In vitro acidification potential and fermentation pattern of cereal grains incubated with inoculum of animals given forage or concentrate based diets

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Abstract

The potential of acidification and fermentation pattern of maize (M1, M2 and M3), barley (B1, B2 and B3) and sorghum (S1, S2 and S3) varieties was studied in three 10 h in vitro incubation series with a low buffered medium using inoculum from rumen contents of sheep given a forage (FI) or a concentrate (CI) diet. From 2 to 10 h average pH with CI was lower than with FI (P<0.001), final pH being 5.25 and 5.76(SEM=0.013), respectively. With CI, incubation pH was lowest (P<0.001) with the three barley varieties from 2 to 6 h, and highest at 8 and 10 h with sorghum, maize recording intermediate values (average pH at 8 h of 5.63, 5.44 and 5.13, respectively). With FI, pH with barley was lowest than with maize or sorghum (average pH at 10 h of 5.52, 5.85 and 5.91, respectively). No pH differences among varieties within cereal species were detected (P>0.05).

The average gas production with CI was always superior (P<0.001) to that with FI (673 vs. 36.8 mL/g OM at 10 h, SEM = 1.02). Concentration of volatile fatty acids (VFA) with CI was higher than FI (31.0 vs. 19.9 mmol/L at 8 h; P<0.001), as well as proportions of butyrate and valerate (P<0.001), whereas proportions of acetate and branched chain VFA were lower (P<0.001), and propionate did not differ among inocula. Among substrates, the volume of gas produced from barley varieties with CI was similar to that from maize (P>0.05), except at 4 and 6 h, when M2 and M1 were lower. No differences among varieties of either barley or maize were detected at any time (P>0.05), but the tannin-free S1 was grouped with maize whereas gas production with S2 and S3 was lower (P<005). After 10 h, barley and maize varieties produced on average 82.5 and
73.0 mL/g OM, whereas S1, S2 and S3 rendered 68.4, 31.1 and 39.7 mL/g OM. With FI, differences between barley and maize were detected after 6 h, resulting in average values of 61.0, 35.3 and 14.1 mL/g OM at 10 h for barley, maize and sorghum. Gas production results were supported by total VFA concentration. The fermentative activity for vitreous starch of a forage inoculum is lower than that promoted by a concentrate diet, thus affecting substrate utilization and rumen environment. Fermentation of starch-rich substrates in a low buffered medium gives a more realistic picture than conventionally buffered conditions.

**Keywords:** cereal grains; pH; inoculum; *in vitro* fermentation.

**Abbreviations:** ADF, acid detergent fibre; ADL, acid detergent lignin; aNDFom, neutral detergent fibre; BCFA, branched chain fatty acids; CI, concentrate inoculum; DM, dry matter; EE, ether extract; FI, forage inoculum; OM, organic matter; SEM, standard error of means; VFA, volatile fatty acids.

1. Introduction

Cereal grains vary in their rate of starch rumen fermentation, which is higher in wheat, oats and barley than maize and sorghum (Herrera-Saldana et al., 1990; O’Brien, 1999). Besides, the presence of phenolic compounds in some varieties of sorghum grains markedly affects their microbial fermentation (Streeter et al., 1990; Kim et al., 2006). Nutritive values of cereal grains are often assumed as common for each vegetal species; however, notable differences reported among varieties (O’Brien, 1999) prevent for such generalisation.

The study of potential rumen microbial fermentation of cereal grains and the comparison of different species and varieties has been approached by in situ incubation (Nocek and Tamminga, 1991; Offner et al., 2003). Since these studies are costly, laborious and time consuming, the *in vitro* gas production technique has been alternatively applied to study the pattern of fermentation of whole grains (Opatpatanakit et al., 1994; Chai et al., 2004; Lanzas et al., 2007) or their starch or fibre components (Chen et al., 1999; Tahir et al., 2013).
However, this technique does not consider the range of rumen pH, below 6.0 and even under 5.5, resulting from the high production of volatile fatty acids (VFA) and lactate by starch fermentation, together with the low salivation promoted by concentrate diets rich in cereal grains (Krause and Oetzel, 2006; Nagaraja and Titgemeyer, 2006). The acidification capacity of cereal grains is associated with their amount of starch, as well as with its chemical nature and its availability for rumen microbes (Van Barneveld, 1999; Offner et al., 2003). Therefore, the study of microbial fermentation of starch rich feeds should require the establishment of low incubation pH, whereas the procedures for the in vitro gas production technique are carried out on incubation under well buffered conditions (Mould et al., 2005a). By reducing the proportion of bicarbonate ion used as buffer in the incubation solution, our group (Amanzougarene and Fondevila, in evaluation) successively adjusted the incubation pH at a range level from 6.50 to 5.75.

