Fitting of pH conditions for the study of concentrate feeds fermentation by the *in vitro* gas production technique

Z. Amanzougarene and M. Fondevila [A]

Departamento de Producción Animal y Ciencia de los Alimentos, Instituto Agroalimentario de Aragón (IA2), Universidad de Zaragoza-CITA. Miguel Servet 177, 50013, Zaragoza, Spain

[A] Corresponding author; Tel. (34) 876 554171; E-mail: mfonde@unizar.es

Short title: Fitting pH for *in vitro* incubation of concentrates

Summary text

Estimation of microbial fermentation of concentrate feeds for ruminants from the *in vitro* gas production technique is biased by the different incubation pH, established around 6.5 when rumen pH actually drops below 6.0 in this type of diets. Adjustment of incubation pH by reducing the buffering of the medium is a simple way to overcome this problem in short-length incubations.

Abstract

Two experiments were conducted to simulate *in vitro* the fermentation conditions under high concentrate feeding by fitting the concentration of bicarbonate ion in the buffer of the incubation solution was assayed in Experiment 1 by adjusting medium pH to 6.50, 6.25, 6.00, 5.75 and 5.50, in two incubation series of 12 h, using barley as reference substrate. The pH diminished linearly (P<0,001) by lowering the buffer, and remained constant throughout 12 h except for treatments 5.75 and 5.50, which pH dropped to 5.51 and 5.31 at 12 h. Gas production decreased linearly with medium pH (P<0.001), the total volume of gas produced after 12 h being highly dependent (P<0.01) of pH at 12 (R^2=0.629), showing the importance of incubation pH for estimation of fermentation of concentrate feeds. In Experiment 2, the effect of pH on direct and indirect gas proportion was studied by inoculating 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mmol of acetic acid, either with or without (water added instead) rumen inoculum in the media. Linear multiple regressions established between the volume of gas produced and both the addition of acetic acid and the bicarbonate ion concentration showed high determination coefficients for water (R^2 = 0.929) and rumen inoculum (R^2 = 0.851). Without inoculum, indirect gas production ranged from 9.4 to 12.4 ml/mmol of acid for medium pH of 5.50 to 6.50. With rumen inoculum, indirect gas was 20.8 ml/mmol acid, although this may be biased by the contribution of inoculum itself to direct fermentation.

Additional keywords: Gas production, pH, bicarbonate buffer, indirect gas, *in vitro.*

Introduction

The rumen pH affects the rate and extent of microbial fermentation, as well as the microbial species involved in the process (Russell and Dombrowski 1980; Hiltner and Dehority 1983), in an extent depending on the balance between production and absorption of volatile fatty acids (VFA), buffer salivary secretion and the self-buffering capacity of feeds (Rymer *et al.* 1998). Under normal feeding conditions, rumen pH with forage diets is generally maintained over a minimum of 6.25, but it transitorily drops to values below 6.0, or even close to 5.5 when high levels of concentrate feeds are given (Hungate 1966).

Several *in vitro* closed batch culture systems have been arranged to estimate the nutritive value of ruminant feeds, simulating the pattern of *in vivo* microbial fermentation (Tilley and Terry 1963; Menke *et al.* 1979; Theodorou *et al.* 1994). These systems have been designed for maintaining the incubation pH between 6.5 and 7.0 by including bicarbonate buffer plus a minor proportion of phosphate buffer (Goering and Van Soest 1970; Mould *et al.* 2005) in the medium. The buffering activity of the incubation solution is established by the equilibrium between the added bicarbonate ion and the CO_2 infused to the medium for ensuring anaerobic conditions. Thus, an incubation solution with 110-mM concentration of bicarbonate (Goering and Van Soest 1970) has a large buffering capacity, and it is able to maintain pH around 6.7-6.8 (Kohn and Dunlap 1998). The VFA resulting from microbial fermentation are buffered by the bicarbonate ion, and then CO_2 is released, contributing as indirect gas (Beuvink and Spoelstra 1992) to the total volume in gas production methods (Menke *et al.* 1979; Theodorou *et al.* 1994) depending on the buffer capacity of the incubation medium.

Whereas this buffering system is suitable for the study of fermentation of fibrous feeds, it is not adapted to high-concentrate conditions. Besides other sources of variation, mostly associated with the nature of the rumen inoculum (Getachew *et al.* 2002), the estimation of the fermentation pattern from the gas production from concentrate feeds is largely biased depending on incubation pH. Bertipaglia *et al.* (2010) reported that the volume of gas produced from a mixed concentrate after 24 h incubation in a semicontinuous system was 0.26 lower at pH 5.8 than 6.5, and a 0.50 lower gas production can be estimated from equations of Opatpanatakit et al. (1994) in the same pH range. The use of other buffers or the acidification of the medium to get the required pH (Grant and Mertens 1992) has been proposed, but the former are often more expensive and the latter rapidly exhausts the buffering capacity (Mould *et al.* 2005). Continuous and semi-continuous incubation systems (Hoover *et al.* 1976; Czerkawski and Breckenridge 1979) have been developed for maintaining pH at a low range through systematic infusion of a buffering solution, but complexity and price of the equipment increase. Using a simple semi-continuous incubation system, Fondevila and Pérez-Espés (2008) and Bertipaglia *et al.* (2010) maintained incubation pH around 6.0 throughout a 24 hour incubation period by reducing the bicarbonate concentration in the buffering solution, according to the calculations by Kohn and Dunlap (1998). However, this has not yet been applied to batch culture systems.

This work studies the possibility to adjust the range of incubation pH during fermentation of a concentrate substrate (Experiment 1). Later, the effect of pH on the direct (produced from microbial fermentation) and indirect (coming from the buffering activity of the medium) contributions to total *in vitro* gas production was addressed, adding acetic acid as a model of fermentation end product (Experiment 2).

