





Statistical options for the analysis of *in vitro* gas production profiles illustrated using rumen liquor as the inoculum[†]

M.S. Dhanoa¹, R. Sanderson², S.J. Lister², R.M. Mauricio³, S. López⁴ , J.L. Ellis¹, C.D. Powell¹ and J. France¹ 

Modelling Animal Systems Research Paper

[†]This article was previously published with an incorrect author name. A notice detailing this has been published and the error corrected in the online PDF and HTML version.

Cite this article: Dhanoa MS, Sanderson R, Lister SJ, Mauricio RM, López S, Ellis JL, Powell CD, France J (2023). Statistical options for the analysis of *in vitro* gas production profiles illustrated using rumen liquor as the inoculum. *The Journal of Agricultural Science* **161**, 686–695. <https://doi.org/10.1017/S0021859623000588>

Received: 8 December 2022
Revised: 17 September 2023
Accepted: 2 November 2023
First published online: 29 November 2023

Keywords:

ante-dependence analysis; modified analysis of variance; multivariate analysis of variance; multivariate distance methodology; residual maximum likelihood

Corresponding author:

J France;
Email: jfrance@uoguelph.ca

¹Department of Animal Biosciences, University of Guelph, Guelph, ON, Canada; ²Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Aberystwyth, UK; ³Department of Biotechnology, Federal University of São João del-Rei (UFSJ), São João del-Rei MG, Brazil and ⁴Instituto de Ganadería de Montaña (IGM), CSIC-Universidad de León, Departamento de Producción Animal, Universidad de León, León, Spain

Abstract

The use of repeated measures analysis of variance (ANOVA) options for the analysis of *in vitro* ruminal fermentation gas production profiles is illustrated. Because of the different variances and covariance structures among profile observations, ordinary ANOVA for more than two-time points is not recommended. To mitigate this problem, the Greenhouse–Geisser epsilon correction can be applied to reduce the degrees of freedom, inflated by violation of the sphericity assumption, for *F* ratio probability calculations. After this correction, the Box–Greenhouse–Geisser ANOVA (modified ANOVA) layout appears similar to the layout of a split-plot design ANOVA with whole plots divided into subplots (incubation time). Any *F* tests in the main plot part are valid but *F* tests involving the time factor from the subplot part need modification because time factor, by its very nature, cannot be allocated at random. Application of multivariate ANOVA, distance multivariate ANOVA, ante-dependence and mixed model analysis are also considered. All these options lend themselves to wide application in the applied biological sciences.

Introduction

To enhance *in vivo* capacity for animal feed evaluation, the use of the *in vitro* gas production (GP) technique has increasingly spread over the last decades. Following on from the manual systems of, e.g. Menke and Steingass (1988) and Theodorou *et al.* (1994), the capacity of the *in vitro* GP system of Theodorou *et al.* (1994) was enhanced by a semi-automation modification (Mauricio *et al.*, 1999). Further progress was made with the arrival of commercial systems such as the ANKOM equipment (ANKOM, 2015). Moreover, in addition to inoculum derived from rumen fluid, further research has shown that inoculum (microbial and fungal) from non-ruminants and also from faecal matter are viable options (El-Meadaway *et al.*, 1998; Mauricio *et al.*, 2001; Nielsen *et al.*, 2002; Lopez *et al.*, 2007). The spectrum of use of the *in vitro* GP technique now includes characterization of human diets and diet components using inocula from pig (Williams *et al.*, 2011) and human faeces (Lu *et al.*, 2022). The effects of substrate and inoculum treatments on *in vitro* GP fermentation profiles have been studied with respect to model parameters and functions of those parameters (France *et al.*, 1993, 2000; Dhanoa *et al.*, 2000). In parallel, analysis of variance (ANOVA) of GP profile data at each time point has also been undertaken. However, ANOVA without accounting for ante-dependence associations will only give gross treatment effects rather than the net effects at those time points in the GP profiles.

Box (1950), citing Wilks (1946), illustrated a compound symmetry test of the hypothesis that all variances (diagonal values) and all covariances (off-diagonal values) are respectively equal (matrix V_0) *v.* the alternative hypothesis that all variances (diagonal values a, b, c) and all covariances (off-diagonal values d, e, f) are (respectively) not equal (matrix V_1). Illustrating for a case of three-point profile-variates:

$$V_0 = \begin{bmatrix} a & d & d \\ d & a & d \\ d & d & a \end{bmatrix} \quad V_1 = \begin{bmatrix} a & d & e \\ d & b & f \\ e & f & c \end{bmatrix}$$

Box (1954a, 1954b) subsequently defined an index of sphericity (ϵ_{Box}) to check for this symmetry, obtained following application of the formula (from Abdi, 2010):

$$\epsilon_{\text{Box}} = \frac{\left(\sum_i s_{ii} \right)}{(\alpha - 1) \sum_{\substack{ij \\ i \neq j}} s_{ij}^2}$$

© The Author(s), 2023. Published by Cambridge University Press. This is an Open Access article, distributed under the terms of the Creative Commons Attribution licence (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted re-use, distribution and reproduction, provided the original article is properly cited.



Here ζ_{ij} are entries (row i , column j) in the population variance-covariance matrix and α is the number of profile samples.

Following the work of Box, Greenhouse and Geisser (1959) proposed a notation for V_1 by assuming that each individual profile is a random vector sampled from a p -variate normal distribution with an arbitrary variance-covariance matrix (Δ as on left side below) with further assumption that the p -variables have the same metric which is necessary if one considers that group profiles have the same shape (Δ on the right side below).

$$\Delta = \begin{bmatrix} \sigma_1^2 & \rho_{12}\sigma_1\sigma_2 & \cdots & \rho_{1p}\sigma_1\sigma_p \\ \rho_{12}\sigma_1\sigma_2 & \sigma_2^2 & \cdots & \rho_{2p}\sigma_2\sigma_p \\ \vdots & \vdots & \ddots & \vdots \\ \rho_{1p}\sigma_1\sigma_p & \rho_{2p}\sigma_2\sigma_p & \cdots & \sigma_p^2 \end{bmatrix} = \begin{bmatrix} \sigma_1^2 & \sigma_{12} & \cdots & \sigma_{1p} \\ \sigma_{12} & \sigma_2^2 & \cdots & \sigma_{2p} \\ \vdots & \vdots & \ddots & \vdots \\ \sigma_{1p} & \sigma_{2p} & \cdots & \sigma_p^2 \end{bmatrix}$$

In the case of growth curve and repeated measures profiles, correlations among the near-time observations tend to be larger compared to observations that are a greater time apart. That means the profile data variance-covariance matrix is likely to be similar to V_1 (or Δ on left) rather than V_0 . This being the case, Greenhouse and Geisser (1959) showed that F ratios involving the time factor variances will become biased and will point to lower F probability values. To mitigate the problem, they proposed a (multiplicative) epsilon (ϵ) correction for degrees of freedoms prior to entering F distribution tables to read F probability values.

