**Use of rumen *in vitro* techniques to perform a preliminary assessment of novel feed ingredients**

The use of in vitro techniques provides a rapid low cost means to assess the nutritive value of forages, individual feeds and mixed rations. However, rumen *in vitro* techniques cannot replicate rumen activity, merely model it. Tilley and Terry (1963) established the *in vitro* determination of diet digestibility. The end point model of Tilley and Terry (1963) has been adapted to measure rumen fermentation kinetics of feeds, feed combinations and the effect of dietary additives by using headspace gas pressure (Pell and Schofield 1993, Theodorou *et al*., 1994)and has been extensively reviewed by Rymer *et al.*; (2005) and Getachew *et al.* (2005).

Absence of a fermentation\*host interaction means that *in vitro* techniques are not confounded by host absorption facilitating a measure of VFA, NH3, CO2 and CH4 production. However, the highly buffered nature of in *vitro* cultures makes detecting effects of treatment on rumen pH challenging. Some of these challenges may be overcome by weakening the buffer strength or increasing the readily fermentable fraction of the substrate to maximise opportunity of observing a treatment effect. Determination of protein degradability *in vitro* is however more complicated. The form of N fed to the culture in the diet will change to be partially (or totally) incorporated into microbial N such that, for a culture Nin=Nout. To be able to calculate diet protein degradability and microbial growth a determination of microbial N is therefore required. Methods exist for measuring *in vitro* microbial protein (Makkar *et al.*, 1982) but further validation is required.

Rumen *in vitro* techniques are ideal for testing feedstuffs, products and scenarios that would be difficult to test in any other way. Such as determining the fermentability/degradability of powders and liquids or implication of a range different silage fermentation stoichiometry on rumen function.

Other applications of rumen *in vitro* methods include:

* Bioavailability of dietary minerals and uptake by the rumen microbial population.
* Impact of including tannin in ruminant diets.
* Dietary factors effecting the rumen survival of *Treponema* spp. and the implications for animal health (interdigital dermatitis).
* Mode of action and rumen effect of essential oils (EO) and resilience of rumen protection methods for PUFA, EO and AA.

Yerby (in press) has taken a novel approach to *in vitro* techniques and employed them to investigate intestinal development in calves. Further, in other species intestinal fluid has been used as an inoculum source and applied to the investigation of caecal/hindgut fermentation in pigs (Anguita *et al.,* 2006) and horses (Bachmann *et al.,* 2020).

Testing novel rumen methane inhibitors that maintain diet degradability and a desirable rumen VFA profile is currently a major research objective. Using rumen *in vitro* techniques then a wide range of chemicals, compounds and natural products can be tested to determine their effect on rumen fermentation. Lower limits for effective response and upper limits to extremis can be determined, leading to the proposal of an effective dose levels for *in vivo* dietary inclusion without out compromising animal health. Inherent to these sorts of experiment is the collection of headspace gas. Total volume and composition can be measured. This is easier with the method of Pell and Schofield (1993) rather than the automated equivalent of Theodorou *et al.* (1994). However, if measuring the pattern of methane evolution is the objective then manual gas production is the more reliable method.

Many *in vivo* studies are limited by animal numbers especially when using rumen fistulates. Whereas *in vitro* studies represent a boundless opportunity of experimental design. Rumen *in vitro* diet-type\*treatment interactions and level treatment inclusion are possible even to extremis without compromising animal health. Increasing the number of interactions or factors increases statistical sensitivity but, the danger comes in designing studies with too many interactions/factors. Interpretation of two or three way interactions do not normally cause too much issue. However, because only your imagination, time to sample and physical capacity are your limitations it could be possible for example to increase the number of factors in a two way design (>4\*4), or overlay a two or three way interaction experiment with a level of addition effect, or nest structures within a relatively simple two or three way design. This would not be wise and can be incredibly difficult to interpret in a meaningful way.

Some feed additives require a lead time of several days to exert their effects on the rumen microbiome. Therefore, adaptation of donor animal to the additive may be required or desired to maximise opportunity of observing an effect *in vitro*. This leads to the proposition of making the rumen fluid the test variable and substrate the standard. These experimental designs work best in conjunction with Latin square designed *in vivo* studies where the effect of different animals is removed. However, experience suggests that the effect being investigated needs to be quite large in order to be observed in a diluted and highly buffered situation of an *in vitro* analysis.

Treatment persistency in the rumen environment can be investigated using continuous culture techniques. These can be carried out in relatively simple reactors or in RUSITEC and permit many of the advantages of batch culture techniques whilst exploring the persistency of treatment effect. Continuous cultures also have the same advantage as batch cultures of being able to test dietary treatments to extremis without compromising animal health but, require more time to feed, deal with waste and prepare artificial saliva making them considerably more time consuming.

Standardised host diet, time of rumen fluid collection and test diet preparation facilitates an evaluation and comparison of TMR from different sources. Diet ingredient interactions within the rumen mean that the fermentation resulting from a TMR is not equal to the sum of its individual components. Using a standardised batch culture technique it is possible to determine how a diet will ferment, degrade, produce VFA (concentration and mix) and methane in the headspace. By extension this has enabled development of a method for evaluating diet compositions for reducing methane emissions.

In conclusion validation of treatment effects observed *in vitro* are ultimately necessary allowing *in vivo* studies to be designed with greater knowledge of treatment effects and limits.

**References**

Anguita M., Canibe N., Perez J.F. and Jensen B.B. (2006). Influence of the amount of dietary fiber on the available energy from hindgut fermentation in growing pigs: Use of cannulated pigs and in vitro fermentation. Journal Animal Science 84(10) 2766-2778.

Bachmann M, Glatter M., Bochnia M., Wensch-Dorendorf M., Greef J.M. Breves G. and Zeyner A. (2020). In vitro gas production from batch cultures of stomach and hindgut digesta of horses adapted to a prebiotic dose of fructooligosaccharides and inulin, Journal of Equine Veterinary Science 90 103020.

Getachew G., DePeters R.J. Robinson P.H. and Fadel J.G. (2005). Use of an in vitro gas production technique to evaluate fermentation of ruminant feeds and its impact on fermentation products. Animal Feed Science and Technology 123-124 (1) 547-559.

Makkar H., Sharma O and Dawra R (1982). Simple determination of microbial protein in rumen liquor. Journal of Dairy Science 65(11) 2170-2173.

Pell A.N. and Schofield P. (1993). Computerised monitoring of gas production to measure forage digestion in vitro. Journal of Dairy Science. 76(4) 1063-1073.

Rymer C., Huntington J.A., Williams B.A. and Givens D.I. (2005). In vitro cumulative gas production techniques: History, methodological considerations and challenges. Animal Feed Science and Technology 123-124(1) 9-30.

Theodorou M.K., Williams B.A. Dhanoa M.S. McAllan A.B and France J. (1994). A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Animal Feed Science and Technology 48(3-4) 185-197.

Tilly J.M.A. and Terry R.A. (1963). A two-stage technique for the *in vitro* digestion of forage crops. Grass and Forage Science 18(2) 104-111.