Different species of rumen bacteria are able to ferment starch in the rumen; further, their activity is also related to the nature of the starch source (McAllister et al., 1990; Klieve et al., 2003). In practical situations, ruminant animals not adapted to high starch diets, such as cattle reared at pasture that are introduced to intensive feeding systems in Mediterranean countries, have a reduced capacity of VFA absorption through the rumen wall that, together with a high production of lactic acid, contributes to the appearance of acidosis. This in vitro work aimed to study the acidification capacity and the fermentation pattern of three varieties of each barley, maize and sorghum grains by using a poorly buffered medium, and to what extent such effects depend on the characteristics of the rumen inoculum, either from a forage or a concentrate diet.

2. Material and methods

2.1. Substrates and inocula
Nine cereal feeds, consisting of three varieties of maize (Dekalb 6815, M1; Dekalb 6667YG, M2; Pioneer 0725, M3), three of sorghum (one white sorghum, S1, and two brown sorghums, S2 and S3; unknown varieties) and three of barley (var. Gustav, B1; var. Signora, B2; var. Graphic, B3), were incubated. All substrates were milled through a sieve of 1 mm using a hammer mill (Retsch Gmbh/SK1/417449). Chemical composition of substrates is given in Table 1.

Four adult Rasa Aragonesa ewes (average weight 71.5 ± 1.7 kg) fitted with a rumen cannula, were used as donor of rumen inoculum. Animals were housed in the facilities of the Servicio de Apoyo a la Experimentación Animal of the Universidad de Zaragoza, Spain. Animal care and procedures for extraction of rumen inoculum 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. At the beginning of the experiment, ewes were fed 600 g of alfalfa hay plus 300 g of straw for two weeks, in order to get a rumen environment characteristic of forage diets (forage inoculum, FI). Then, diet was changed to 500 g of concentrate (0.60 barley, 0.20 corn, and 0.20 soybean meal) plus 300 g of alfalfa hay for another two weeks, as a typical concentrate-fed animals (concentrate inoculum, CI). Rumen contents (approximately 300 ml) were extracted before the morning feeding, pooled, filtered through cheesecloth and transferred to the laboratory in thermos bottles preheated to 39° C.

2.2 Incubation procedures

The in vitro incubation of substrates followed the procedure of Theodorou et al. (1994), in glass bottles (116 mL total volume) containing 500 mg of substrate. The bottles were filled with 80 mL of incubation solution, which consisted of 0.10 rumen inoculum and 0.90 of an incubation mixture containing (mL/L): 474 mL distilled water; 238 mL of buffer solution made up with 1.9 g/L sodium bicarbonate (NaHCO₃) and 0.1 g/L ammonium bicarbonate ((NH₄)HCO₃), calculated to establish a minimum pH of 5.50 (Kohn and Dunlap, 1998) and assumed as an almost pH-free
medium (Amanzougarene and Fondevila, in evaluation); 238 mL of macro-minerals solution made up with 5.7 g disodium hydrogen phosphate (Na$_2$HPO$_4$), 6.2 g potassium di-hydrogen orthophosphate (KH$_2$PO$_4$), and 0.6 g magnesium sulphate (MgSO$_4$·7H$_2$O); and 50 mL of reducing solution made up with 47.5 mL distilled water, 2 mL 1N NaOH and 313 mg HCl-cysteine. Micro-minerals solution and resazurin were not included in the incubation medium (Mould et al., 2005a). Ingredients were mixed and allowed to be reduced under a CO$_2$ atmosphere. Bottles were filled with the incubation solution under a CO$_2$ stream, sealed with rubber caps and aluminium rings, and incubated at 39 °C. Three 10 h runs of incubation with five bottles per treatment were carried out with each type of inoculum (FI and CI). On each incubation run, three additional bottles without substrate were also included as blanks, for subtracting the contribution of inoculum to overall gas production. Pressure was recorded every two hours of incubation, by means of an HD 2124.02 manometer fitted with a TP804 pressure gauge (Delta Ohm, Caselle di Selvazzano, Italy). Pressure readings were converted into volume by a pre-established linear regression equation between the pressure recorded in the same bottles under the same conditions and known air volumes (n= 103; $R^2= 0.996$). The gas volume recorded for each incubation time was expressed per unit of incubated organic matter (OM). One bottle per treatment was opened every 2 h and at the end of each incubation run, for measuring incubation pH (CRISON micropH 2001, Barcelona, España). Incubation medium in the bottle opened after 4 and 8 h was sampled and stored at -20°C for volatile fatty acids (VFA) analysis (2 mL sample, collected over 0.5 mL of a deproteinizing mixture of 0.5M PO$_4$H$_3$ with 2 mg/mL 4-methyl valeric acid) and lactate (2 mL).

