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# Effect of the addition of malate on *in vitro* rumen fermentation of cereal grains

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Batch cultures of mixed rumen micro-organisms were used to study the effects of different concentrations of malate (Rumalato®; Norel & Nature S.A., Barcelona, Spain; composed of disodium malate–calcium malate (0·16:0·84, w/w)) on the fermentation of four cereal grains (maize, barley, wheat and sorghum). Rumen contents were collected from four Merino sheep fed lucerne hay *ad libitum* and supplemented with 300 g concentrate/d. Rumalato® was added to the incubation bottles to achieve final concentrations of 0, 4, 7 and 10 mM-malate. Gas production was measured at regular intervals up to 120 h. Malate increased ($P<0·01$) the average fermentation rate of all substrates, and the lag time decreased ($P<0·05$) linearly with increasing concentrations of malate for all substrates, with the exception of sorghum. In 17 h incubations, the final pH and total volatile fatty acid production increased ($P<0·001$) linearly for all substrates as malate concentration increased from 0 to 10 mM. Propionate and butyrate production increased ($P<0·05$), while the value of the acetate : propionate ratio and L-lactate concentrations decreased ($P<0·05$) linearly with increasing doses of malate. Malate treatment increased ($P<0·05$) the $CO_2$ production and decreased the production of $CH_4$, although this effect was not significant ($P>0·05$) for maize. Malate at 4 and 7 mM increased ($P<0·05$) optical density of the cultures measured at 600 nm for maize, with no differences for the other substrates. The results indicate that malate may be used as a feed additive for ruminant animals fed high proportions of cereal grains, because it increased pH and propionate production and decreased $CH_4$ production and L-lactate concentrations; however, in general, no beneficial effects of 10 compared with 7 mM-malate were observed.

**Rumen: Malate: Batch cultures: Cereal grains**

Antimicrobial compounds are routinely incorporated into ruminant animal diets to improve production efficiency. However, in recent years there has been an increasing concern regarding the use of antibiotics in ruminant animal diets due to the progressive increase of antibiotic resistance among pathogenic micro-organisms. For this reason, the European Union has banned many of these additives, and has recently presented a new regulation that would phase out the authorisations of the four antibiotic feed additives that are still on the European Union market by January 2006. As a consequence, there is an urgent need to develop alternatives to the use of antibiotics as growth promoters. However, compared with the efforts to study the effects of antibiotic compounds (mainly ionophores) on rumen fermentation, little research has been conducted to evaluate the possible alternatives to these compounds (Martin, 1998).

Some authors (Callaway & Martin, 1996; Newbold *et al.* 1996) have suggested that organic acids (aspartate, fumarate, malate) could provide an alternative to currently used antimicrobial compounds. Many of the experiments conducted on the effects of malate on rumen fermentation have been carried out *in vitro*. In most of these studies (Martin & Streeter, 1995; Callaway & Martin, 1996; Carro *et al.* 1999), malate treatment resulted in changes in final pH, $CH_4$ and volatile fatty acid concentrations that are analogous to the effects of ionophores. However, the mode of action of malate appears to be completely different, and in contrast with antimicrobial compounds, it seems to stimulate rather than inhibit some specific rumen bacterial populations (Nisbet & Martin, 1993). Malate is a key intermediate in the production of succinate or propionate in some rumen bacteria and therefore could stimulate propionate production. In fact, propionate production was increased by adding malate to *in vitro* cultures (Martin & Streeter, 1995; Callaway & Martin, 1996) or semi-continuous fermenters (Carro *et al.* 1999).

**Abbreviations:** OD600, optical density at 600 nm; OM, organic matter.

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Malate supplementation in ruminant animal diets has been shown to improve average daily gain and feed efficiency in steers (Sanson & Stallcup, 1984; Martin *et al.* 1999) and to increase milk persistency and feed efficiency in dairy cows (Stallcup, 1979; Kung *et al.* 1982). In contrast, no effects of malate on rumen digestion and rumen microbial efficiency were found by Montaño *et al.* (1999) in steers fed a diet containing 770 g steam-flaked barley, 100 g hay, 60 g cane molasses and 40 g yellow grease (fats and oils from cooking)/kg; Kung *et al.* (1982) reported no effect of malate on diet digestibility and N retention in steers fed a diet based on whole-shelled maize–maize silage (50:50, w/w) *ad libitum*. These contrasting results could be due to differences in the composition of the diet and/or to the dose of malate fed to animals. The objective of the present study was to evaluate the effects of different doses of malate on the *in vitro* rumen fermentation of cereal grains (maize, barley, wheat and sorghum).