Greenhouse and Geisser (1959) further discussed how the distribution of F values is affected by various elements of the variance-covariance matrix and hence developed their epsilon correction to reduce degrees of freedoms before calculating F probability values:

$$\epsilon = \frac{p^2(\bar{\sigma}_{tt} - \bar{\sigma}_{..})^2}{(p-1)} \left[\sum \sum \sigma_{t.}^2 - 2p \sum \bar{\sigma}_{t.}^2 + p^2 \bar{\sigma}_{..}^2 \right] \quad (1)$$

In this equation, $\sigma_{t.}$ are the elements of the matrix Δ , $\bar{\sigma}_{tt}$ is the mean of the diagonal terms, $\bar{\sigma}_{t.}$ is the mean of the t th row (or t th column), $\bar{\sigma}_{..}$ is the grand mean, and p is the number of profile samples. For any p -time point profile, the lower bound of epsilon will be $\epsilon = 1/(p-1)$ and it is independent of variance-covariance matrix values (the authors suggest its use when profile time-points are greater than the design units net of profile samples; design units = number of profile samples \times number of replicates). However, actual epsilon value based on profile sample data needs to be calculated (using Eqn (1) above and the method described in Abdi, 2010) if net design units or subjects are greater than the profile time points (Greenhouse and Geisser, 1959).

When the epsilon estimate is close to unity, the Greenhouse-Geisser correction tends to be conservative because of their ϵ value being an underestimate whilst the correction of Huynh and Feldt (1976) tends to overestimate ϵ (Abdi, 2010). Therefore, recommended practice is to use the Greenhouse-Geisser correction if $\epsilon < 0.75$ but use the Huynh-Feldt correction if $\epsilon > 0.75$. The formula for the Huynh-Feldt correction (from Abdi, 2010) is

$$\hat{\epsilon} = \frac{s(\alpha-1)\hat{\epsilon} - 2}{(\alpha-1)[s-1 - (\alpha-1)\hat{\epsilon}]}$$

where $\hat{\epsilon}$ is the estimate for ϵ ; s the number of subjects; and α the number of profile samples.

The objective of our work was to illustrate the use of a modified ANOVA procedure (modANOVA) using the Genstat procedure AREPMEASURES (Payne, 2022), which produces an ANOVA for repeated measurements. The procedure modANOVA incorporates the refinements proposed by both Box (1950) and Greenhouse and Geisser (1959), which are discussed above. Various aspects of relevant methods are simply described but more mathematical details can be found in the cited references. Along with modANOVA, other approaches such as ante-dependence analysis, multivariate ANOVA or residual maximum likelihood parameter estimation in mixed models, provide suitable alternatives for the analysis of repeated measures structured data for the purpose of treatment comparisons. Raw GP profile data are repeated measures in nature, and these alternatives can be applied for elucidating the effects of substrate, additives, treatments or inocula on rumen fermentation kinetics. It should be stated that modelling the GP profile trends of design unit curves remains a valuable tool for the enhancement of experimental information and scientific knowledge in general.

Materials and methods

Handling of GP data profiles resembles, as for growth curves, repeated measurements and sometimes multivariate analysis. There are choices that can be made for the comparison of substrate and inoculum treatment effects. Some of the options available are as follows. These are implemented herein using the statistical software Genstat (VSN International, 2022). Note that the R Project software and SAS, among others, also provide these options.

Option 1

The GP curve for each design unit over the entire incubation period (usually until the upper asymptote is quasi-reached) is modelled with the use of some appropriate function and thereafter ANOVA can be used to compare treatments on the basis of fitted model parameters and measures derived from them. For this purpose, the specially designed model of France *et al.* (1993) or classic growth functions can be used. For the analysis of non-standard GP profiles, recent publications by Powell *et al.* (2020) and Dhanoa *et al.* (2021) cover analysis of monophasic, multiphasic, hybrid-phasic and numerical modelling. Model fitting and ANOVA can be combined by the use of grouped nonlinear regression (Heitjan, 1989), also called parallel curve analysis or simply regression-ANOVA.

Option 2

Using raw GP data, modANOVA based on the methodologies of Box (1950) and Greenhouse-Geisser (1959) for dealing with the effects of unequal variances, covariances and correlations among repeated profile measurements, is a suitable option unlike the use of ordinary ANOVA without accounting for correlations among repeated profile measurements taken for all the experimental units (= sum of replicates for all profile samples = subjects). The modANOVA layout will be similar to that of split-plot design ANOVA (sometimes called split-plot-in-time) but there are important differences with respect to the subplot part F -ratios (see above). The Genstat procedure AREPMEASURES (Payne, 2022) implements the above modifications and produces an analysis of variance for repeated measurements and growth profiles as is the case with *in vitro* cumulative GP data.

Option 3

Another methodology for ANOVA-like analysis is ante-dependence analysis (Gabriel, 1962; Kenward, 1987), which also deals with auto and serial correlation in the inter-profile curve trend. Correlation order-based tests of treatments can point to any emerging or short-term and overall effects. Once the ante-dependence order is determined, e.g. using the Genstat procedure ANTORDER (Ridout and Payne, 2022), this analysis is essentially equivalent to standard ANOVA for a given time point with (for example) previous one or two time points as covariates. More detailed analysis is provided by using Genstat procedure ANTTEST (Payne and Ridout, 2022). Both the procedures ANTORDER and ANTTEST allow actual times of profile samples to be used, thus obviating the need for data to be recorded at equal time points.

Option 4

Using recorded raw data

For the analysis of one or more dependent variables as affected by multiple predictors, multivariate ANOVA (MANOVA; Chatfield and Collins, 1986) is a standard method. Growth or repeated measurement profiles can be analysed with the use of MANOVA (Payne and Arnold, 2022) and it does not require the assumption of sphericity. However, if the time points of the profiles are greater than the main plot residual degrees of freedom ($DF = N - g$; where N = subjects or design units and g = profile samples) then MANOVA cannot be used. This situation is quite common with GP, growth and repeated measurement profile data. However, ante-dependence analysis (Option 3 above) and mixed model analysis (Option 5 below) can still be used. Furthermore, as an alternative, Option 4b via the use of the inter-data-unit-based distance matrix is also available.