2.3. Chemical analyses

Substrates were analysed following the procedures of AOAC (2005) for dry matter (DM; ref. 934.01), organic matter (OM; ref. 942.05), crude protein (CP; ref. 976.05) and ether extract (EE; ref. 2003.05) analysis. Concentration of neutral detergent fibre (aNDFom) was analysed as
described by Mertens (2002) in an Ankom 200 Fibre Analyser (Ankom Technology, New York), using α-amylase and sodium sulphite, and results are expressed exclusive of residual ashes. The acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined as described by AOAC (2005) and Robertson and Van Soest (1981), respectively. 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). The total phenolic (TP) and tannin (TT) content was analysed following the colorimetric method of Makkar et al. (1993) using the Folin-Ciocalteau reagent and with tannic acid (MERCK Chemicals, Madrid, Spain) as the reference standard. The frozen samples of incubation media were thawed and centrifuged at 20,000 g for 15 minutes for their analysis of lactate and VFA. The VFA were determined by gas chromatography on an Agilent 6890, apparatus equipped with a capillary column (HP-FFAP Polyethylene glycol TPA, 30 m x 530 µm id). The lactate concentration was determined by the colorimetric method proposed by Barker and Summerson (1941).

2.4. Statistical analysis

Results were analysed by ANOVA using the Statistix 10 software package (Analytical Software, 2010). The effect of the type of inoculum, the cereal sources and their interaction on the gas production, pH, total VFA concentration, VFA profile and lactate were studied as factors for each time of sampling, considering the interaction inoculum x incubation run as block. The differences were considered significant when P<0.05, and a trend for significance was considered when 0.05≤P<0.10. The Tukey test (P<0.05) was used for the multiple comparison between means. The relationship among results from the different parameters was studied by linear or multiple regression analyses, at a level of significance of 0.05.

3. Results
At the start of the incubation series, the mean inoculum pH was 6.35 ± 0.16 and 6.73 ± 0.19 for the concentrate (CI) and forage (FI) inocula, respectively (n=3), and dropped to average values of 5.82 and 5.99, respectively, in the first two hours of incubation (SEM=0.005). During the whole incubation period, average pH with CI was lower than that of FI ($P<0.001$), differences increasing up to 0.51 pH units at 10 h incubation (pH of 5.25 and 5.76 for CI and FI; SEM=0.013). Since the interaction inoculum x substrate resulted significant at 2, 8 and 10 h of incubation ($P<0.01$), the pH pattern followed with each substrate is presented separately for each inoculum.