## Materials and methods

### *Substrates and experimental procedure*

Samples of maize, barley, wheat and sorghum were ground through a 1 mm screen and fermented *in vitro* with buffered rumen contents. Rumen contents were obtained from four rumen-cannulated Merino sheep fed forage (medium-quality lucerne hay) *ad libitum* and 300 g concentrate/d administered in two equal portions at 09.00 and 18.00 hours. Concentrate was based on barley–maize–soyabean meal (39:40:23, by weight on a fresh matter basis). The chemical composition of foods and cereal grains is given in Table 1. Rumen contents of each sheep were obtained 2 h after the morning feed of concentrate and strained through four layers of cheesecloth into an Erlenmeyer flask with an $O_2$-free headspace. Particle-free rumen contents were mixed with the buffer solution of Goering & Van Soest (1970) in a proportion 1:4 (v/v) at 39°C under continuous flushing with $CO_2$. Samples of 500 mg of each cereal grain were accurately weighed into 125 ml serum bottles (Laboratorios Ovejero S.A., León, Spain). Rumalato® (Norel & Nature S.A., Barcelona, Spain) was added to achieve final malate concentrations of 0, 4, 7 and 10 mM. Rumalato® is a commercial product composed of disodium malate–calcium malate (0·16:0·84, w/w). The cost of Rumalato® is €2·90/kg. Bottles were pre-warmed (39°C) prior to the addition of 50 ml buffered rumen contents into each bottle under $CO_2$ flushing. Bottles were sealed with rubber stoppers and Al caps and incubated at 39°C. All samples were incubated with the rumen contents of each sheep so that each treatment was conducted in quadruplicate.

### *Fermentation kinetics from gas production curves*

Gas production was measured in four bottles per substrate and per malate treatment at 2, 4, 6, 9, 12, 16, 21, 26, 31, 36, 48, 60, 72, 96 and 120 h using the pressure transducer technique (Theodorou *et al.* 1994). After 120 h incubation, the fermentation was stopped by swirling the bottles in ice, the bottles were opened and their contents were transferred to previously weighed filter crucibles. The residue of incubation was washed with 50 ml hot distilled water, dried at 50°C for 48 h and the apparent disappearance of substrate was calculated. The residue was then analysed for ash to calculate the organic matter (OM) apparent disappearance. For each sheep, two blanks were included to correct the gas production values for gas release from endogenous substrates.

### *Fermentation variables*

A total of sixteen bottles (four bottles for each malate concentration) were incubated for each substrate. Bottles were withdrawn from the incubator 17 h after inoculation (corresponding to a passage rate from the rumen of 6 % per h) and total gas production was measured using a calibrated syringe. A gas sample was removed from each bottle and stored in a Haemoguard Vacutainer (Terumo Europe N.V., Leuven, Belgium) before analysis for $CH_4$ and $CO_2$ concentration. The fermentation was then stopped by swirling the bottles on ice. Bottles were uncapped and the pH was measured immediately with a pH meter. Bottles were emptied into centrifuge tubes and centrifuged (600 ***g***, 4°C, 10 min) to eliminate feed particles. Supernatant fluid (1 ml) was added to 1 ml deproteinising solution (metaphosphoric acid–crotonic acid (10·00:0·06, w/v)) for volatile fatty acid analysis and another 5 ml were added to 5 ml HCl for $NH_3$-N analysis. Samples were stored at −20°C. A sample of the supernatant fraction was taken to analyse concentrations of L-lactate by an enzymatic–colorimetric method using a diagnostic kit (Sigma, Madrid, Spain). Optical density at 600 nm (OD600) was used as an index of the size of the bacterial population supernatant fraction of the culture. Samples of the supernatant fraction were diluted 1:25 with saline solution (9 g NaCl/l) before measurement of OD600 with a

**Table 1.** Chemical composition (g/kg DM) of ingredients of sheep diet and cereal grains incubated *in vitro*

| | Organic matter | Crude protein (N × 6·25) | Neutral-detergent fibre | Acid-detergent fibre |
|---|---|---|---|---|
| Diet ingredients | | | | |
| Lucerne hay | 912 | 158 | 472 | 301 |
| Concentrate | 916 | 198 | 151 | 46·8 |
| Cereal grains | | | | |
| Maize | 985 | 89·6 | 119 | 23·2 |
| Barley | 974 | 116 | 176 | 42·5 |
| Wheat | 983 | 103 | 142 | 28·6 |
| Sorghum | 982 | 114 | 106 | 31·8 |

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spectrophotometer (Spectronic 20D; Milton Roy (UK) Ltd, Wokingham, Berks., UK). Finally, the contents of the centrifuge tube were transferred to previously weighed filter crucibles. The residue of incubation was washed with 50 ml hot distilled water, dried at 50°C for 48 h and analysed for ash to calculate the OM apparent disappearance. For each sheep, two blanks were included to correct the gas production values for gas release from endogenous substrates.