Using the data-based distance matrix

Analysis of multivariate distance methodology devised by Gower and Krzanowski (1999) is implemented in Genstat via the procedure MVAOD (Payne and White, 2021). This option provides a breakdown of the sums of squared distances between the design units, similar to that provided for sums of squares in an analysis of variance. The squared distances between the units in a symmetric matrix are either input or calculated from the profile data. The methodology implemented in MVAOD can be regarded as providing an alternative to MANOVA for units whose attributes are not all continuous variables. This method permits profile samples greater than the design residual degrees of freedom. For treatment comparison, a permutation-based probability calculation for treatment effects is available.

Option 5

Residual maximum likelihood (REML), an algorithm for variance parameter estimation in linear mixed models (Patterson and Thompson, 1971), can also handle repeated measurement (hence GP) profile data. In the statistical software Genstat, mixed model features for analysing repeated measurements using REML include: (i) time points variate, (ii) treatments formula, (iii) additional random terms formula, (iv) dealing with equally spaced or irregular time-points, (v) model for correlation within subject across time, (vi) allowance for heterogeneity across

time, and (vii) additional uniform correlation within subject (for details see VSN International, 2022).

Data set 1

Gliricidia trees provide a browse plant and source of fodder for ruminants in the tropics. The data set reported by Lister *et al.* (2000) embraced 25 Gliricidia provenances originating from nine countries (one provenance unidentified) but were grown at a single site in Honduras (El Zamorano at 14° 1' N, 87° 2' W). Full details are given in Lister *et al.* (2000) where near-infrared spectra-based analysis is described. Samples from these trees were incubated *in vitro* for up to 140 h using ovine rumen fluid as the inoculum, and cumulative fermentation GP was recorded at 3, 6, 9, 15, 19, 24, 27, 33, 38, 44, 51, 59, 69, 82, 96, 120 and 140 h (17 time points, see observed values in Fig. 1). France *et al.* (1993) used some of the GP curves to test the fit of the Mitscherlich equation to GP data over the incubation time span, so that effects of country of origin on the model parameters (asymptotic GP or half-life from fractional fermentation rate) could be assessed. This data set is used to demonstrate modANOVA using AREPMEASURES (Option 2). For this option, the number of time points must be less than the degrees of freedom for the experimental error applicable to whole unit comparisons (in this particular case equal to the total number of provenances minus the number of different countries of origin, i.e. $25 - 10 = 15$). Thus, only 14 incubation times could be used, and GP values recorded at 69, 120 and 140 h were excluded from the original data set to run the repeated measures ANOVA (Option 2).

Data set 2

These data were generated in three experiments reported by Mauricio *et al.* (2006) using a semi-automatic *in vitro* GP technique (Mauricio *et al.*, 1999). In each experiment, the inoculum used was bovine rumen fluid and 15 GP profiles were obtained by recording cumulative GP at 2, 4, 6, 8, 10, 12, 16, 19, 24, 30, 36, 48, 60, 72 and 96 h of incubation. In Experiment 1, which used five replicates and four treatments (cutting age: 56, 84, 112 and 140 d), the substrate was *Andropogon gayanus* hay. The data (Data Set 2a) are used for MANOVA and distance-MANOVA (Option 4a and 4b, respectively). Experiment 2 examined four tropical grasses: elephant grass (*Pennisetum purpureum*), sugar cane (*Saccharum officinarum*) and two hybrids of sorghum with Sudan grass (*Sorghum bicolor* × *Sorghum sudanense*) using three replicates. These data (Data Set 2b) are used for ante-dependence analysis with Genstat procedure ANTTEST (Option 3). Experiment 3 examined silages of sunflower (*Helianthus annuus*) cut when 100% of the grains were mature. Four genotypes were considered (Rumbossol 91, Victoria 627, Victoria 807 and Mycogen 93338) and three replicates per genotype were used. These data (Data Set 2c) are used for mixed model analysis using the REML algorithm (Option 5).

Results

Modelling the trends of design units generated by repeated measurements adds to the information contained in the raw data in terms of kinetics, model parameters and meaningful functions of those parameters (France *et al.*, 1993; Powell *et al.*, 2020; Dhanoa *et al.*, 2021). This is illustrated in Fig. 1 for Data Set 1,

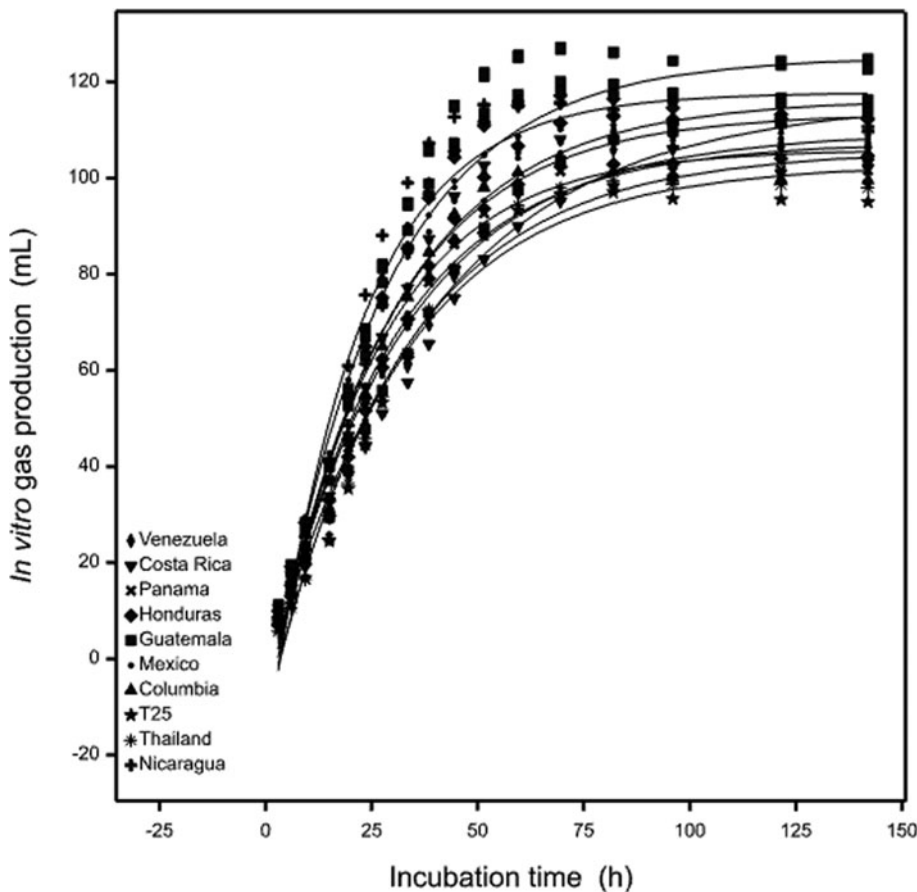


Figure 1. *In vitro* gas production profiles of 25 *Gliricidia* provenances with country of origin (as described by Lister *et al.*, 2000; T25 has no provenance).