Incubation pH from 2 to 6 h incubation with CI (Figure 1a) was lowest ($P<0.001$) with the three varieties of barley, but differences among sorghum and maize were not manifested until 8 and 10 h, when pH was highest with sorghum and recording intermediate and minimum values with maize and barley varieties (average pH at 8 h of 5.63, 5.44 and 5.13 for sorghum, maize and barley, respectively). No differences in pH among varieties of each cereal species were detected with CI ($P>0.05$), except at the end of incubation (10 h), when pH for sorghum S1 was lower than for S2, and that of M3 lower than for M1 (5.42 vs. 5.61 and 5.16 vs. 5.32, respectively; $P<0.05$), whereas the pH recorded at 10 h with M1 and S1 did not differ ($P>0.05$). When cereals were incubated using FI (Figure 1b), the pH from 2 to 10 h incubation was reduced at a lower extent than with CI. At 8 and 10 h incubation, pH with barley varieties (on average, 5.68 and 5.52, respectively) was lowest than with maize (5.93 and 5.85 at 8 and 10 h) or sorghum (5.96 and 5.91). No differences in incubation pH were detected among varieties within cereals, nor between sorghum and corn varieties except with M1 at 6 and 8 h incubation ($P>0.05$).

Between inocula, once corrected for the gas production in blank bottles the average gas production recorded with CI was always superior ($P<0.001$) to the gas production recorded with FI (Figure 2), with differences increasing with time from 8 to 31 mL/g OM (average gas production of 67.3 and 36.8 mL/g OM for CI and FI at 10 h incubation; SEM = 1.02. The pattern of gas production among cereal varieties of each cereal species differed for each inoculum ($P= 0.001$), and for an easier understanding results are reported separately for CI and FI. When CI was used (Figure 3a),
the volume of gas produced from barley varieties was similar to that from maize ($P > 0.05$), except at 4 and 6 h incubation, when M2 and M1 gave lower values than the barley. No differences among varieties of either barley or maize were detected at any incubation time ($P > 0.05$). In contrast, sorghum varieties showed a different behaviour ($P < 0.05$): whereas S1 was grouped with maize varieties, S2 and S3 produced a lower amount of gas. As a result, after 10 h incubation, barley and maize varieties produced on average 82.5 and 73.0 mL/g OM, whereas S1, S2 and S3 rendered 68.4, 31.1 and 39.7 mL/g OM, respectively. However, with FI (Figure 3b) no differences between barley and maize substrates were detected up to 6 h, and afterwards substrates ranked as: B1, B3, B2 > M1, M2 > M3, S1, S3 > S2 ($P < 0.05$), resulting in average values of 61.0, 38.9, 22.9 and 1.5 mL/g OM at 10 h for each mentioned group.

At both sampling times CI showed a higher ($P < 0.001$) concentration of VFA (22.8 vs. 14.8 mmol/L at 4 h and 31.0 vs. 19.9 mmol/L at 8 h) than FI, as well as higher ($P < 0.001$) proportions of butyrate (0.107 vs. 0.052 and 0.105 vs. 0.053) and valerate (0.009 vs. 0.006 at both 4 and 8 h) and lower ($P < 0.001$) proportions of acetate (0.479 vs. 0.542 and 0.469 vs. 0.531) and branched chain VFA (sum of isobutyrate and isovalerate, BCFA; 0.013 vs. 0.020 and 0.012 vs. 0.019), whereas propionate did not differ among inocula (average proportion of 0.177 and 0.191 at 4 and 8 h).

Since the inoculum x substrate interaction was significant only for butyrate proportion at 4 h ($P = 0.009$) and for acetate and butyrate proportions at 8 h ($P < 0.001$), only substrate means for total VFA and molar VFA are shown in Tables 2 and 3, and the implications of such interactions are explained in text. On average, the VFA concentration at 4 h (Table 2) was higher ($P = 0.001$) with the barley variety B3 than all maize and sorghum varieties, and propionate proportion was higher ($P < 0.001$) for barley than sorghum varieties, with maize showing intermediate values. In contrast, acetate proportion was higher ($P < 0.001$) in sorghum than barley varieties. Concerning butyrate proportion at 4 h ($P = 0.009$), S2 showed a higher proportion than B1, B2, M1 and M2 when incubated with CI, but no substrate differences were recorded with FI. The BCFA proportion after 4 h of incubation was higher ($P = 0.004$) in S3 than in B2, B3, M2 and M3, whereas no
differences were recorded in valerate proportion. No differences were detected among varieties of the same cereal species for total VFA concentration or any VFA molar proportion.