### *Analytical procedures*

DM, ash and N were determined according to the Association of Official Analytical Chemists (1995). Neutral- and acid-detergent fibre analyses were carried out according to Van Soest *et al.* (1991) and Goering & Van Soest (1970) respectively. $NH_3$ concentration was determined by a modified colorimetric method (Weatherburn, 1967). Volatile fatty acids were determined in centrifuged samples (1 ml) by GC as previously described (Carro *et al.* 1999). $CH_4$ and $CO_2$ were analysed with a GC (Shimadzu GC 14B; Shimadzu Corporation, Kyoto, Japan) equipped with a thermal conductivity detector and a column packed with Carboxen 1000 (Supelco, Madrid, Spain). The carrier gas was He and peaks were identified by comparison with standards of known composition. The volume of gas produced (ml) was corrected for standard conditions ($10^5$ Pa, 298 K), and the amounts of $CH_4$ and $CO_2$ produced (mmol) were calculated by multiplying the gas produced (mmol) by the concentration of each gas in the analysed sample.

### *Calculations and statistical analyses*

The amount of volatile fatty acids produced was obtained by subtracting the amount present initially in the incubation medium from that determined at the end of the incubation period. Gas production values were fitted with time to the exponential model:

$$\text{gas} = A(1 - e^{(-c(t-lag))}),$$

where $A$ is the asymptotic gas production and $c$ is the fractional degradation rate. The variables $A$, $c$ and $lag$ were estimated by an iterative least squares procedure using the PROC NLIN of the Statistical Analysis Systems Institute (version 6, 1989; SAS Institute Inc., Cary, NY, USA). The effective degradability of substrate OM (g/kg OM incubated) was estimated assuming a rumen particulate outflow of 0·06 per h, according to the equation proposed by France *et al.* (2000). The average fermentation rate (ml gas/h) was defined as the average gas production rate between the start of the incubation and the time at which the cumulative gas production was half of its asymptotic value, and was calculated as:

$$\text{rate}Ac/(2(\ln 2 + c \;\; lag)).$$

Data for each type of cereal grain were analysed as a one-way ANOVA with four concentrations of malate (0, 4, 7 and 10 mM). The sums of squares were further partitioned by orthogonal polynomial contrast to study linear effects of treatment. Comparisons between treatment means were tested by the least significant difference method. All statistical analyses were performed using the GLM procedure of the Statistical Analysis Systems program (version 6, 1989; SAS Institute Inc., Cary, NC, USA).

## Results

The effects of malate on gas production variables and OM effective degradability are shown in Table 2. Asymptotic gas production ($A$) increased linearly ($P<0·01$) as the concentration of malate increased from 0 to 10 mM when maize, wheat and sorghum were incubated, whereas no effect of malate ($P>0·05$) was detected for barley. Whereas fractional rate of degradation ($c$) was not affected ($P>0·05$) by malate treatment, lag time decreased ($P<0·05$) linearly with increasing levels of malate for all substrates with the exception of sorghum. Malate increased ($P<0·01$) the average fermentation rate of all substrates, with no differences due to the dose of malate. For barley, OM effective degradability was greater ($P=0·027$) when malate was present, but no differences were observed for the other substrates.

The effects of malate on *in vitro* rumen fermentation of maize, barley, wheat and sorghum are shown in Tables 3, 4, 5 and 6 respectively. For all substrates, final pH increased linearly ($P<0·001$) as malate concentration increased, the greatest values corresponding to 10 mM-malate. Whereas no treatment effects ($P>0·05$) were observed for OM apparent disappearance when barley, wheat and sorghum were incubated, OM apparent disappearance for maize was increased ($P<0·05$) by 4 and 7 mM-malate (Table 3). The addition of malate increased ($P<0·05$) the $CO_2$ production for all substrates and decreased ($P<0·05$) the production of $CH_4$ for barley, wheat and sorghum. There were no differences ($P>0·05$) either in $CO_2$ or in $CH_4$ production between malate at 7 and 10 mM.