Material and methods

Two *in vitro* experiments were carried out. Maintenance and extraction procedures of rumen inoculum from donor animals were approved by the Ethics Committee for Animal Experimentation. Care and management of animals agreed with the Spanish Policy for Animal Protection RD 53/2013, which complies with EU Directive 2010/63 on the protection of animals used for experimental and other scientific purposes. About 300 ml of rumen contents from four rumen cannulated sheep (71.5 ± 1.7 kg body weight) of the Servicio de Experimentación Animal, University of Zaragoza) daily given 600 g alfalfa hay plus 300 g barley straw were extracted immediately before the morning feeding, pooled, filtered through cheesecloth and transferred to the laboratory in thermos bottles preheated to 39° C.

Fermentation pattern of barley at different incubation pH (Experiment 1)

Two incubation series were carried out in two consecutive days, using pooled inoculum from the ewes mentioned above. A total of 35 glass bottles, seven for each of 5 experimental treatments, were used on each series. Bottles (116 mL total volume) were filled under anaerobic conditions with 8 mL rumen inoculum and 72 mL of an incubation solution made up with (ml/L) 238 buffer solution, 238 macrominerals solution (5.7 g Na_2HPO_4, 6.2 g KH_2PO_4 and 0.6 g $MgSO_4.7\ H_2O$ per L), 474 distilled water and 50 reduction solution (47.5 mL distilled water, 2 mL 1N NaOH and 313 mg HCl-cysteine), following the Theodorou *et al.* (1994) procedures. Microminerals and resazurin were not added (Mould *et al.* 2005). The experimental treatments consisted on the adjustment of medium pH to 6.50, 6.25, 6.00, 5.75 and 5.50 by reducing the proportion of bicarbonate ion (from sodium bicarbonate and ammonium bicarbonate) in the buffer solution, according to the Kohn and Dunlap (1998) calculations (Table 1). An amount of 500 mg of barley grain (var. Graphil) ground to 1 mm particle size was included as substrate. Barley grain was chosen as a reference substrate because of its acidification properties, even assuming differences for extrapolating results to the fermentation pattern of other feeds. Bottles were sealed under a CO_2 stream and incubated at 39º C for 12 h. Gas pressure produced in each bottle was recorded every two hours (at 2, 4, 6, 8, 10 and 12 h of incubation) with a HD 2124.02 manometer fitted with a TP804 pressure gauge (Delta Ohm, Caselle di Selvazzano, Italy), and one bottle of each treatment was opened immediately after to determine the incubation pH (CRISON micropH 2001, Barcelona, Spain). Readings were converted into volume by a pre-established linear regression equation between the pressure recorded in the same bottles under the same incubation conditions and known injected air volumes (n= 103; R^2= 0.996), and the gas volume recorded for each incubation time was expressed per unit of incubated organic matter (OM). The evolution of gas production was estimated as the average of the two bottles maintained for 12 h on each incubation series.

In vitro acidification (Experiment 2)

In a second experiment, acetic acid as a model of VFA produced by microbial fermentation was added over the incubation solution, to estimate the contribution of gas produced by buffering under *in vitro* conditions (indirect gas). Thirty bottles (116 mL total volume) were filled with 80 mL of incubation solution but substituting rumen liquid with the same proportion of distilled water (without inoculum), and the medium was adjusted to the same pH as in Experiment 1 (6.50, 6.25, 6.00, 5.75 and 5.50). Increasing volumes of 1M acetic acid (0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mL) were added to mimic microbial fermentation, resulting in acetic acid concentrations of 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mM, in two incubation series. One bottle for each acetic acid concentration and incubation pH was incubated at 39ºC. The volume of gas produced was measured after 30 min and then bottles were opened and the final pH measured. Further, in another approach within Experiment 2, the same experimental design was used, but in this case rumen inoculum was included in the incubation solution instead of distilled water, in two series of incubation with duplicate bottles. The average of the two bottles for each treatment was considered as the experimental unit, resulting 12 data (six doses of acetic acid in two incubation series) on each of the five incubation pH.

Chemical and statistical analyses

The AOAC (2005) procedures were followed to determine dry matter (DM; method reference 934.01) and OM (method reference 942.05) of the barley substrate. Total starch content was determined enzymatically from samples ground to 0.5 mm using a commercial kit (Total Starch Assay Kit K-TSTA 07/11, Megazyme, Bray, Ireland).

Simple and multiple linear regressions were established in Experiments 1 and 2 to establish relationship among the different parameters studied using the Statistix 10 software package (Analytical Software 2010). In both experiments, the average gas volume resulting from two bottles for each treatment on each incubation series was considered as the experimental unit, except for the incubation with water (without inoculum) in Experiment 2, where a single bottle was considered. In Experiment 1, polynomial results were also analysed by ANOVA, considering the average of the two bottles of the same treatment on each incubation time as the experimental unit and the series as a block, and polynomial (linear and quadratic) contrasts were established when differences between pH levels for each incubation time (n=10) or between times for each pH (n=12) reached significance. In Experiment 2, a Split-plot design was followed, with the incubation series as a block, the concentration of bicarbonate as main plot and the added concentration of acetic acid as subplot. Differences were considered significant if $P \leq 0.05$, and as a trend to significance when $0.05 < P \leq 0.10$. When differences were significant, means were compared by the Tukey t-test.