After 8 h, a higher ($P>0.001$) VFA concentration was detected with barley varieties compared with S2 and S3, and B2 was also higher than S1, whereas those of maize recorded intermediate values (Table 3). As for 4h, no differences were detected among varieties of the same cereal species. Propionate proportion in barley varieties and M1 was higher ($P<0.001$) than in sorghums, but M2 and M3 did not differ from S3. With FI as inoculum, fermentation of sorghum varieties rendered a higher proportion of acetate than the three barleys, those of maize recording intermediate values, whereas no substrate differences were detected with CI. Concerning butyrate, its proportion was lowest for barley when incubated with CI, but it did not differ among substrates with FI.

No differences among inocula were detected in lactic acid concentration at 4 h but it was higher ($P=0.001$) with CI than FI at 8 h incubation (2.31 vs. 1.33 mM; SEM=0.225). Among substrates, lactate concentration at 4 h was higher in barley varieties than in those of sorghum and M2 and M3 ($P<0.001$, data not shown), whereas after 8 h lactate concentration in barley varieties were highest (average values of 3.92, 3.94, 3.97, 0.75, 0.96, 1.17, 0.35, 0.26 and 0.26 mM for B1, B2, B3, M1, M2, M3, S1, S2 and S3; SEM= 0.478; $P<0.001$).

4. Discussion

When ruminants at pasture are introduced to intensive systems based on cereal-rich diets there is an acute drop of rumen pH associated to the higher extent of fermentation (Krause and Oetzel, 2006; Nagaraja and Titgemeyer, 2006). Our aim in the present work was to determine the magnitude of fermentation differences between concentrate- and forage-type environments, considering the potential application of results to practical situations of feeding transition. In such scenario, it is worth knowing the potential of acidification of the different substrates, since this
may determine either an easier or a more difficult adaptation of the rumen, thus minimising at possible the risk of acidosis (Broudiscou et al., 2014).

The amount of starch of the tested varieties of cereal species agrees with that reported by FEDNA (2010), being higher in both sorghum and corn than in barley. Rumen microbial fermentation of different cereal species and varieties within a certain species has been previously compared at a buffered pH (O’Brien, 1999; Chai et al., 2004; Lanzas et al., 2007). Since other factors such as the geographical site or the year of growth also affect the result (Opatpanatakit et al., 1994; O’Brien, 1999), this work was rather focused to the different potential of acidification of the rumen environment and its effect on microbial utilisation, in order to estimate which can be better suited to a gradual adaptation of a fibrolytic to an amylolytic microbiota. Most in vitro approaches to this had been limited because studies were mostly carried out on a well-buffered medium, and therefore results can hardly be extrapolated to practical situations. However, at the same time results from this experiment are difficult to compare with others in literature, since approaches to gas production allowing for a relatively free incubation pH are scarce (Opatpanatakit et al., 1994). In incubation conditions with buffered medium, Chai et al. (2004) observed that gas production from maize after 6 h was 0.36 that of barley, whereas such proportion increased to 1.06 after 12 h incubation. Lanzas et al. (2007) observed higher fractional rate of gas production with barley than maize and sorghum varieties (on average, 0.24, 0.15 and 0.06 h⁻¹), showing interspecies ranges between 0.22 and 0.29, 0.09 and 0.17, and 0.05 and 0.08, respectively. Opatpanatakit et al. (1994) used the McDougall (1948) artificial saliva, which allows for a more acute drop of pH. They also observed highest gas production with barley, intermediate with maize and lowest with sorghum (on average, 222, 138 and 104 mL/g DM, respectively), under pH values after 7 h incubation ranging from 5.7 to 6.1 for barley, 6.5 to 6.9 for maize and 6.5 to 6.8 for sorghum.

The effect of the source of rumen fluid on the in vitro fermentation has been recently demonstrated by Broudiscou et al. (2014), and it is widely accepted that forage based diets
maintain higher incubation medium pH than diets including a major proportion of concentrate.