With all substrates, malate treatment increased ($P<0·001$) linearly total volatile fatty acid production, the greatest values being found at 7 and 10 mM-malate. Malate treatment increased ($P<0·05$) acetate, propionate and butyrate productions and decreased ($P<0·001$) the value of acetate:propionate ratio for all substrates. There was no significant change ($P>0·05$) in the $NH_3$-N concentration with added malate for barley, wheat and sorghum, but 4, 7 and 10 mM-malate decreased ($P<0·05$) $NH_3$-N concentrations when maize was incubated. All concentrations of malate decreased the concentration of L-lactate, resulting in a linear decrease ($P<0·05$) for all substrates with increasing levels of malate.

For maize, the OD600 was increased ($P<0·05$) by adding 4 and 7 mM-malate, but no effect was observed for the other substrates. 10 mM-Malate did not affect ($P>0·05$) the OD600 for maize and sorghum, but decreased ($P<0·05$) the OD600 for barley and wheat.

## Discussion

The incubation with malate resulted in shorter lag times of

**Table 2.** Influence of different concentrations of malate on gas production variables (*A*, *c* and *lag*), average fermentation rate (AFR), and organic matter (OM) effective degradability for maize, barley, wheat and sorghum incubated in batch cultures of mixed rumen micro-organisms for 120 h*

(Mean values for four samples)

| | Malate (mM) | | | | | Statistical significance of the treatment effect: *P* | |
|---|---|---|---|---|---|---|---|
| Substrate and variable | 0 | 4 | 7 | 10 | SED | C *v.* Malate† | Linear‡ |
| Maize | | | | | | | |
| *A* (ml) | 192[a] | 201[b] | 201[b] | 201[b] | 2·1 | 0·001 | 0·003 |
| *c* ($h^{-1}$) | 0·0802 | 0·0773 | 0·0772 | 0·0748 | 0·00242 | NS | NS |
| *lag* (h) | 2·95[b] | 2·65[ab] | 2·47[a] | 2·44[a] | 0·149 | 0·006 | 0·006 |
| AFR (ml/h)§ | 8·36[a] | 8·76[b] | 8·89[b] | 8·71[b] | 0·128 | 0·003 | 0·018 |
| OM effective degradability (%) | 44·0 | 44·6 | 44·8 | 44·4 | 0·54 | NS | NS |
| Barley | | | | | | | |
| *A* (ml) | 185 | 189 | 188 | 191 | 2·6 | NS | NS |
| *c* ($h^{-1}$) | 0·0895 | 0·0900 | 0·0887 | 0·0873 | 0·00299 | NS | NS |
| *lag* (h) | 1·99[c] | 1·79[bc] | 1·39[ab] | 1·31[a] | 0·202 | 0·014 | 0·004 |
| AFR (ml/h)§ | 9·57[a] | 10·0[b] | 10·3[b] | 10·4[b] | 0·119 | 0·001 | 0·001 |
| OM effective degradability (%) | 47·2[a] | 48·3[ab] | 49·1[b] | 49·0[b] | 0·74 | 0·027 | 0·029 |
| Wheat | | | | | | | |
| *A* (ml) | 183[a] | 198[b] | 196[b] | 198[b] | 1·8 | 0·001 | 0·001 |
| *c* ($h^{-1}$) | 0·1058 | 0·1024 | 0·1040 | 0·0986 | 0·00290 | NS | NS |
| *lag* (h) | 2·30[b] | 2·27[ab] | 2·21[ab] | 1·91[a] | 0·169 | NS | 0·046 |
| AFR (ml/h)§ | 10·4[a] | 11·0[b] | 11·1[b] | 11·2[b] | 0·14 | 0·001 | 0·001 |
| OM effective degradability (%) | 50·5 | 50·1 | 50·8 | 50·7 | 0·47 | NS | NS |
| Sorghum | | | | | | | |
| *A* (ml) | 192[a] | 199[b] | 203[b] | 204[b] | 2·2 | 0·001 | 0·001 |
| *C* ($h^{-1}$) | 0·0710 | 0·0707 | 0·0680 | 0·0664 | 0·00220 | NS | NS |
| *lag* (h) | 3·71 | 3·63 | 3·22 | 3·17 | 0·288 | NS | NS |
| AFR (ml/h)§ | 7·19[a] | 7·44[b] | 7·60[b] | 7·52[b] | 0·086 | 0·001 | 0·002 |
| OM effective degradability (%) | 39·0 | 39·5 | 39·8 | 40·0 | 0·48 | NS | NS |

C, Control.
[a,b,c]Mean values within a row with unlike superscript letters were significantly different: $P<0·05$.
* For details of procedures, see p. 182.
† Orthogonal contrast, C *v.* Malate: comparison between control and malate treatments.
‡ Orthogonal polynomials, linear effects of malate dose.
§ Values for 500 mg substrate incubated.