with the lines showing the fitted curves when the diminishing returns equation of Mitscherlich (1909) is fitted. Results obtained with grouped nonlinear regression (Option 1) are detailed in Table 1. The accumulated analysis of variance from grouped regression shows that the function (Mitscherlich) used to fit the data accounted for most of the variability (93.2%). The inclusion of the effect of γ -intercept increased this percentage to 95.4%,

whereas the effects of x -axis curve position and curve shape only increased this latter value to a marginal extent. Option 1 allows us to evaluate the suitability of the model to fit the data over incubation time and to determine the effects of the main source of variation among curves (in this case country of origin of the provenances) on the parameters defining the intercepts, position and shape of the GP curves. Other options (2–5) can

Table 1. Grouped regression ANOVA when modelling *in vitro* gas production profiles using the Mitscherlich (1909) equation (Option 1, Data Set 1; Lister *et al.*, 2000)

Change *	df	Sum of squares	Mean square	F value	P value	Adjusted R ²	s.e.
+model fit ^{†1}	2	530 848.26	265 424.13	4635.45	<0.001	93.2	9.58
+A (origin) ^{†2}	9	13 063.01	1451.45	25.35	<0.001	95.4	7.88
+B (origin) ^{†3}	9	1721.72	191.30	3.34	<0.001	95.6	7.69
+C (origin) ^{†4}	9	1294.76	143.86	2.51	0.008	95.7	7.57
Residual	395	22 617.55	57.26				
Total	424	569 545.30	1343.27				

df, degrees of freedom; F value, variance ratio; P value, F ratio probability; SE = standard error of observations.
 Response variable: GP = cumulative gas production for 25 *Gliricidia* provenances originating in 10 different countries (locations).
 Explanatory variable: t = incubation time (h).
 Fitted equation: $GP = A + B \times C^t \equiv A + B \times e^{-kt}$,
 where A = upper asymptote; B is a parameter related to the position of the curve in the x -axis; $C = e^{-k}$ (constrained to $C < 1$), k = fractional rate (parameter determining the curve shape).
 Accumulated analysis of variance (from grouped regression).
 * Change: '+' indicates addition of individual factor intercepts and origin slopes to overall Mitscherlich equation fit.
 †1 model fit = overall Mitscherlich equation fit, i.e. effect of incubation time on GP using the equation.
 GP = $A + B \times R^t$, so that the adjusted R^2 for this factor is the percentage of variance explained by the model.
 †2 A (origin) = γ -intercepts for each origin, i.e. effect of country of origin on the parameter A.
 †3 B (origin) = x -axis curve position, i.e. effect of country of origin on the parameter B.
 †4 C (origin) = separate nonlinear curves or curve shape, i.e. effect of country of origin of the parameter C.

Table 2. Modified Box–Greenhouse–Geisser analysis of variance (modANOVA; Option 2) with Data Set 1 (gas production profiles for 25 *Gliricidia* provenances originated from 10 different countries, each profile comprises gas production recordings at 14 incubation times)^a

Source of variation	df	Sum of squares	Mean square	F value	P value
Among subject stratum					
Country of origin (O)	9	10 522.26	1169.14	2.07	0.102
Residual (error for whole unit comparisons)	15	8458.12	563.87	36.86	
Within subject stratum (among subunits within whole units) ^b					
Incubation time (T)	13	432 927.36	33 302.10	2176.78	<0.001
Interaction O × T	117	3230.32	27.61	1.80	0.096
Residual (error for subunits comparisons)	195	2983.26	15.30		
Total	349	458 121.32			

df, degrees of freedom; F value, variance ratio; P value, F ratio probability.

^aThe analysis considers the 25 *Gliricidia* provenances as the whole units or subjects, and the 14 incubation times as the subunits within the whole units.

^bThe df in the within subject stratum were multiplied by the correction factor (Greenhouse–Geisser $\epsilon = 0.1241$) before calculating F probabilities (if no epsilon correction is applied the P value would be 0.00014428).

be used for the analysis of GP profile data, and those outlined in this work should serve that purpose.

A modified analysis of variance (modANOVA; Option 2) is illustrated in Table 2 using Data Set 1 (limited to 14 time points). The variance–covariance matrix for these data showed variances within each time increasing from 1.87 (at 3 h) to 105.4 (at 38 h) then decreasing to 31.1 at 96 h of incubation; with a mean value of 54.5. The average covariance and correlation between incubation times were 39.2 and 0.719, respectively. However, there was a large variation in covariance, minimum (1.4) with values at 3 h and maximum (up to 100.8) with values at 33 or 38 h of incubation. Box's test (both χ^2 and F test) indicates that compound symmetry of the variance–covariance matrix cannot be assumed ($P < 0.001$). Thus, data can be analysed by repeated measures ANOVA without assuming sphericity, using the Greenhouse–Geisser method. The derived Greenhouse–Geisser correction factor for the data ($\epsilon = 0.124$). The modified Box–Greenhouse–Geisser analysis of variance with the adjustment accounting for the value of epsilon is given in Table 2. The analysis showed no significant effect of the country of origin of the *Gliricidia* provenance on average GP ($P = 0.102$), which was confirmed in a multiple comparisons of means using the Student–Newman–Keuls test (Thomas, 1973). Although the *Gliricidia* forage tree provenances were from different countries, samples for *in vitro* GP were collected from trees grown at a single site. The site's soil attributes and the local environment appear to have had a defining effect on the nutritional quality of these trees. The effect of incubation time is large because these GP profiles were recorded as cumulative GP increased steadily as incubation time lengthened. If interest is in the net values at the time-points then the first difference of the trends of the profile curve may be used instead, as suggested by Box (1950).

Ante-dependence analysis (Option 3) is demonstrated in Table 3 using Data Set 2b. Application of the procedures ANTORDER and ANTEST brings out details about the serial- or auto-correlation structure–property of the data set profiles. First, the extent of serial correlation between zero-, first- and second-order (or higher) is determined (first part of Table 3). After pairwise testing of serial order, pooled order evidence is garnered for the overall order (order 2 in this case; the second part of Table 3) for ante-dependence analysis using ANTTEST. Assuming order 2, the third part of Table 3 details treatment

effects (3 columns after the time column) at each data profile step. These three columns give cumulative treatment effects up to each relevant time point. In this case, treatment effect significance is established from 6 h of incubation (incubation time number 3 in the sequence) onwards.

