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The potential of *Megasphaera elsdenii* isolates to control ruminal acidosis

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ABSTRACT

The objectives were: (a) to select the most effective lactate-utilizing *Megasphaera elsdenii* (*Me*) isolates (strains) from mixed rumen cultures (Phase 1) and (b) to determine *in vivo* the efficacy of the two most promising isolates from Phase 1 to control ruminal acidosis (Phase 2). In Phase 1 batch cultures of nine strains of lactate-utilizing *Me* were isolated and compared with the *Me* type strain ATCC 25940 and a membrane-filtered supernatant of a centrifuged culture (Control). Culture concentration in the incubation tubes was *ca* 5×10^5 cfu's/ml. In Phase 2 forage-fed rumen-fistulated sheep were drenched intraruminally 100 ml containing 10^{11} cfu's of the promising *Me* isolates CH4 and CH7, following abrupt administration of 1 kg maize meal and 300 g maltose per sheep, and compared with Control sheep drenched with a placebo. For *Me* isolates CH3, CH4 and CH7 rumen pH remained below pH = 5.0 for <3 h/24 h compared to >5 h/24 h for the type strain and the Control ($p<0.001$) (Phase 1). At +6 h lactic acid concentration increased to 24 mmol/l in the case of the type strain and 38.5 mmol/l for Control, compared to respectively 14 mmol/l for *Me* isolate CH3 and <5 mmol/l for *Me* isolates CH4 and CH7 ($p<0.001$). Lactic acid concentration for all isolates decreased to <5 mmol/l at +10 h, but for Control it progressively increased to 79 mmol/l at +24 h. In Phase 2, rumen pH for Control was <5 from 8 h to 24 h post-drenching, for *Me* isolate CH7 <5.5 from 4 h to 24 h, but always >5.0 ($p<0.001$), whereas for *Me* isolate CH4 rumen pH was >5.5 for the total post-drenching period. Lactic acid concentration post-drenching consistently remained <10 mmol/l for the two isolates, whereas it progressively increased to >55 mmol/l at +10 h for Control ($p<0.001$). Forage intake of Control sheep decreased by 16–18% from pre- to post-drenching and for *Me* isolate administered sheep by only 3–9% ($p<0.05$). It is concluded that the selected *Me* isolates are promising in the control of ruminal acidosis.

1. Introduction

Ruminal or lactic acid acidosis in ruminants may be chronic with negative consequences to production and health, or acute often resulting in death. The condition is associated with a sudden excess intake of readily fermentable carbohydrates during transition from forage to concentrate diets. The disorder is characterized by an accumulation of organic acids, especially

Abbreviations: cfu, colony forming units; LSD, least significant difference; *Me*, *Megasphaera elsdenii*; SARA, sub-acute ruminal acidosis; SD, standard deviation; SE, standard error; SEM, standard error of the mean; T, treatment; Ti, time; TMR, total mixed ration; VFA, volatile fatty acids.

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lactic acid in the rumen (Dawson and Allison, 1988) because lactic acid producers outweigh lactic acid utilizers (Slyter, 1976) and grow faster (Oetzel, 2003). Logically, therefore, the condition may be prevented or alleviated if high numbers of effective lactate-utilizing organisms can be administered into the rumen at the time of transition from forage to concentrate diets. *Megasphaera elsdenii* (*Me*) and *Selenomonas ruminantium* are prominent lactate-utilizing species in the rumen. Of the two, *Me* is more important because it utilizes 0.65–0.95 of the lactate available in the rumen (Counotte et al., 1981), shows preference for lactate as substrate (Russell and Baldwin, 1978; Marounek et al., 1989), but also uses glucose and maltose and therefore competes with lactate producers for substrate. *S. ruminantium* on the other hand is unlikely to be effective as it undergoes catabolite repression (Russell and Baldwin, 1978) and is comparatively acid-intolerant (Mackie and Gilchrist, 1979). Therefore, investigations into the capability of strains of *Me* to utilize lactic acid may be the starting point.

The success of the approach in practice, however, has been variable, primarily because the isolates (strains) used have not shown high growth rates and biomass production, have not preferentially used D- and L-lactate as carbon source (even in the presence of glucose and maltose) and could not maintain multiplication at low rumen pH (Horn et al., 2009). In addition, such isolates should not be sensitive to ionophores. Some isolates nevertheless displayed characteristics in the right direction: Kung and Hessian (1995) found that strain *Me* B 159 prevented the accumulation of lactic acid when challenged with highly fermentable carbohydrates; Robinson et al. (1992) demonstrated that the addition of strain *Me* (407A) increased intake by 24% compared to Control in steers; strain *Me* NIAH 1102 preferred lactate above glucose as substrate (Hino et al., 1994) and strain *Me* JDB301 maintained rumen stability (pH > 6.0) for days following acute grain feeding (Wiryawan and Brooker, 1995).