**Table 3.** Influence of different concentrations of malate on *in vitro* fermentation of maize (17 h) by mixed rumen micro-organisms in batch cultures*

(Mean values for four samples)

| | Malate (mM) | | | | | Statistical significance of the treatment effect: *P* | |
|---|---|---|---|---|---|---|---|
| | 0 | 4 | 7 | 10 | SED | C *v.* Malate† | Linear‡ |
| pH | 6·14[a] | 6·17[ab] | 6·18[b] | 6·27[c] | 0·015 | 0·001 | 0·001 |
| OM apparent disappearance (%) | 79·4[a] | 82·1[b] | 81·8[b] | 80·8[ab] | 0·93 | 0·022 | NS |
| $CH_4$ (mmol) | 1·10 | 1·07 | 1·02 | 1·01 | 0·072 | NS | NS |
| $CO_2$ (mmol) | 4·54[a] | 4·72[ab] | 5·11[b] | 5·13[b] | 0·240 | 0·041 | 0·016 |
| VFA (mmol) | | | | | | | |
| Acetate | 1·59[a] | 1·66[ab] | 1·73[b] | 1·69[b] | 0·038 | 0·009 | 0·015 |
| Propionate | 1·38[a] | 1·57[b] | 1·65[c] | 1·62[bc] | 0·030 | 0·001 | 0·001 |
| Butyrate | 0·277[a] | 0·287[ab] | 0·317[b] | 0·355[c] | 0·0165 | 0·011 | 0·001 |
| Others§ | 0·058[ab] | 0·062[b] | 0·061[ab] | 0·054[a] | 0·0034 | NS | NS |
| Total VFA | 3·31[a] | 3·58[b] | 3·76[c] | 3·72[bc] | 0·075 | 0·001 | 0·001 |
| Acetate:propionate (mol:mol) | 1·49[b] | 1·38[a] | 1·36[a] | 1·36[a] | 0·012 | 0·001 | 0·001 |
| $NH_3$-N (mg/l) | 74·1[b] | 58·3[a] | 56·3[a] | 60·8[a] | 3·32 | 0·001 | 0·003 |
| L-Lactate (mg/l) | 203[b] | 148[ab] | 118[a] | 103[a] | 26·8 | 0·005 | 0·004 |
| Optical density (600 nm) | 0·106[a] | 0·125[b] | 0·123[b] | 0·108[a] | 0·0055 | 0·020 | NS |

C, control; OM, organic matter; VFA, volatile fatty acids.
[a,b,c]Mean values within a row with unlike superscript letters were significantly different: $P<0·05$.
* 500 mg ground maize were incubated with 50 ml diluted rumen contents; for further details of procedures, see p. 182.
† Orthogonal contrast, C *v.* Malate: comparison between control and malate treatments.
‡ Orthogonal polynomials, linear effects of malate dose.
§ Calculated as the sum of isobutyrate, isovalerate and valerate acids.

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**Table 4.** Influence of different concentrations of malate on *in vitro* fermentation of barley (17 h) by mixed rumen micro-organisms in batch cultures*

(Mean values for four samples)