Multivariate analysis of variance and covariance (MANOVA; Option 4a) using recorded raw GP data (Data Set 2a) is illustrated in Table 4. The procedure analyses the data variates by ANOVA first as *y*-variates, and then as covariates in order to obtain the sums of squares and products (SSP) matrices. The SSP matrices are then adjusted for the covariates (e.g. using matrix manipulation in Genstat's CALCULATE directive; VSN International, 2022), and latent root and vector decompositions are done before the test statistics are calculated (using CALCULATE). MANOVA calculates Wilks lambda (with approximate F test) and the Pillai–Bartlett trace among other test statistics (Chatfield and Collins, 1986). Wilks lambda test demonstrates the strength of the relationship between the dependent variable (MANOVA allows more than one dependent or *y*-variable) and the predictor variables (the lower the value of lambda the better the explained variation). The actual lambda value indicates the proportion of total variation not explained by the MANOVA model; this is rather different from an ANOVA F test which tests the explained part of total variance. The lambda test provides an overall test of the significance of treatment effects. Rao's F probability provides an expansion of Wilks's criterion (Rao, 1951). If the probability is ≤ 0.05 then significant differences among treatment means are indicated. The profile sample times need to be equal for MANOVA. If data are recorded at unequal time points, then it will be necessary to calculate interpolated data at equal time points (Dhanoa *et al.*, 2021).

Multivariate analysis of design inter-unit distances (MVAOD; Option 4b) using Data Set 2a is demonstrated in Table 5. Data variates may not all be continuous and distributional requirements may not be fully complied with for standard MANOVA (Option 4a). Additionally, MVAOD provides an alternative when the size of profile samples is greater than net design units. In MANOVA, actual data values are used but the alternative methodology of MVAOD uses design inter-unit distances across all profile samples generating a dissimilarity matrix. Instead of the sum of squares of actual data values as in simple ANOVA, in this analysis, the sum of squared distances is used. The total

Table 3. Ante-dependence analysis (Option 3) using Data Set 2b sequential comparison of ante-dependence structures

	Unadjusted χ^2 -statistic	Adjustment factor	Adjusted χ^2 -statistic	df	P value	
Order 0 v order 1	563.02	0.538	303.17	13	<0.001	
Order 1 v order 2	148.53	0.455	67.51	12	<0.001	
Order 2 v order 3	48.51	0.370	17.97	11	0.082	
Order 3 v order 4	58.08	0.286	16.59	10	0.084	
	Unadjusted χ^2 -statistic	Adjustment factor	Adjusted χ^2 -statistic	df	P value	
Order 0 v order 4	818.15	0.409	334.36	46	<0.001	
Order 1 v order 4	255.13	0.367	93.55	33	<0.001	
Order 2 v order 4	106.59	0.335	35.70	21	0.024	
Order 3 v order 4	58.08	0.286	16.59	10	0.084	
Test for change at each time* ¹				Overall test up to each time* ²		
Time	Statistic	df	P value	Statistic	df	P value
1	1.482	3	0.686	1.482	3	0.686
2	1.828	3	0.609	3.332	6	0.766
3	17.442	3	<0.001	23.047	9	0.006
4	1.468	3	0.690	23.866	12	0.021
5	18.580	3	<0.001	43.478	15	<0.001
6	30.687	3	<0.001	75.643	18	<0.001
7	5.034	3	0.169	80.245	21	<0.001
8	4.425	3	0.219	84.320	24	<0.001
9	38.977	3	<0.001	124.543	27	<0.001
10	0.734	3	0.865	124.762	30	<0.001
11	8.227	3	0.042	132.839	33	<0.001
12	7.570	3	0.056	140.262	36	<0.001
13	3.343	3	0.342	143.356	39	<0.001
14	1.442	3	0.696	144.532	42	<0.001

Assessment of the order of ante-dependence for repeated measures data (GENSTAR ANTORDER procedure)

Comparison of ante-dependence structures with maximum order (4).

From the above two tests an ante-dependence order 2 is appropriate for ANTTEST procedure.

Overall tests of treatment terms assuming an order 2 of ante-dependence structure (GENSTAT ANTTEST procedure).

Tests 'at each time (*¹)' and 'test up to each time (*²)' illustrate emerging effects of the treatments.

Overall test using data from all the times: statistic 164.289, df 48, $P < 0.001$.

Table 4. Multivariate analysis of variance (MANOVA; Option 4a) using *in vitro* gas production (GP) profiles of Data Set 2a
Test statistics and probabilities from permutation tests.

Source of variation	df	Wilks lambda	Rao F	F df1	F df2	F P value	Permutation* P value
Cutting age	3	0.0001132	3.03	45	7	0.068	0.047
Source of variation	df	Pillai-Bartlett trace			Permutation* P value		
Cutting age	3	2.759			0.011		

The GP recorded at each incubation time constituted one of the y -variables included in this MANOVA (for a total of 15 y -variables).

* Permutation probabilities based on 999 random permutations.

squared distance between the units is partitioned into components that can be explained by each of the terms (e.g. treatment factors) in the design-based model. The advantage of this distance-based analysis is that it can handle various types of data. The units may often be described by a collection of attributes

ranging from continuous measurements to categorical variables, such as the presence or absence of a particular feature. Output from MVAOD looks similar to the one-way ANOVA output but there is no F test available, instead treatment significance probability is calculated from 999 random permutations. The distance

Table 5. Multivariate analysis of design inter-unit distances (MVAOD; Option 4b) for Data Set 2a. Analysis of design inter-unit distances

Term	df	Sum of squared distance		P value*
Cutting age	3	1.043		0.001
Residual	16	0.628		
Total	19	1.671		
Cutting age (d)	56	84	112	140
56	0.00000			
84	0.07243	0.00000		
112	0.03173	0.03341	0.00000	
140	0.24843 ^a	0.29608 ^a	0.15255 ^a	0.00000

* Probabilities determined from 999 random permutations.

Distances among treatment levels.

^aThese distances are relatively large.

matrix from this analysis can be used for further multivariate analyses, e.g. by converting the distances to similarities and then using them as input for a principal coordinates analysis (see PCO directive of Genstat; VSN International, 2022). Summary distances among treatment means shown at the end of Table 5 can be used to look for apparent differences (in this case distances between treatment four and other treatments are relatively large).

