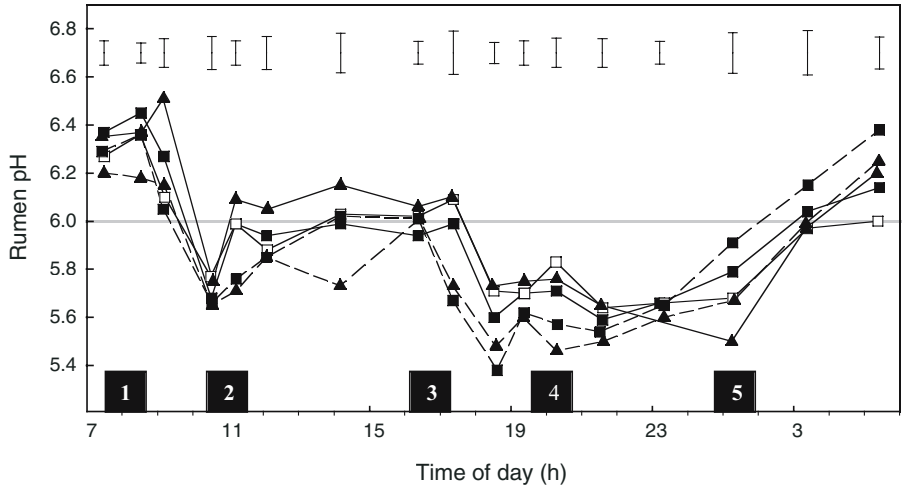

Figure 1. Diurnal pattern of rumen pH in cows grazing perennial ryegrass – based pastures alone at low (■—) or high (—) allowances or at low allowance and receiving a grain pellet (■- - -), hay cube (▲—) or grain/hay cube (▲- - -). Bar blocks along the 'time' axis indicate priority sampling times; open blocks indicate transition sampling times for comparison of microbial population as they change with time and different dietary conditions. The error bars indicate the s.e.d. for comparing between dietary treatments at each time. Based on Williams et al. [45].