Despite the magnitude of the drop of pH caused by fermentation was more pronounced, the incubation environment promoted by giving a concentrate diet to the donor animals was more favourable for cereal fermentation than that induced by a fibrous diet, as expected (Menke and Steingass, 1988; Mould et al., 2005b). Throughout the 10 h incubation period, pH was more stable with the forage inoculum, probably because of its lower capability to ferment starch and the lower extent of fermentation caused by the lack of adaptation of microbial communities to starch substrates (Trei et al., 1970; Nagadi et al., 2000). In any case, pH in maize and sorghum incubations with FI was around 6.0, considered as a threshold for viability of most non-amylolytic rumen bacterial species (Russell and Dombrowski, 1980), and even reached 5.5 after 10 h incubation with barley. With inoculum from a concentrate diet, pH dropped at a higher extent as the incubation proceeded and reached lower values with all substrates, allowing for clearer differences among substrates. This was also manifested in a higher VFA concentration, as noted by Calsamiglia et al. (2008), but also in butyrate proportion at the expense of that of acetate and BCFA.

The accumulated volume of gas recorded from the blanks of inocula (without substrate) after 10 h incubation with FI and CI were 20.7 and 31.0 mL (from 80 mL, data not shown), produced by fermentation of solubles suspended in the medium. Once this amount was subtracted from the total recorded volume, the volume of gas produced from substrates was also higher with CI than FI as inoculum, in 1.3 to 1.4-fold for the three barleys, from 1.8 to 2.0-fold for M1 and M2, and from 2.3 to 3.0 for M3, S1 and S3. It has to be considered that, despite the source of inoculum, the pH along all the 10 h incubation was below 6.0, being lowest with barley varieties, with which ranged from 5.9 to 5.5 with FI and from 5.7 to 5.0 with CI, seriously challenging microbial activity at the last part of incubation. In fact, the extent of fermentation in terms of gas production was lower than what has been reached in well-buffered media from cereal grains (Chai et al., 2004; Lanzas et al., 2007; Tahir et al., 2013). In vivo, Offner and Sauvant
(2004) established a linear relationship between pH in the range from 6.4 to 5.7 and rumen
digestion of starch; however, Shriver et al. (1986) did not observe differences in in vitro
digestibility of non-structural carbohydrates of a concentrate feed between incubation pH of 5.8
and 7.0. Further, the contribution of fermentation acids produced to the volume of gas is lower at
low pH, because of the reduced contribution of the indirect gas from the buffer (Amanzougarene
and Fondevila, in evaluation), and therefore the magnitude of differences among inocula are
expected to be higher in the case of barleys.

Compared with corn and sorghum, it is expected that the starch of barley was degraded
more rapidly (Opatpanatak et al., 1994; Lanzas et al., 2007). Whereas it is apparent that barley
produced more gas and at a faster rate than maize and sorghum with FI, differences between
barley and corn were lower when the inoculum came from a highly concentrate diet. In this
sense, it is worth noting that fermentation of both corn and sorghum varieties is clearly low when
FI was used, suggesting that the microbial population must be adapted to the more recalcitrant
type of starch in these cereals. The different structure of starch among cereal species, or even
among varieties (Van Barneveld, 1999), concretely the structure of the endosperm and the
proportion of amylose (Huntington, 1997; Offner et al., 2003) may affect the rate and extent of
starch fermentation, thus explaining differences in the volume of gas produced and VFA
concentration between substrates. Besides, the protein matrix in maize and sorghum limits the
access of rumen microbes to starch granules (McAllister et al., 1993; Rooney and Pflugfelder,
1986). The white sorghum S1, with a low phenolic and tannin content, reached similar values than
maize varieties when incubated with inoculum from a concentrate diet. However, with FI, the gas
production from maize varieties, mainly M3, as well as that from S1, were reduced at a higher
extent than those of barleys, indicating that such inoculum is less capable to ferment the vitreous
starch and the protein matrix in the endosperm of the former cereals (Philippeau and Michalet-
Doreau, 1997; Ramos et al., 2009). Further, differences among varieties were apparent for
sorghum, probably due to their different contents of phenolics and tannins (Table 1). With CI, gas
production from S1 was higher than that from the two brown varieties S3 and S2, because of the lower content of tannins and other phenolics in the former (Hibberd et al., 1985; Streeter et al., 1990; Opatpatanakit et al., 1994), but with FI differences between S1 and S3 were minimised. In the case of S2, the gas production was almost nil when incubated with FI.