Residual maximum likelihood-based mixed model analysis (REML; Option 5) using Data Set 2c is illustrated in Table 6. Any of the available options for the analysis of linear mixed models can be used when applying the Genstat REML directive, including the one for repeated measurements which is relevant for the analysis of *in vitro* cumulative GP profile data. In GP data the replicated design units are the substrate (replicated) samples and are treated as the random effects along with treatments as the fixed effects. Times of the profile readings can be unequal but need to be one of the REML model inputs along with many other model modifying options (for details see VSN International,

2022). In Table 6, information and options of the REML model are listed before the listing of tests for fixed effects. These tests include the Wald statistic (Ward and Ahlquist, 2018) along with the *F* statistic and *F* probability. The fixed effects part also includes information about deleting some terms from the REML model. Here REML suggests dropping the time and treatment interaction term will make a significant change. Like ANOVA and modANOVA (Option 2) superscripts can be calculated for treatment differences (Bonferroni multiple comparisons are used herein; Hsu, 1996). The Akaike information criterion (= 725.31; Snipes and Taylor, 2014) and Schwarz (Bayesian) information criterion (= 940.30; Verbyla, 2019) are measures of REML model goodness of fit, calculated in Genstat using the VAIC procedure (Payne and Cave, 2022). These coefficients are a simple way of ranking the models.

Discussion

In vivo digestibility and *in situ* nylon bag methods (e.g., Blaxter *et al.*, 1956; Ørskov and McDonald, 1979) provided means of assessing animal feed quality but due to the limited capacity of these procedures, laboratory methods were sought to find *in vitro* quality measurements. Menke and Steingass (1988) developed the initial *in vitro* GP technique to speed up forage matter quality testing using rumen liquor as the inoculum. Semi-automated and automated methods of measuring cumulative GP subsequently followed (e.g., Mauricio *et al.*, 1999; ANKOM, 2015). Non-invasive use of many animal species (including humans) led to the use of faecal matter-based inocula. Given the ever-increasing use of *in vitro* GP to evaluate a variety of plant products, food processing by-products, animal feeds and human diets, and such a variety of methods for generating cumulative GP profiles, rapid analysis of GP profile data and sufficient statistical analysis options are needed. Options outlined in this work should serve that purpose. There is always the application of substrate and inoculum treatments that need statistical

Table 6. Residual maximum likelihood-based mixed model analysis (REML; Option 5) for *in vitro* gas production profiles using Data Set 2c (gas production at 2, 4, 6, 8, 10, 12, 15, 18, 21, 24, 30, 36, 48, 60, 72 and 96 h incubation)

●Tests for fixed effects

Fixed effect	Wald statistic	Numerator df	F statistic	Denominator df ^a	P value
Genotype	53.67	3	17.89	8.0	<0.001
Incubation time	26 404.02	15	1760.27	120.0	<0.001
(Genotype × time)	386.65	45	8.59	120.0	<0.001
					Mean
Victoria 627					53.10 ^a
Victoria 807					60.96 ^{ab}
Mycogen					65.89 ^{bc}
Rumbosol					75.08 ^c

^aDenominator degrees of freedom for approximate *F*-tests are calculated using algebraic derivatives ignoring fixed/boundary/singular variance parameters.

REML variance components analysis.

Response variate: gas production (GP).

Fixed model: constant + genotype + incubation time + (genotype × time).

Random model: (genotype × replicates × time) – used as residual term.

Number of units: with three replicates per genotype (4 × 3 × 16) = 192.

Bonferroni test

Akaike information coefficient 725.31.

Schwarz Bayes information coefficient 940.30.

Multiple comparisons between Genotype means.

evaluation for the detection of any significant effects. In addition to modelling the profile-related time dimension of subject curves and the analysis of model parameters and their functions, direct analyses of raw profile data under five methodology options are presented in this paper that can be applied even when individual design unit trends may have different shapes.

Results from the five options, as implemented in the statistical software Genstat, show flexibility of methodology choice and ease of application. It is worth re-iterating that the statistical analyses proposed herein can be implemented in any other statistical software or programming language commonly used (e.g., R Project, SAS, SPSS). The choice of analytical approach (Options 2–5 delineated herein) and successful application depend on the experimental design of the GP study, profile samples and replication of any GP experiment. Some profiles hold valuable kinetic information, so the modelling option (i.e. Option 1) is useful either as the preferred option or as a supplemental one for obtaining further information. Modelling can also identify multiphasic features of the response profile that allow split-curve information to be generated.

In addition to recording the amount of gas produced, the status of substrate degradation can provide further useful information. By recording remaining substrate dry matter (DM) at various stages of fermentation and the final DM remaining, *in vitro* GP can be a suitable surrogate for *in vivo* digestibility (Lowman *et al.*, 1999), *in situ* substrate degradation in the rumen (Sileshi *et al.*, 1996) or *in vivo* extent of feed degradation in the rumen (France *et al.*, 2000). The study of substrate degradation kinetics in the rumen proper is complicated by the particle fractions lost with digesta passage, and an advantage of GP is that substrate can only disappear via microbial degradation (Dhanao *et al.*, 2000). France *et al.* (1993) linked GP information to that obtained from the rumen proper, proposing a formula for calculating ruminal extent of substrate degradation while accounting for relevant passage rate and hence the losses. Another facet of the GP technique is fluid from the incubation bottles can be used to determine the volatile fatty acid composition of the test feed and hence the $C_2:C_3$ ratio (Morvay *et al.*, 2011). In GP studies, sufficient replicates are required to derive robust and representative GP profiles and reliable fermentation kinetics that can be extrapolated to the rumen itself. In addition, replication is required to analyse the effects of substrate and inoculum on fermentation kinetics, or for the comparison among experimental treatments applied in each study.