| | Malate (mM) | | | | SED | Statistical significance of the treatment effect: *P* | |
|---|---|---|---|---|---|---|---|
| | 0 | 4 | 7 | 10 | | C *v.* Malate† | Linear‡ |
| pH | 6·17[a] | 6·24[b] | 6·26[bc] | 6·29[c] | 0·016 | 0·001 | 0·001 |
| OM apparent disappearance (%) | 84·5[ab] | 84·1[a] | 86·4[b] | 83·2[a] | 0·93 | NS | NS |
| $CH_4$ (mmol) | 1·16[b] | 1·09[ab] | 1·08[ab] | 1·03[a] | 0·052 | 0·019 | NS |
| $CO_2$ (mmol) | 4·60[a] | 5·12[b] | 5·00[b] | 5·28[b] | 0·163 | 0·003 | 0·005 |
| VFA (mmol) | | | | | | | |
| Acetate | 1·69[a] | 1·73[ab] | 1·74[b] | 1·72[ab] | 0·021 | 0·033 | NS |
| Propionate | 1·40[a] | 1·52[b] | 1·61[c] | 1·67[d] | 0·026 | 0·001 | 0·001 |
| Butyrate | 0·357[a] | 0·410[b] | 0·407[b] | 0·449[b] | 0·0218 | 0·005 | 0·003 |
| Others§ | 0·080 | 0·084 | 0·089 | 0·086 | 0·0047 | NS | NS |
| Total VFA | 3·52[a] | 3·75[b] | 3·84[bc] | 3·93[c] | 0·0424 | 0·001 | 0·001 |
| Acetate:propionate (mol:mol) | 1·53[d] | 1·45[c] | 1·39[b] | 1·33[a] | 0·019 | 0·001 | 0·001 |
| $NH_3$-N (mg/l) | 116 | 115 | 112 | 112 | 4·6 | NS | NS |
| L-Lactate (mg/l) | 179[b] | 125[a] | 131[a] | 126[a] | 16·7 | 0·004 | 0·018 |
| Optical density (600 nm) | 0·113[b] | 0·111[b] | 0·111[b] | 0·097[a] | 0·0035 | NS | 0·002 |

[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different: $P<0·05$.
C, control; OM, organic matter; VFA, volatile fatty acids.
* 500 mg ground barley were incubated with 50 ml diluted rumen contents; for furhter details of procedures, see p. 182.
† Orthogonal contrast, C *v.* Malate: comparison between control and malate treatments.
‡ Orthogonal polynomials, linear effects of malate dose.
§ Calculated as the sum of isobutyrate, isovalerate and valerate acids.

gas production and greater average fermentation rate for all substrates (Table 2). These results would indicate a stimulatory effect of malate on fermentation, presumably due to changes in bacterial populations and/or in their activity. Nisbet & Martin (1993) showed that adding malate to *in vitro* cultures stimulated the growth of *Selenomonas ruminantium* in a medium that contained lactate. *S. ruminantium* is a common Gram-negative rumen bacterium that can account for up to 51 % total viable bacterial counts in the rumen of animals fed on cereal grains (Caldwell & Bryant, 1966). Malate is a key intermediate in the succinate–propionate pathway, which is used by *S. ruminantium* to synthesise succinate and propionate (Martin, 1998). In this pathway, malate is dehydrated to fumarate, fumarate is reduced to succinate and succinate is decarboxylated to propionate. In fact, *S. ruminantium* has been implicated as being the micro-organism primarily responsible for succinate decarboxylation in the rumen,

**Table 5.** Influence of different concentrations of malate on *in vitro* fermentation of wheat (17 h) by mixed rumen micro-organisms in batch cultures*

(Mean values for four samples)

| | Malate (mM) | | | | SED | Statistical significance of the treatment effect: *P* | |
|---|---|---|---|---|---|---|---|
| | 0 | 4 | 7 | 10 | | C *v.* Malate† | Linear‡ |
| pH | 6·12[a] | 6·13[ab] | 6·16[b] | 6·21[c] | 0·015 | 0·002 | 0·001 |
| OM apparent disappearance (%) | 88·9 | 89·4 | 89·8 | 89·3 | 0·77 | NS | NS |
| $CH_4$ (mmol) | 1·20[b] | 1·11[a] | 1·10[a] | 1·10[a] | 0·038 | 0·011 | NS |
| $CO_2$ (mmol) | 4·71[a] | 5·08[b] | 5·15[bc] | 5·38[c] | 0·119 | 0·001 | 0·015 |
| VFA (mmol) | | | | | | | |
| Acetate | 1·78[a] | 1·84[b] | 1·93[c] | 1·85[b] | 0·016 | 0·001 | 0·001 |
| Propionate | 1·49[a] | 1·63[b] | 1·71[c] | 1·71[c] | 0·024 | 0·001 | 0·001 |
| Butyrate | 0·356[a] | 0·370[ab] | 0·388[ab] | 0·406[b] | 0·0188 | 0·050 | 0·019 |
| Others§ | 0·088 | 0·103 | 0·093 | 0·090 | 0·0060 | NS | NS |
| Total VFA | 3·72[a] | 3·94[b] | 4·12[c] | 4·06[bc] | 0·052 | 0·001 | 0·001 |
| Acetate:propionate (mol:mol) | 1·50[c] | 1·43[b] | 1·42[b] | 1·38[a] | 0·015 | 0·001 | 0·001 |
| $NH_3$-N (mg/l) | 105 | 99·2 | 97·3 | 103 | 3·98 | NS | NS |
| L-Lactate (mg/l) | 216[b] | 147[ab] | 145[ab] | 131[a] | 37·3 | 0·036 | 0·049 |
| Optical density (600 nm) | 0·133[b] | 0·135[b] | 0·133[b] | 0·113[a] | 0·0039 | NS | 0·001 |