The higher total VFA and lactate production with barley varieties are the cause of their higher acidification capacity, as it has been previously reported by Russell and Hino (1985) and Broudiscou et al. (2014). In fact, a strong correlation was observed in this work between incubation pH and lactate concentration at 8 h incubation \( (R^2 = 0.98) \). Differences in the molar proportion of VFA indicate that the fermentation profile of cereal grains differ according to the type and availability of starch, as well as with the phenolic and tannin content of sorghum varieties, in agreement with the gas production pattern.

5. Conclusions

Among the studied cereal species, the extent and rate of fermentation of barley grains were higher than that of maize varieties, contributing to the magnitude of the drop of medium pH in the first hours of incubation, and there were no major variation among the studied varieties of each species. However, despite of the minor differences among varieties of barley and maize, care must be taken in generalising experimental results to a species level. The response of sorghum varieties is largely dependent of their content in tannins and phenolic compounds, and whereas sorghum S1 behaved similarly to the maize substrates, fermentation of brown sorghums S2 and S3 depended on their amount of these secondary compounds. Fermentation of barley and maize grains is increased when inoculum from a concentrate-based diet is used, which contributes to a faster drop of pH that a forage inoculum. However, the extent of differences is higher among maize varieties, supporting that microbial community of a forage inoculum is less able to ferment vitreous types of starch. Despite the origin of inoculum, incubation pH was more stable with
sorghum substrates, but gas production from S2, with a higher proportion of phenolic compounds was almost nil with forage inoculum, indicating a higher sensibility of this microbial community.

In any case, despite it seems clear that a rapid establishment of an acidic rumen environment is useful to maximise the rumen utilisation of cereals in ruminants given high concentrate diets, the impact of such situation in overall health status of the animals and consequently their productive performances were not evaluated with this experiment, and will deserve further consideration.

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**Table 1.** Chemical composition (g/kg DM) of incubated substrates (varieties of maize, sorghum and barley).

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<td>B1</td>
<td>978</td>
<td>105</td>
<td>24</td>
<td>672</td>
<td>173</td>
<td>56</td>
<td>17.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>978</td>
<td>112</td>
<td>18</td>
<td>635</td>
<td>152</td>
<td>44</td>
<td>9.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>977</td>
<td>91</td>
<td>18</td>
<td>651</td>
<td>189</td>
<td>55</td>
<td>9.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1: M1, M2 and M3 are varieties of maize; S1, S2 and S3 are varieties of sorghum; B1, B2 and B3 are varieties of barley.

2: OM: organic matter; CP: crude protein; EE: ether extract; aNDFom: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; TP: total phenolics; TT: total tannins.
Table 2. Average total volatile fatty acids concentration (VFA, mM) and molar VFA proportions (mmol/mmol) recorded at 4 h incubation of varieties of maize, sorghum and barley in a low buffered medium.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>VFA</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
<th>BCFA</th>
<th>Valerate</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>17.0&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.513&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.179&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.079&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.016&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.007</td>
</tr>
<tr>
<td>M2</td>
<td>17.6&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.514&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.178&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.079&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.016&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.007</td>
</tr>
<tr>
<td>M3</td>
<td>17.8&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.510&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.180&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.080&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.015&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.007</td>
</tr>
<tr>
<td>S1</td>
<td>17.8&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.520&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.164&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>0.082&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.018&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.008</td>
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<tr>
<td>S2</td>
<td>16.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.526&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.159&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.083&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.019&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.009</td>
</tr>
<tr>
<td>S3</td>
<td>17.5&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.519&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.161&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.082&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.021&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.010</td>
</tr>
<tr>
<td>B1</td>
<td>20.8&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.499&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.191&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.079&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>0.008</td>
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<tr>
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<td>21.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.501&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.191&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.078&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.016&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.008</td>
</tr>
<tr>
<td>B3</td>
<td>23.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.496&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.195&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.080&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.015&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.008</td>
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<tr>
<td>SEM&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.02</td>
<td>0.0036</td>
<td>0.0035</td>
<td>0.0010</td>
<td>0.0010</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