The statistical analysis methodologies identified and illustrated in this communication are for ANOVA-like comparison of substrate and inoculum treatments in any study-design generated GP profile. For a single-phase GP curve, grouped regression is straightforward to implement and to make treatment comparisons in terms of trend shape and position of the GP curve. However, this will become more complicated if GP curves consist of multiphasic subsections, which appear more likely to arise when using voided dung rather than rumen fluid as the inoculum. For modelling identifiable bi-phasic and tri-phasic GP curves, Dhanoa *et al.* (2021) used single- and double-node connected functions (either a doubled-up same function or hybrid pairs). Modified ANOVA for repeated measures splits variance between and within plots and considers the structure of the variance-covariance matrix to assess the statistical significance of treatments and time effects. Ante-dependence analysis is useful to detect the critical incubation times at which differences between treatments become relevant. Multivariate ANOVA (i.e. MANOVA) integrates the information

gathered at all incubation times to analyse the comparison among treatments. Too many profile samples over the plateau part of GP curves may not add much to curve information, likewise over the lag phase, but can make MANOVA use difficult. So, some profile samples may be best left out from the selected analysis. If possible, GP profile readings should be taken at equal time intervals (it is a requirement for Options 2–4a described above), otherwise interpolation is needed to obtain equal time intervals from the trends of each design unit as described in Dhanoa *et al.* (2021). The MANOVA option, like modANOVA, requires that the number of profile time points should be less than the number of design units (the other options do not have this requirement). Option 4b (MVAOD) is an alternative for a multivariate analysis when time intervals are unequal. Finally, repeated measures designs can be analysed as mixed models using REML methodology, a useful tool to discriminate the effects of experimental treatments from that attributed to the incubation time. The choice of one or another option, or the implementation of a variety of them for the analysis of a given data set, will depend on the objectives of each particular study.

The profile data analysis options described in this communication lend themselves to wider applications. In the *in vitro* GP method, a second profile set can be obtained by analysing bottle contents, at the end of incubation, for volatile fatty acids (e.g. acetate and propionate). Profile data responses are common in the agricultural, medical, environmental and other applied sciences. In animal science, for example, sets of diet proteins, fatty acids, minerals, amino acids, etc. should be analysed in totality because of their inter-correlations (multicollinearity). MANOVA and mixed model options are applicable. If comparing monthly climate data at two or more geo-sites using a number of years' data, then the options modANOVA, MANOVA, MVAOD and REML may help. The chemical composition of dairy cow milk at various stages of lactation could be handled using the options described herein. Diurnal patterns of water-soluble carbohydrates in crops are a feature much studied by plant scientists. For designed experiments to test selected treatments, many covariates in addition to the response variate may be recorded, so ANOVA (with or without covariates) tends to be used. Instead of covariates, the response variate might be observed multiple times leading to repeated measurement profile data and options described here will be needed.

Conclusion

The options described herein allow analysis of raw GP data in addition to application of the curve fitting approach. Results from the ante-dependence methodology are indicative of further insights into one's data that should lead to better understanding and improved inference from the relevant experiment. This option, in particular, can be used in conjunction with the other options to extract additional information from the experimental data. The options described are suited to wider application in the applied biological sciences generally.

Author contributions. Conceptualization: MSD, SL and JF; methodology: MSD, RS, SJL, RMM, SL and JF; formal analysis: MSD, RS, SL, JLE, CDP and JF; writing – original draft preparation: MSD and JF; writing – review and editing: MSD, RS, SJL, RMM, SL, JLE, CDP and JF. All authors have read and agreed to the published version of the manuscript.

Funding statement. This work was funded in part by the Canada Research Chairs program, grant number 045867 (Natural Sciences and Engineering Research Council of Canada, Ottawa).

Competing interests. None.

Ethical standards. Not applicable.