Results

Experiment 1

The barley used as substrate in Experiment 1 had an OM and starch content of 977 and 651 g/kg, respectively. For the study of the fermentation pattern of barley as substrate as affected by the adjusted medium pH, the pH of the rumen inoculum was 6.62 and 6.50 in the incubation series 1 and 2, respectively. At every sampling time, a linear decrease ($P < 0.001$) of medium pH was observed with the decrease of the buffering capacity (pH values of 6.37, 6.28, 5.97, 5.51 and 5.31, s.e.m. = 0.095, for medium pH 6.50, 6.25, 6.00, 5.75 and 5.50 after 12 h incubation), as it can also be seen within each incubation time in Fig. 1. Besides, pH at 4 h tended (P = 0.081) to drop quadratically with the level of bicarbonate, this pattern being significant at 6 h (P = 0.006) because of the lack of differences between media 5.75 and 5.50 (pH of 5.90 and 5.85, respectively). The different concentration of bicarbonate ion in the buffer allowed to reach the expected pH (± 0.1 units) for treatments 6.25, 6.00, 5.75 and 5.50 at 4, 6, 8 and 10 h of incubation (6.35, 6.08, 5.83 and 5.46, respectively; Fig. 1). From these times of incubation onwards, medium pH was maintained within the range provided with treatments 6.25 and 6.00, while it dropped slightly respect to the expected values with treatments 5.75 and 5.50 (final pH after 12 h of 5.51 and 5.31, respectively). Besides, at 4 h of incubation only treatments 5.75 and 5.5 achieve pH values of less than 6.0. Regarding medium 6.50, from the beginning of incubation the pH was maintained within the range planned for the experiment (pH 6.53 at 2 h), with minor modifications of 0.1 pH units until 10 h of incubation, slightly decreasing afterwards to pH 6.37.

The volume of gas produced decreased linearly (P< 0.05) with the pH of the incubation medium at every time of incubation (173, 156, 141, 136 and 130 mL/g OM, s.e.m. = 4.16, for treatments 6.50, 6.25, 6.00, 5.75 and 5.50 after 10 h), as it is showed in Fig. 2. This effect also showed a quadratic pattern (P = 0.012) at 12 h, because of the increased differences between the volume of gas produced with the medium at pH 6.50 (217 mL/g OM), and to a lesser extent at pH 6.25 (184 mL/g OM), compared to the increases in gas production with the other treatments at this time of incubation (158, 152, and 145 mL/g OM for pH 6.00, 5.75, and 5.5 respectively). In this sense, from 8 h afterwards, treatments which stabilized incubation pH at 0.2 units or more above 6.0 (pH 6.50 and 6.25) maintained a positive trend to increase the volume of gas produced, whereas the volume of gas produced in this period tended to decrease in treatments which medium was maintained at pH 6.0 or below (Fig. 2). A relationship between the production of gas at 12 h and the pH of the medium was detected, defined by a significant coefficient of determination not only at the end of the incubation period (R^2 = 0.629; P = 0.004), but also between the final volume at 12 h and pH recorded at previous incubation times, especially 6 h (R^2 = 0.836; P < 0.001).

Experiment 2

In a first approach to estimate the contribution of gas produced by buffering under *in vitro* conditions, acetic acid was added over the incubation solution without inoculum, that was substituted by the same volume of distilled water. A general linear regression equation was established to estimate either the medium pH or the volume of gas produced (mL) at 30 min according to the added concentration of acetic acid (mmol) and corrected for the adjusted medium pH by including the bicarbonate concentration in the buffer (mmol), as follows:

pH = 5.969 (0.0362) + 0.060 (0.0037) HCO_3^- - 0.711 (0.0979) acetic acid ;

n= 60; SD= 0.1270; R^2= 0.840; P< 0.001 (1)

gas = 3.004 (0.1549) + 0.139 (0.0157) HCO_3^- + 10.859 (0.4106) acetic acid ;

n= 60; SD= 0.5431; R^2= 0.929; P< 0.001 (2)

The inclusion of bicarbonate concentration in the relationship improved the adjustment for both pH and gas (P < 0.001). As a further approach, when the magnitude of the drop of pH from the initial medium pH after 30 min was correlated with the addition of acetic acid, the relationship reached a R^2= 0.669. When studied by ANOVA, the final pH after 30 min incubation (data not shown) linearly decreased with the treatment pH (P = 0.007), as expected, also falling with the added concentration of acetic acid (P < 0.001). The interaction acetic acid x bicarbonate concentration in the volume of gas produced (P = 0.033) indicates that the increase of this parameter with acetic acid was inversely related with the medium pH, as it is reflected in Fig. 3.

In order to avoid the possible bias of the differences in the buffering capacity of the experimental media on pH and gas production, equations among the volume of gas and the added acetic acid were also adjusted for each medium (Table 2), confirming that the regression coefficients (increase of the volume of gas produced per unit of added acid) decreased with the concentration of bicarbonate ion in the medium, from treatments 6.50 and 6.25 to 5.75 and 5.50. The volume of gas was inversely proportional to the adjusted incubation pH, decreasing from that observed at pH 6.50 in a proportion of 0.032, 0.137, 0.221 and 0.244 at pH 6.25, 6.00, 5.75 and 5.50. In all cases, determination coefficients were over 0.91 (P < 0.001).

In another approach, when rumen inoculum was included in the incubation (no water added), the pH of the initial inoculum was 6.86 and 7.16 for each incubation series. Linear relationships established between either pH or gas (mL) and bicarbonate and acetic acid concentration (mmol), were:

$$pH = 5.961\ (0.0622) + 0.070\ (0.0063)\ HCO_3^- - 1.138\ (0.1650)\ \text{acetic acid}\ ;$$

$$n = 59;\ SD = 0.2183;\ R^2 = 0.743;\ P < 0.001 \qquad (3)$$

$$gas = 2.047\ (0.4414) + 0.222\ (0.0446)\ HCO_3^- + 20.779\ (0.4414)\ \text{acetic acid}\ ;$$

$$n = 60;\ SD = 15.481;\ R^2 = 0.851;\ P < 0.001 \qquad (4)$$

Comparison of results from *in vitro* incubation with rumen inoculum by ANOVA gave similar results than when water was included instead. The final pH after 30 min incubation linearly decreased (P < 0.001) with both the incubation pH and the added acetic acid. Again, the interaction acetic acid x bicarbonate concentration in the volume of gas produced (P = 0.002) showed that the increase of this parameter differed with the medium, as it is reflected in Fig. 4.