<sup>a</sup>, <sup>b</sup>, <sup>c</sup> Different superscripts within a column indicate means differences (P<0.05)

1: M1, M2 and M3 are varieties of maize; S1, S2 and S3 are varieties of sorghum; B1, B2 and B3 are varieties of barley

2: BCFA: branched-chain fatty acids (sum of isobutyrate + isovalerate)

3: SEM: standard error of the means
Table 3. Average total volatile fatty acids concentration (VFA, mM) and molar VFA proportions (mmol/mmol) recorded at 8 h incubation of varieties of maize, sorghum and barley in a low buffered medium.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>VFA</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
<th>BCFA</th>
<th>Valerate</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>25.2</td>
<td>0.495&lt;sup&gt;de&lt;/sup&gt;</td>
<td>0.196&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.082&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.014</td>
<td>0.007</td>
</tr>
<tr>
<td>M2</td>
<td>26.0</td>
<td>0.504&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>0.188&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>0.080&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.014</td>
<td>0.007</td>
</tr>
<tr>
<td>M3</td>
<td>25.2</td>
<td>0.499&lt;sup&gt;bcde&lt;/sup&gt;</td>
<td>0.193&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.081&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.014</td>
<td>0.007</td>
</tr>
<tr>
<td>S1</td>
<td>23.8</td>
<td>0.510&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.177&lt;sup&gt;de&lt;/sup&gt;</td>
<td>0.084&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.015</td>
<td>0.007</td>
</tr>
<tr>
<td>S2</td>
<td>21.6</td>
<td>0.522&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.164&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.081&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.018</td>
<td>0.009</td>
</tr>
<tr>
<td>S3</td>
<td>21.5</td>
<td>0.506&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.178&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>0.083&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.018</td>
<td>0.008</td>
</tr>
<tr>
<td>B1</td>
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<td>0.077&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.015</td>
<td>0.008</td>
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<tr>
<td>B2</td>
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<td>0.486&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>0.015</td>
<td>0.008</td>
</tr>
<tr>
<td>B3</td>
<td>27.4</td>
<td>0.492&lt;sup&gt;de&lt;/sup&gt;</td>
<td>0.204&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.077&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.014</td>
<td>0.008</td>
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<tr>
<td>SEM&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.18</td>
<td>0.0030</td>
<td>0.0035</td>
<td>0.0012</td>
<td>0.0009</td>
<td>0.0005</td>
</tr>
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</table>

<sup>a, b, c</sup> Different superscripts within a column indicate means differences (P<0.05)

1: M1, M2 and M3 are varieties of maize; S1, S2 and S3 are varieties of sorghum; B1, B2 and B3 are varieties of barley

2: BCFA: branched-chain fatty acids (sum of isobutyrate + isovalerate)

3: SEM: standard error of the means
Figure 1. Pattern of incubation pH according to the different varieties of maize (M1 ■, M2 ▲, M3 ▼, solid line), sorghum (S1 ■, S2 ▲, S3 ●, dashed line) and barley (B1 ▼, B2 ▲, B3 ○; dotted line) when incubated in a low buffered medium with inoculum from a concentrate diet (CI, Fig. 2a) or from a forage diet (FI, Fig. 2b). Upper bars show standard error of means.
Figure 2. Average pattern of gas production from the substrates incubated in a low buffered medium with inoculum from a concentrate diet (CI, •) or a forage diet (FI, O). Upper bars show standard error of means.
Figure 3. Pattern of gas production from the different varieties of maize (M1 ■, M2 ▲, M3 ●, solid line), sorghum (S1 □, S2 △, S3 ○, dashed line) and barley (B1 ■■, B2 ▲, B3 ○; dotted line) when incubated in a low buffered medium with inoculum from a concentrate diet (CI, Fig. 3a) or from a forage diet (FI, Fig. 3b). Upper bars show standard error of means.