[a,b,c]Mean values within a row with unlike superscript letters were significantly different: $P<0·05$.
C, control; OM, organic matter; VFA, volatile fatty acids.
* 500 mg ground wheat were incubated with 50 ml diluted rumen contents; for further details of procedures, see p. 182.
† Orthogonal contrast, C *v.* Malate: comparison between control and malate treatments.
‡ Orthogonal polynomials, linear effects of malate dose.
§ Calculated as the sum of isobutyrate, isovalerate and valerate acids.

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**Table 6.** Influence of different doses of malate on *in vitro* fermentation of sorghum (17 h) by mixed rumen micro-organisms in batch cultures*
(Mean values for four samples)

| | Malate (mM) | | | | | Statistical significance of the treatment effect: *P* | |
|---|---|---|---|---|---|---|---|
| | 0 | 4 | 7 | 10 | SED | C *v.* Malate† | Linear‡ |
| pH | 6·19[a] | 6·24[ab] | 6·27[b] | 6·34[c] | 0·022 | 0·001 | 0·001 |
| OM apparent disappearance (%) | 75·9 | 76·9 | 76·0 | 73·4 | 1·87 | NS | NS |
| $CH_4$ (mmol) | 1·04 | 0·964 | 0·959 | 0·953 | 0·0631 | 0·048 | 0·048 |
| $CO_2$ (mmol) | 4·44[a] | 5·06[b] | 5·13[b] | 4·99[b] | 0·181 | 0·002 | 0·002 |
| VFA (mmol) | | | | | | | |
| Acetate | 1·57[a] | 1·59[ab] | 1·61[ab] | 1·64[b] | 0·023 | 0·028 | 0·011 |
| Propionate | 1·35[a] | 1·47[b] | 1·53[c] | 1·60[d] | 0·021 | 0·001 | 0·001 |
| Butyrate | 0·236[a] | 0·241[a] | 0·259[a] | 0·294[b] | 0·0152 | 0·045 | 0·003 |
| Others§ | 0·051 | 0·056 | 0·052 | 0·049 | 0·0048 | NS | NS |
| Total VFA | 3·20[a] | 3·36[b] | 3·46[b] | 3·58[c] | 0·049 | 0·001 | 0·001 |
| Acetate:propionate (mol:mol) | 1·51[d] | 1·42[c] | 1·38[b] | 1·35[a] | 0·009 | 0·001 | 0·001 |
| $NH_3$-N (mg/l) | 81·4 | 75·4 | 76·7 | 77·0 | 5·45 | NS | NS |
| L-Lactate (mg/l) | 143[b] | 130[ab] | 114[a] | 121[ab] | 10·3 | 0·032 | 0·031 |
| Optical density (600 nm) | 0·107 | 0·114 | 0·119 | 0·102 | 0·0089 | NS | NS |

[a,b,c,d]Mean values within a row with unlike superscript letters were significantly different: $P<0·05$.
C, control; OM, organic matter; VFA, volatile fatty acids.
* 500 mg ground sorghum were incubated with 50 ml rumen contents; for further details of procedures, see p. 182.
† Orthogonal contrast, C *v.* Malate: comparison between control and malate treatments.
‡ Orthogonal polynomials, linear effects of malate dose.
§ Calculated as the sum of isobutyrate, isovalerate and valerate acids.

and therefore for most of the propionate production (Wolin & Miller, 1988). In the present study, the supplementation with malate increased the propionate production with all substrates. Unlike other additives, such as ionophores, which increase propionate at the expense of acetate (Russell & Strobel, 1989), organic acids, malate in particular, can be converted into propionate and acetate following different pathways (Demeyer & Henderickx, 1967). Thus, although the value of the acetate:propionate ratio decreased ($P<0·05$) by the addition of all three concentrations of malate compared with control values, the supplementation with malate did not decrease the production of acetate, in agreement with the results reported by other authors (Russell & Van Soest, 1984; Callaway & Martin, 1997; Carro *et al.* 1999).