Linear relationship were also established for each medium pH between gas production and added acetic acid (Table 3). As expected, when rumen inoculum was included in the incubation solution, the experimental variability showed by the magnitude of the error term (standard deviation of equations from 1.03 to 1.63) was higher, and the determination coefficient (R^2 from 0.78 to 0.95) lower, than when water was included instead (Table 2). The regression coefficients (proportion at which gas is produced for every unit of acetic acid added to the medium) on each case in relation to medium pH 6.50 were reduced in 0.113, 0.256, 0.333 and 0.396 in media 6.25, 6.00, 5.75 and 5.50, respectively.

Discussion

The apparent contradiction of using rumen contents from sheep fed on a fibrous diet as inoculum for the study of concentrate fermentation is justified by our interest in ensuring a wide range of pH. An inoculum from concentrate-fed animals may compromise both to have an initial pH of 6.50 (in Experiments 1 and 2, pH of inocula ranged from 6.50 to 7.1) and to maintain a high pH throughout the incubation period. It should be considered that this work was not focused to get absolute fermentation results to extrapolate to practical situations, but to modulate incubation pH and estimate origin and magnitude of indirect gas. Despite the importance of the nature of inoculum on microbial fermentation (Martinez et al., 2010; Broudiscou et al., 2014), buffering of major VFA (acetate and propionate) renders the same amount of indirect CO_2 (Beuvink and Spoelstra, 1992). Results from our laboratory (Amanzougarene et al., in evaluation in Anim. Prod. Sci.) showed that, despite gas production from the same barley substrate with either forage or concentrate inoculum after 10 h incubation was 0.36 times greater with the latter, differences in VFA molar pattern of *in vitro* fermentation (acetate:propionate:butyrate ratios of 0.52:0.20:0.06 *vs.* 0.47:0.21:0.10) rendered similar estimated contributions to indirect gas (1.50 *vs.* 1.44 mmol CO_2 per mmol of VFA produced).

The buffering capacity of the incubation media was mainly given by bicarbonate ion (HCO_3^-), included at concentrations adjusted to reach the desired incubation pH (Kohn and Dunlap, 1998). Bicarbonate is considered as the most prevalent ruminal buffer (Counotte *et al.* 1979; Erdman 1988), and is the basis of most *in vitro* media used for fermentation studies (Goering and Van Soest 1970; Menke *et al.* 1979; Theodorou *et al.* 1994). The incubation medium also includes phosphate ion as buffer, in a concentration that varies slightly according to the method (Mould *et al.* 2005), and in our case it was 0.016 moles/L. According to the estimations of Beuvink and Spoelstra (1992), phosphate contribution to total buffering capacity is 0.18 at pH 6.9, and drops to 0.07 at pH 6.5 and 0.00 at pH 6.0. Following the calculations of Kohn and Dunlap (1998), the contribution of phosphate ion to total buffering capacity in our work ranged from 0.04 to 0.06 at a pH between 6.50 and 5.50, even after reducing the concentration of bicarbonate buffer for adjusting pH. Therefore, it can be assumed that acids produced were buffered by bicarbonate, thus producing CO_2 as indirect gas.

Under incubation protocols of most *in vitro* gas production systems, pH is generally fixed to 6.7 - 5.9, and rarely drops below 6.5 during the fermentation process. Thus, the incubation conditions are far from those generally occurring in practical conditions when animals are given high proportions of starch-rich concentrate feeds, preventing from direct extrapolation of results to common feeding practices. By reducing the concentration of bicarbonate ion (Table 1) it is possible to maintain incubation pH within a desired range for at least 12 h of incubation, provided that the bicarbonate concentration is adjusted for maintaining a pH of 6.00 or above, as it has been previously observed in a semi-continuous incubation system by Fondevila and Pérez-Espés (2008) and Bertipaglia *et al.* (2010), although this has not been approached in a closed batch system. In Experiment 1 such stable pH was maintained for 10 h, although it dropped afterwards when pH was adjusted to 5.75 or below. However, it has to be considered that for the study of high-starch feeds such interval could be enough assuming that an important proportion of microbial fermentation takes place within this range (Mould *et al.* 2005; Lanzas *et al.* 2007; Bertipaglia *et al.* 2010). In any case, the contribution of the negative effect of medium pH to microbial fermentative activity and the volume of gas produced from a given substrate cannot be directly quantified from this experimental design, since differences in the concentration of bicarbonate in the medium alters the contribution of indirect gas to total volume.

In Experiment 2, acetic acid was chosen to mimic the release of microbial fermentation products to the medium, in a range of acid concentrations within the normally observed VFA produced in *in vitro* trials. This acid was used as model of acidification for being the most abundant VFA in rumen fermentation, although propionic acid is more characteristic of what represents a high-concentrate rumen environment. Despite it has been suggested that acidification capacity differs among VFA depending on their pKa (Theodorou *et al.* 1998), differences among acetic, propionic and butyric acids are scarce (pKa of 4.76; 4.87 and 4.82, respectively), and all of them are well below the current range of rumen pH. In this way, Beuvink and Spoelstra (1992) and Rymer and Givens (1998) did not found differences in acidification capacity between the different VFA on molar basis.

From results of acetic acid addition to the media with either water or rumen inoculum, indirect gas production tends to diminish at low medium pH since the buffering capacity of bicarbonate becomes lower, as discussed. Thus, the comparison of substrates fermentation at pH 6.0 or below could be established in terms of direct gas. However, in such case the production of propionic acid would not be detected, since it does not render direct gas (Beuvink and Spoelstra 1992). Results by Bertipaglia *et al.* (2010) show that *in vitro* acetate:propionate:butyrate proportions resulting from fermentation of concentrates does not greatly change with incubation pH, resulting in 57:36:8 at pH 6.5 and 57:34:9 at pH 5.8, and therefore stoichometrical calculation of the gas (either direct or indirect) produced should not be affected when incubation pH is reduced. In any case, the combination of gas production with the study of the VFA profile would help to clarify the magnitude of the underestimation caused by differences in propionate proportion.