References

- Abdi H (2010) The Greenhouse–Geisser correction. In Salkind N (ed.), *Encyclopedia of Research Design*. Thousand Oaks, CA: Sage, 10 pp.
- ANKOM (2015) *Gas Production System Operator's Manual*. Macedon, NY, USA: Ankom Technology.
- Blaxter KL, Graham NMCC and Wainman FW (1956) Some observations on the digestibility of food by sheep, and on related problems. *British Journal of Nutrition* **10**, 69–91.
- Box GEP (1950) Problems in the analysis of growth and wear curves. *Biometrics* **6**, 362–389.
- Box GEP (1954a) Some theorems on quadratic forms applied to the study of analysis of variance problem; I: effect of inequality of variance in the one way-classification. *Annals of Mathematical Statistics* **25**, 290–302.
- Box GEP (1954b) Some theorems on quadratic forms applied to the study of analysis of variance problem; II: effect of inequality of variance and of correlation in the one way classification. *Annals of Mathematical Statistics* **25**, 484–498.
- Chatfield C and Collins AJ (1986) *Introduction to Multivariate Analysis*, revised Edn. London: Chapman & Hall.
- Dhanoa MS, López S, Dijkstra J, Davies DR, Sanderson R, Williams BA, Sileshi Z and France J (2000) Estimating the extent of degradation of ruminant feeds from a description of their gas production profiles observed *in vitro*: comparison of models. *British Journal of Nutrition* **83**, 131–142.
- Dhanoa MS, López L, Powell CD, Sanderson R, Ellis JL, Murray J-AMD, Garber A, Williams BA and France J (2021) An illustrative analysis of atypical gas production profiles obtained from *in vitro* digestibility studies using fecal inoculum. *Animals* **11**, 1069.
- El-Meadaway A, Mir Z, Mir PS, Zaman MS and Yanke LJ (1998) Relative efficacy of inocula from rumen fluid or faecal solution for determining *in vitro* digestibility and gas production. *Canadian Journal of Animal Science* **78**, 673–679.
- France J, Dhanoa MS, Theodorou MK, Lister SJ, Davies DR and Isac D (1993) A model to interpret gas accumulation profiles associated with *in vitro* degradation of ruminal feeds. *Journal of Theoretical Biology* **163**, 99–111.
- France J, Dijkstra J, Dhanoa MS, López S and Bannink A (2000) Estimating the extent of degradation of ruminant feeds from a description of their gas production profiles observed *in vitro*: derivation of models and other mathematical considerations. *British Journal of Nutrition* **83**, 143–150.
- Gabriel KR (1962) Ante-dependence analysis of an ordered set of variables. *Annals of Mathematical Statistics* **33**, 201–212.
- Gower JC and Krzanowski WJ (1999) Analysis of distance for structured multivariate data and extensions to multivariate analysis of variance. *Applied Statistics* **48**, 505–519.
- Greenhouse SW and Geisser S (1959) On methods in the analysis of profile data. *Psychometrika* **24**, 95–112.
- Heitjan D (1989) Inference from grouped continuous data: a review (with discussion). *Statistical Science* **4**, 164–179.
- Hsu JC (1996) *Multiple Comparisons Theory and Methods*. London: Chapman & Hall.
- Huynh H and Feldt LS (1976) Estimation of the Box correction for degrees of freedom from sample data in randomized block and split plot designs. *Journal of Educational Statistics* **1**, 69–82.
- Kenward MG (1987) A method for comparing profiles of repeated measurements. *Applied Statistics* **36**, 296–308.
- Lister SJ, Dhanoa MS, Stewart JL and Gill M (2000) Classification and comparison of Gliricidia provenances using near infrared reflectance spectroscopy. *Animal Feed Science and Technology* **86**, 221–238.
- López S, Dhanoa MS, Dijkstra J, Bannink A, Kebreab E and France J (2007) Some methodological and analytical considerations regarding application of the gas production technique. *Animal Feed Science and Technology* **135**, 139–156.
- Lowman RS, Theodorou MK, Hyslop JJ, Dhanoa MS and Cuddeford D (1999) Evaluation of an *in vitro* batch culture technique for estimating the *in vivo* digestibility and digestible energy content of equine feeds using equine faeces as the source of microbial inoculum. *Animal Feed Science and Technology* **80**, 11–27.
- Lu S, Flanagan BM, Mikkelsen D, Williams BA and Gidley MJ (2022) *In vitro* fermentation of onion cell walls and model polysaccharides using human faecal inoculum: effects of molecular interactions and cell wall architecture. *Food Hydrocolloids* **124**, 107257.
- Mauricio RM, Mould FL, Dhanoa MS, Owen E, Channa KS and Theodorou MK (1999) A semi-automated *in vitro* gas production technique for ruminant feedstuff evaluation. *Animal Feed Science and Technology* **79**, 321–330.
- Mauricio RM, Owen E, Mould FL, Givens I, Theodorou MK, France J, Davies DR and Dhanoa MS (2001) Comparison of bovine rumen liquor and bovine faeces as inoculum for an *in vitro* gas production technique for evaluating forages. *Animal Feed Science and Technology* **89**, 33–48.
- Mauricio RM, Tomich TR, Filho AR, Reis RB, Goncalves LC, Borges I and Dhanoa MS (2006) Comparison between *in vitro* gas production and *in situ* degradation for evaluating rumen dry matter degradation of maize grain and tropical grasses. In Sandoval-Castro CA, Hovell FDdeB, Torres Acosta F and Ayala-Burgos A (eds), *Herbivores: Assessment of Intake, Digestibility and the Roles of Secondary Compounds*. BSAP Publ. No. 34. Nottingham, UK: Nottingham University Press, pp. 69–72.
- Menke K and Steingass H (1988) Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Animal Research and Development* **28**, 7–55.
- Mitscherlich EA (1909) The law of minimum and the law of decreasing soil yield (in German: das gesetz des minimums und das gesetz des abnehmenden bodenertrages). *Landwirtschaftliche Jahrbücher* **38**, 537–552.
- Morvay Y, Bannink A, France J, Kebreab E and Dijkstra J (2011) Evaluation of models to predict the stoichiometry of volatile fatty acid profiles in rumen fluid of lactating Holstein cows. *Journal of Dairy Science* **94**, 3063–3080.
- Nielsen BB, Zhu W-Y, Dhanoa MS, Trinci AP and Theodorou MK (2002) Comparison of the growth kinetics of anaerobic gut fungi on wheat straw in batch culture. *Anaerobe* **8**, 216–222.
- Ørskov ER and McDonald I (1979) The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. *Journal of Agricultural Science, Cambridge* **92**, 499–503.
- Patterson HD and Thompson R (1971) Recovery of inter-block information when block sizes are unequal. *Biometrika* **58**, 545–554.
- Payne RW (2022) Procedure AREPMEASURES Produces an analysis of variance for repeated measurements. In Genstat Reference Manual (Release 22). Available at <https://genstat.kb.vsnico.uk/knowledge-base/arepmeas/>
- Payne RW and Arnold GM (2022) Procedure MANOVA Performs multivariate analysis of variance and covariance. In Genstat Reference Manual (Release 22). Available at <https://genstat.kb.vsnico.uk/knowledge-base/manova/>
- Payne RW and Cave VM (2022) Procedure VAIC Calculates the Akaike and Schwarz (Bayesian) information coefficients for REML. In Genstat Reference Manual (Release 22). Available at <https://genstat.kb.vsnico.uk/knowledge-base/vaic/>
- Payne RW and Ridout MS (2022) Procedure ANTTEST Calculates overall tests based on a specified order of ante-dependence. In Genstat Reference Manual (Release 22). Available at <https://genstat.kb.vsnico.uk/knowledge-base/anttest/>
- Payne RW and White RP (2021) Procedure MVAOD Does an analysis of distance of multivariate data. In Genstat Reference Manual (Release 22). Available at <https://genstat.kb.vsnico.uk/knowledge-base/mvaod>
- Powell CD, Dhanoa MS, Garber A, Murray J-AMD, López S, Ellis JL and France J (2020) Models based on the Mitscherlich equation for describing typical and atypical gas production profiles obtained from *in vitro* digestibility studies using equine faecal inoculum. *Animals* **10**, 308.
- Rao CR (1951) An asymptotic expansion of the distribution of Wilks criterion. *Bulletin of the International Statistical Institute* **33**, 177–181.
- Ridout MS and Payne RW (2022) Procedure ANTORDER Assesses order of ante-dependence for repeated measures data. In Genstat Reference Manual (Release 22). Available at <https://genstat.kb.vsnico.uk/knowledge-base/antorder/>

- Sileshi Z, Owen E, Dhanoa MS and Theodorou MK** (1996) Prediction of *in situ* rumen dry matter disappearance of Ethiopian forages from an *in vitro* gas production technique using a pressure transducer, chemical analyses or *in vitro* digestibility. *Animal Feed Science and Technology* **61**, 73–87.
- Snipes M and Taylor DC** (2014) Model selection and Akaike information criteria: an example from wine ratings and prices. *Wine Economics and Policy* **3**, 3–9.
- Theodorou MK, Williams BA, Dhanoa MS, McAllan AB 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**, 185–197.
- Thomas DAH** (1973) Multiple comparisons among means, a review. *The Statistician* **22**, 16–42.
- Verbyla AP** (2019) A note on model selection using information criteria for general linear models estimated using REML. *Australia and New Zealand Journal of Statistics* **61**, 39–50.
- VSN International** (2022) *Genstat Reference Manual (Release 22)*. Hemel Hempstead, UK: VSN International.
- Ward MD and Ahlquist JS** (2018) *Maximum Likelihood for Social Science: Strategies for Analysis*. Cambridge: Cambridge University Press.
- Wilks SS** (1946) Sample criteria for testing equality of means, equality of variances, and equality of covariances in a normal multivariate distribution. *Annals of Mathematical Statistics* **17**, 257–281.
- Williams BA, Mikkelsen D, le Paih L and Gidley MJ** (2011) *In vitro* fermentation kinetics and end-products of cereal arabinoxylans and (1,3;1,4)- β -glucans by porcine faeces. *Journal of Cereal Science* **53**, 53–58.