The increase in the production of butyrate found with all four substrates is in agreement with the results of Callaway & Martin (1996), when cracked maize was fermented *in vitro*, and with those of Kung *et al.* (1982), when dairy cows received 70, 105 or 140 g malate/d. In contrast, other authors have reported no effect of malate on butyrate production in rumen fermentations *in vitro* using batch cultures (Martin & Streeter, 1995) and semi-continuous fermenters (Carro *et al.* 1999). As *S. ruminantium* ferments carbohydrates to lactate, acetate, propionate and $CO_2$ (Melville *et al.* 1988), the increase in butyrate might only be due to an increase in the concentration and/or activity of butyrate-producing bacteria.

When animals are fed high concentrations of cereal grains, lactate can accumulate in the rumen. In fact, moderate concentrations of L-lactate were observed in our present incubations of cereal grains at 0 mM-malate (Tables 3, 4, 5 and 6). However, L-lactate concentrations decreased with the addition of malate, probably due to lactate utilisation by *S. ruminantium*. Nisbet & Martin (1991) reported that different concentrations of malate (0·03–10·00 mM) stimulated L-lactate uptake by *S. ruminantium* HD4 in a dose-response fashion, although no significant differences between malate at 5·00 and 10·00 mM were detected. In agreement with these results, no differences in L-lactate concentration at concentration of malate >4 mM were observed in the present experiment. As only L-lactate was measured in the present study, the effects of malate on D-lactate concentrations were not investigated. In addition to decreasing L-lactate concentrations, addition of malate increased $CO_2$ production for all substrates. $CO_2$ is an endproduct of lactate fermentation to propionate via the succinate–propionate pathway. Therefore, as suggested by Callaway & Martin (1996), malate may act to buffer rumen contents by a dual mechanism of increased lactate utilisation and $CO_2$ production by *S. ruminantium*. The increase observed in the final pH (Tables 3, 4, 5 and 6), particularly with 7 and 10 mM-malate, is consistent with these observations.

The conversion of glucose to acetate, propionate and butyrate in the rumen results in overall net release of reducing power. Much of this is used by methanogenic bacteria to reduce $CO_2$ to $CH_4$, but H can also be used as a substrate in fumarate reduction (Russell & Wallace, 1988). Nisbet & Martin (1991) hypothesised that malate might act as an electron sink for H, before dehydration of malate to fumarate. As H is used to reduce fumarate, there is a decrease in the availability of H for methanogenesis in the rumen, which could explain the observed decrease in $CH_4$ production when substrates were incubated with malate. The 7–8 % decrease in $CH_4$ formation found in the present study is consistent with the response observed by other authors (Callaway & Martin, 1996; López *et al.* 1999) for similar doses of organic acids (malate and fumarate).

There was a great variation among substrates in the recovery of malate as propionate. For maize, 88, 71 and 44 % added malate was recovered as propionate for 4, 7

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and 10 mM-malate treatments respectively (Table 3). However, recoveries with the other substrates (Tables 4, 5 and 6) were considerably lower (mean values 59, 54 and 46 % for 4, 7 and 10 mM-malate respectively). Malate can also appear as succinate, which can accumulate in rumen contents before being converted into propionate (Evans & Martin, 1997). The low recoveries observed for 10 mM-malate (44, 50, 41 and 46 % for maize, barley, wheat and sorghum respectively) could be due to an incomplete fermentation of malate after 17 h of incubation, although previous research (Russell & Van Soest, 1984; Callaway & Martin, 1997) has shown that malate at a concentration of 7·5mM is completely fermented by rumen micro-organisms *in vitro* within 10–24 h. In any case, the greater recoveries found for maize in comparison with the other substrates might indicate that malate utilisation *in vitro* could depend on the incubated substrate. In fact, although the effects of malate were similar for all the substrates, maize showed the greatest response. The lower $NH_3$-N concentrations observed when maize (Table 3) was incubated with 4 and 7 mM-malate could be due to a greater utilisation by rumen micro-organisms. In agreement with this hypothesis, the OD600 in these cultures was greater ($P < 0.05$) than in those at 0 and 10 mM-malate. In contrast, no differences in the $NH_3$-N concentration due to malate were observed for the other three substrates.

The results of the present study suggest that malate has a beneficial effect on *in vitro* rumen fermentation of cereal grains by increasing volatile fatty acid concentrations (acetate, propionate and butyrate) and final pH, and by decreasing lactate concentrations and $CH_4$ production. Some of these effects are dose-dependent, but in general, no beneficial effects of 10 compared with 7 mM-malate were observed. If these effects are confirmed *in vivo*, malate would provide an effective alternative to currently used antimicrobial compounds in animals fed high-concentrate diets.

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