The gas production estimation when water was included instead of rumen inoculum in Experiment 2 (equation 2) allows considering the concentration of acetic acid added as an index of indirect gas released from the buffer at any incubation pH. However, this contribution to total gas gives a general coefficient despite the buffering capacity of the media. The inclusion in the equation of the concentration of bicarbonate ion in the medium improved the adjustment. In the same way, the intercept, which reflects the gas produced when no acid was added, comes from the equilibrium initially established between the bicarbonate buffer and the carbonic acid produced, which releases CO_2. In our experimental conditions, 10.9 mL of gas was produced from each mmol of acid. However, low-buffered media (especially those at pH 5.75 and 5.50) may have not enough concentration of bicarbonate to buffer all the added acetic acid, thus giving a biased estimation of indirect gas. In equations between the volume of gas and the addition of acetic acid adjusted for each medium (Table 2), coefficients of regression indicate the production of gas per unit of acid added, that is, the indirect gas. Since a limited concentration of bicarbonate buffer in the medium may affect the response, and assuming that medium 6.50 is capable to completely buffer the incubation pH in our conditions, the indirect gas should be estimated directly from equation for medium 6.50 in 12.4 mL per mmol of acid added. With media including 0.099 to 0.117 M HCO_3^-, Rymer *et al.* (1998) estimated between 12 and 15 mL/mmol of acid, but the buffering capacity of these solutions were in all cases quite higher than in the media used in this experiment (Table 1). According to Table 2, the production of indirect gas was inversely proportional to the adjusted incubation pH. Therefore, the direct gas would become more important in total gas production as the incubation pH drops from 6.50. However, direct gas does not consider the contribution of propionate to overall fermentation, and therefore the microbial utilisation of substrates should be underestimated at a higher extent.

When in Experiment 2 rumen inoculum was included in the medium, response in gas production to the addition of acetic acid was higher, resulting on average 20.8 mL gas per mmol for all media (equation 4), which exactly match with the value reported by Beuvink and Spoelstra (1992) in a medium with a final CO_3H^- concentration of 0.78 M. It has to be considered that the buffering capacity of the inoculum itself reduces differences among media. On the contrary, a drop of medium pH to 6.00 and below, should reduce microbial fermentative activity (Hungate 1966; Russell and Dombrowski 1980), thus contributing to enhance the negative effect of acidification. This is reflected in the linear relationship established between gas production and added acetic acid for each medium pH (Table 3). The proportion at which gas is produced for every unit of acetic acid added to the medium on each case in relation to medium pH 6.50 was reduced at a higher extent than with water instead of rumen inoculum. This would suggest that the effect of a lower microbial activity at medium below 6.00 should contribute to reduce indirect gas production, already depressed by a low inclusion of bicarbonate. In the opposite sense, the direct gas produced by fermentation of the soluble nutrients from the inoculum itself positively contributes to gas volume, partly counterbalancing this effect.

Conclusions

For the study of concentrate feeds for simulating intensive feeding conditions, the *in vitro* incubation pH was adjusted to values around or below 6.00 by reducing the concentration of bicarbonate ion in the incubation solution, and it was maintained within the expected range for at least 10 h when pH was adjusted to 5.75 and 5.50. The importance of this is evidenced by the differences observed in the pattern of gas production from a single substrate incubated at a range of pH from 6.50 to 5.50. However, indirect gas is reduced when concentration of bicarbonate in the buffer is lowered. In our experimental conditions, indirect gas can be estimated when using water instead of rumen inoculum, in 9.4 to 12.4 mL per mmol acid produced at incubation pH 5.50 to 6.50. These values increase to 20.8 ml/mmol when rumen inoculum is included in the medium, although this may be biased by the contribution of inoculum itself to direct fermentation.

Acknowledgements

This work was supported by the Spanish Government through the MINECO project AGL 2013-46820, with the participation of the Department of Industry and Innovation of the Government of Aragón, and the European Social Fund. Z. Amanzougarene was granted by the Centre International de Hautes Études Agronomiques Méditerranéennes (CIHEAM). There are no conflicts of interests.

References

Analytical software (2010) ´Statistix 10 for Windows´ (Analytical Software; Tallahasee, FL)

AOAC (2005) 'Official Methods of Analysis (18th ed)' (Association of Official Analytical Chemists: Gaithersburg. MD)

Bertipaglia LMA, Fondevila M, van Laar H, Castrillo C (2010) Effect of pelleting and pellet size of a concentrate for intensively reared beef cattle on in vitro fermentation by two different approaches. *Animal Feed Science and Technology* **159**, 88-95. doi:10.1016/j.anifeedsci.2010.05.010

Beuvink JM, Spoelstra SF (1992) Interactions between substrate, fermentation end-products, buffering systems and gas production upon fermentation of different carbohydrates by mixed rumen microorganisms *in vitro*. *Applied Microbiology Technology* **37**, 505-509. doi:10.1007/BF00180978

Broudiscou LP, Offner A, Sauvant D (2014) Effects of inoculum source, pH, redox potential and headspace di-hidrogen on rumen *in vitro* fermentation yields. *Animal* **8**, 931-937. doi:10.1017/S1751731114000640

Counotte GHM, Van't Klooster AT, Van der Kuilen J, Prins RA (1979) An analysis of the buffer system in the rumen of dairy cattle. *Journal of Animal Science* **49**, 1536-1544. doi: 10.2134/jas1979.4961536x

Czerkawski JW, Breckenridge G (1977) Design and development of a long-term rumen simulation technique (RUSITEC). *British Journal of Nutrition* **38**, 371-384. doi: http://dx.doi.org/10.1079/BJN19770102

Erdman RA (1988) Dietary buffering requirements of the lactating dairy cow: A review. *Journal of Dairy Science* **71**, 3246-3266. doi: http://dx.doi.org/10.3168/jds.S0022-0302(88)79930-0

Fondevila M, Perez-Espés B (2008) A new in vitro system to study the effect of liquid phase turnover and pH on microbial fermentation of concentrate diets for ruminants. *Animal Feed Science and Technology* **144**, 196-211. doi: 10.1016/j.anifeedsci.2007.10.013

Getachew G, Crovetto GM, Fondevila M, Krishnamoorthy U, Singh B, Spanghero M, Steingass H, Robinson PH, Kailas MM (2002) Laboratory variation of 24 h in vitro gas production and estimated metabolizable energy values of ruminant feeds. *Animal Feed Science and Technology* **102**, 169-180. doi: 10.1016/S0377-8401(02)00212-02

Goering HK, van Soest PJ (1970) 'Forage fiber analyses (apparatus, reagents, procedures and some applications)' (USDA Agriculture Handbook No. 379. USDA-ARS: Washington DC)

Grant RJ, Mertens DR (1992) Influence of buffer pH and raw corn starch addition on in vitro fibre digestion kinetics. *Journal of Dairy Science* **75**, 2762-2768. doi: http://dx.doi.org/10.3168/jds.S0022-0302(92)78039-4

Hiltner P, Dehority BA (1983) Effect of soluble carbohydrates on digestion of cellulose by pure cultures of rumen bacteria. *Applied and Environmental Microbiology* **46**, 642–648.

Hoover WH, Crooker BA, Sniffen CJ (1976) Effects of differential solid-liquid removal rates on protozoa numbers in continuous cultures of rumen contents. *Journal of Animal Science* **43**, 528–534. doi: 10.2134/jas1976.432528x

Hungate RE (1966) 'The rumen and its microbes' (Academic Press: NY)

Kohn RA, Dunlap TF (1998) Calculation of the buffering capacity of bicarbonate in the rumen and *in vitro*. *Journal of Animal Science* **76**, 1702-1709. doi: /1998.7661702x

Lanzas C, Fox DG, Pell AN (2007). Digestion kinetics of dried cereal grains. *Animal Feed Science and Technology* **136**, 265-280. doi: 10.1016/j.anifeedsci.2006.09.004

Martinez ME, Ranilla MJ, Tejido ML, Saro C, Carro MD (2010) The effect of the diet fed to donor sheep on *in vitro* methane production and ruminal fermentation of diets of variable composition. *Animal Feed Science and Technology* **158**, 126-135. doi:10.106/j.anifeedsci.2010.04.005.

Menke KH, Raab l, Salewski A, Steingass H, Fritz D, Schneider W (1979) The estimation of the digestibility and metabolizable energy content of ruminant feeding stuffs from the gas production when they are incubated with ruminal liquor. *Journal of Agricultural Science, Cambridge* **97**, 217-222. doi: http://dx.doi.org/10.1017/S0021859600086305

Mould FL, Morgan R, Kliem KE, Krystallidou E (2005) A review and simplification of the in vitro incubation medium. *Animal Feed Science and Technology* **123-124**, 155-172. doi: 10.1016/ j.anifeedsci.2005.05.002

Opatpatanakit Y, Kellaway RC, Lean IJ, Annison G, Kirby A (1994) Microbial fermentation of cereal grains *in vitro*. *Australian Journal of Agricultural Research* **45**, 1247-1263. doi: 10.1071/AR9941247

Russell JB, Dombrowski DB (1980) Effect of pH on the efficiency of growth by pure cultures of rumen bacteria in continuous culture. *Applied and Environmental Microbiology* **39**, 604–610.

Rymer C, Givens DI (1998) Relationship between the rates of production of gas and VFA during *in vitro* incubation s of high temperature dried grass. *Proceedings of the British Society of Animal Science Annual Meeting 1998*, p. 55.

Rymer C, Moss AR, Deaville ER, Givens DI (1998) Factors affecting the amount of indirect gas produced by the *in vitro* gas production technique. In: '*In vitro* techniques for measuring nutrient supply to ruminants' (Eds. ER Deaville, E Owen, AT Adesogan, C Rymer, JA Huntington, TLJ Lawrence), pp. 89-91 (BSAS Occ. Publ. No. 22)

Theodorou MK, Williams BA, Dhanoa MS, McAllan AB, 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**, 185-1 97. doi:10.1016/0377-8401(94)90171-6

Theodorou MK, Lowman RS, Davies ZS, Cuddeford D, Owen E (1998) Principles of techniques that rely on gas measurements in ruminant nutrition. In: '*In vitro* techniques for measuring nutrient supply to ruminants' (Eds. ER Deaville, E Owen, AT Adesogan, C Rymer, JA Huntington, TLJ Lawrence), pp. 55-63 (BSAS Occ. Publ. No. 22)

Tilley JMA, Terry RA (1963) A two stage technique for the in vitro digestion of forage crops. *Journal of the British Grassland Society* **18**, 104-111.

Table 1. Bicarbonate concentration (g/L) included in the buffer solution for adjusting the incubation pH and concentration (g/L) in the final medium, together with final concentration of bicarbonate ion (mol/L) in the incubation medium.

adjusted pH	$NaHCO_3$ in buffer	(NH_4) HCO_3 in buffer	$NaHCO_3$ in medium	(NH_4) HCO_3 in medium	medium HCO_3^- conc.
6.50	18.30	1.90	3.92	0.41	0.058
6.25	10.30	1.07	2.21	0.23	0.032
6.00	5.70	0.60	1.22	0.13	0.018
5.75	3.17	0.25	0.68	0.05	0.010
5.50	1.91	0.12	0.41	0.03	0.006

Table 2. Fitted equations of gas production (*y*; mL) and acid concentration in the medium (*x*; mmol) for each incubation pH when water was included instead of rumen inoculum (n=12). Values in brackets are standard error of coefficients.

medium pH	equation	SD[1]	R^2	Probability
6.50	y = 4.360 (0.2721) + 12.436 (0.8988) x	0.5317	0.945	< 0.001
6.25	y = 3.983 (0.3381) + 12.032 (1.1168) x	0.6607	0.913	< 0.001
6.00	y = 3.889 (0.2329) + 10.731 (0.7692) x	0.4551	0.946	< 0.001
5.75	y = 3.597 (0.2128) + 9.6903 (0.7028) x	0.4158	0.945	< 0.001
5.50	y = 3.287 (0.1277) + 9.405 (0.4216) x	0.2494	0.978	< 0.001

[1]: SD: standard deviation

Table 3. Fitted equations of gas production (*y*; mL) and acid concentration in the medium (*x*; mmol) for each incubation pH with rumen inoculum (n=12). Values in brackets are standard error of coefficients.

medium pH	equation	SD[1]	R^2	Probability
6.50	y = 3.502 (0.8355) + 26.633 (2.7596) x	1.6326	0.893	< 0.001
6.25	y = 3.051 (0.5128) + 23.613 (1.6936) x	1.0019	0.951	< 0.001
6.00	y = 3.653 (0.7738) + 19.803 (2.5559) x	1.5121	0.843	< 0.001
5.75	y = 3.836 (0.8502) + 17.751 (2.8082) x	1.6614	0.780	< 0.001
5.50	y = 2.742 (0.5250) + 16.093 (1.7340) x	1.0259	0.886	< 0.001

[1]: SD: standard deviation

Fig. 1. Pattern of incubation pH according to the fitted pH based on the concentration of bicarbonate ion in the incubation solution (pH 6.5, ○; pH 6.25, ●; pH 6.00, ■; pH 5.75, ◆; pH 5.50, ▲). For each time of incubation, upper bars show standard error of means.

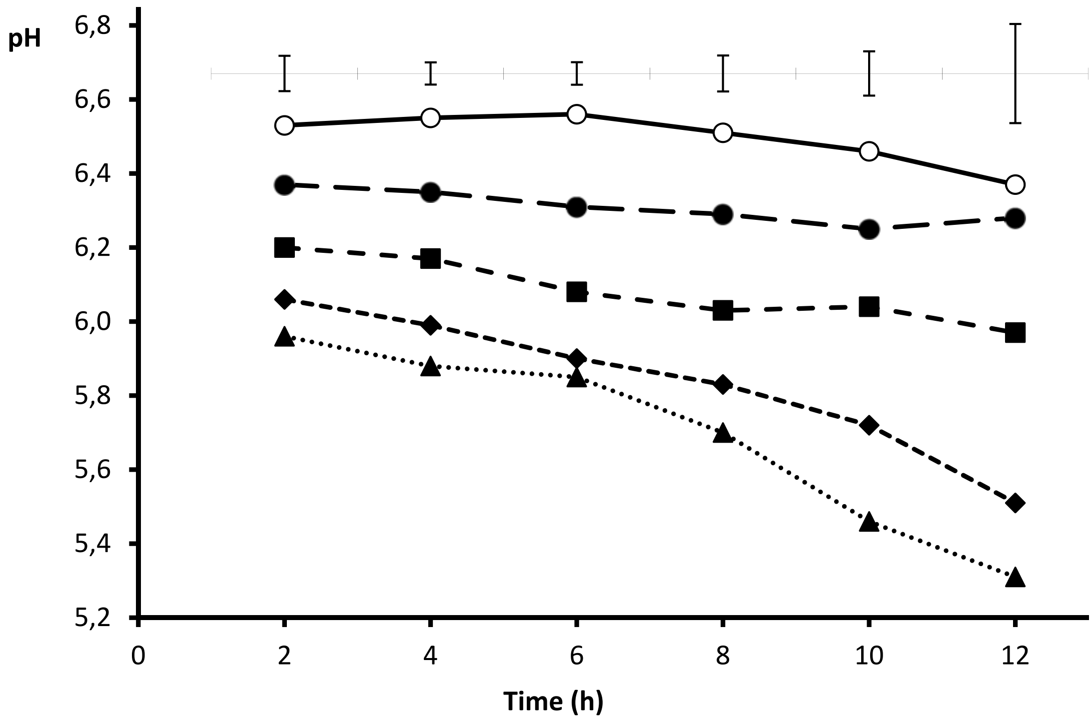

Fig. 2. Pattern of the volume of gas produced *in vitro* (ml/g OM) according to the planned pH based on concentration of bicarbonate ion in the incubation solution (pH 6.5, ○; pH 6.25, ●; pH 6.00, ■; pH 5.75, ◆; pH 5.50, ▲). For each time of incubation, upper bars show standard error of means.

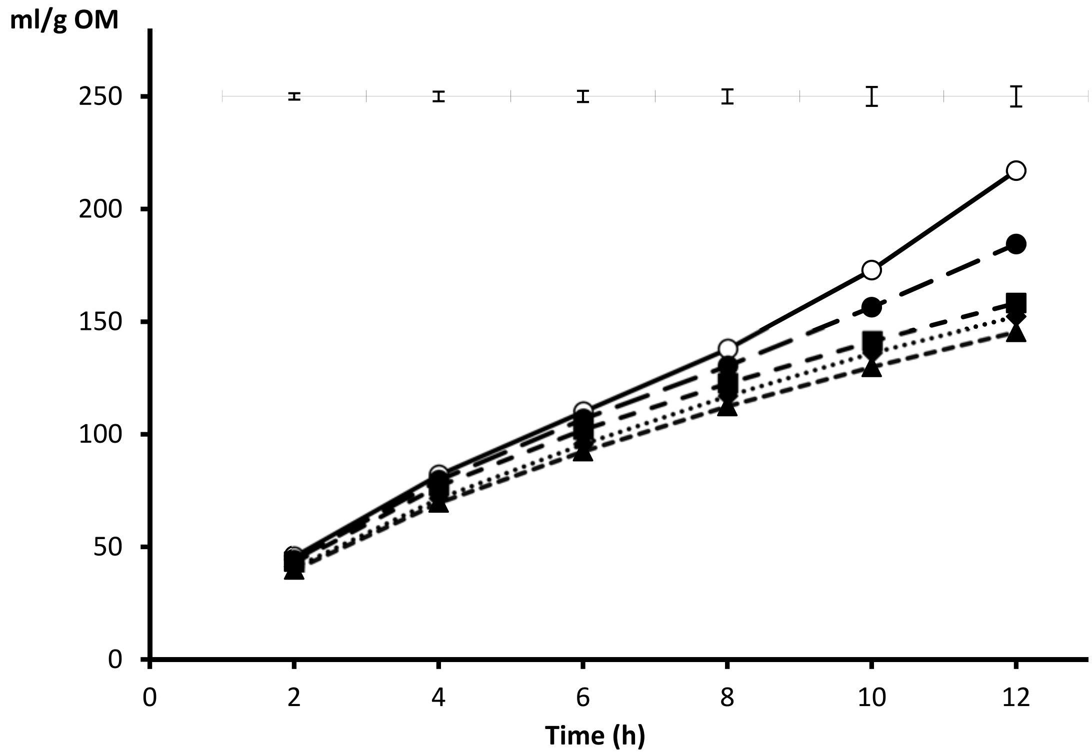

Fig. 3. Gas production (mL) from addition of acetic acid (mmol) after 30 min with water instead of rumen inoculum, according to the planned pH based on concentration of bicarbonate ion in the incubation solution (pH 6.5, ○; pH 6.25, ●; pH 6.00, ■; pH 5.75, ◆; pH 5.50, ▲). Values are the average of two series of incubation (s.e.m.= 0.204).

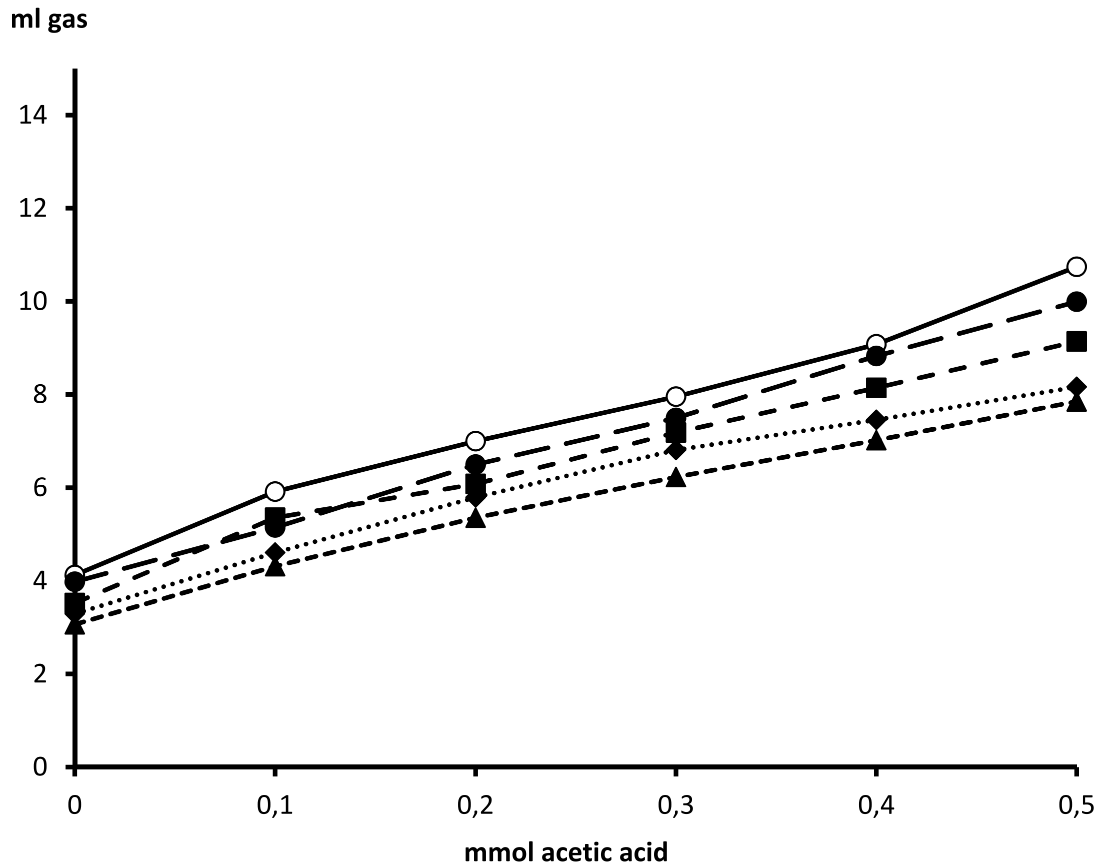

Fig. 4. Gas production (mL) from addition of acetic acid (mmol) after 30 min with rumen inoculum, according to the planned pH based on concentration of bicarbonate ion in the incubation solution (pH 6.5, ○; pH 6.25, ●; pH 6.00, ■; pH 5.75, ◆; pH 5.50, ▲). Values are the average of two series of incubation (s.e.m.= 0.743).