In this study promising *Me* isolates were evaluated *in vitro* and *in vivo* as to their potential to prevent lactic acid acidosis or minimize the effects thereof.

The study was done in two phases. The objective in Phase 1 was to select *Me* isolates which were the most effective in metabolizing lactic acid in mixed rumen cultures. The objective in Phase 2 was to determine the efficacy of preventing lactic acid accumulation *in vivo* or minimizing its effect, using the two most promising *Me* isolates from Phase 1.

2. Materials and methods

2.1. Phase 1

Batch cultures of nine isolates of lactate-utilizing strains in their exponential growth phase were added to strained rumen fluid, obtained from a forage-fed rumen-cannulated sheep. These isolates were stringently selected by pH-auxostat enrichment and tested to meet the characteristics described above (Horn et al., 2009). The isolate-containing supernatant was diluted fivefold with pre-warmed (39 °C), CO_2-saturated buffer solution, which was similar to that described by Maeng (1975), except that the amounts of $Na_2HPO_4{\cdot}12H_2O$ and KH_2PO_4 were respectively 17.9 g/l and 6.8 g/l. A culture of the type strain *Me* ATCC 25940 was included for comparative purposes, in addition to a treatment consisting of strained rumen fluid diluted with membrane-filtered supernatant of a centrifuged culture, *i.e.* containing no culture (Control). The batch culture isolate code and treatment acronym were as follows: 170692A9 (A), 220792A4 (B), 110892A8 (C), 110892A13 (D), 110992C13 (E), ATCC 25940 (F), CH3 (G), CH6 (H), CH7 (I), CH4 (J) and Control (K).

The batch culture treatments (6 repetitions per treatment) were incubated for 12 h in glass fermentation tubes (100 ml inoculum/tube) at 39 °C, while being continuously flushed with CO_2. The pH of each incubation was measured and samples were taken for lactic acid determination, just prior to the addition of maltose to the medium. On commencement of incubation 20 ml of a maltose solution was added to each fermentation flask as energy medium. The concentration of culture inocula in the incubation tube thereafter was *ca* 5×10^5 colony forming units (cfu's)/ml. The pH of each culture was subsequently measured hourly for 12 h, and again at 24 h. Samples for lactic acid determination were respectively taken at 0 h, 2 h, 4 h, 6 h, 8 h, 10 h and 12 h and measured by an enzyme method, which measures both D- and L-lactate isomers (Test combination 1112 821, Boehringer Mannheim GmbH, Mannheim) (Horn et al., 2009).

Statistical analysis was by Genstat (2000) employing the MIXED model of SAS with variables treatment (T), time (Ti) and interaction ($T \times$ Ti). Treatment means were separated using Fischer's protected t-test. Least significant difference (LSD) was accepted at the 5% level of probability.

2.2. Phase 2

Animal ethics approval was obtained from the Agricultural Research Council Animal Ethics Committee.

Eighteen rumen-cannulated sheep (45 kg; SD = 2.5 kg) were randomly divided into three groups of six each and each group was assigned to one of three treatments. One group was drenched intra-ruminally with one of the *Me* isolates (dosage size: 100 ml containing 10^{11} cfu's or *ca* 10^6 cfu's/ml final concentration in the rumen), the second group with the other *Me* isolate at the same dosage size, whereas the third group (Control) received the cell-free filtrate of 50% of each of the two isolate treatments on day of drenching (denoted day 0). The isolates were obtained from batch grown cells harvested during the late exponential phase.

The experiment was conducted over two periods: (a) because the metabolism facility had only 12 units for individual feeding and rumen sampling and (b) to prevent possible cross contamination effects between the two *Me* isolates. In the

Table 1
Dietary composition of the forage diet fed to sheep pre- and post-drenching with respectively *Me* isolate CH4 or CH7 or the cell-free filtrate (Control).

	DM[a], g/kg
Eragrostis teff hay	924
Fish meal	30
Urea	5
Molasses	30
$CaH_4(PO_4)_2$	5
NaCl	5
Mineral and vitamin premix[b]	1

[a] DM = dry matter.
[b] *Composition:* Vit. A, 6×10^6 IU; Vit. B1, 3 g; antioxidant, 3.5 g; iron, 30 g; copper, 12 g; zinc, 50 g; cobalt, 1 g; magnesium, 200 g; manganese, 40 g; iodine, 1 g; selenium, 0.1 g; monensin, 22 mg.

first period the one isolate was compared with the Control and 2 weeks later the other isolate. Thus, period was introduced as variable in the statistical model.

The sheep were fed a forage-based diet *ad lib* for 21 days prior to day 0 and again post-drenching, the composition of which is shown in Table 1.

Intake was measured from day −4 through day 0 to day +4. The forage diet was removed 12 h before drenching and on day 0 each sheep was offered 1 kg maize meal while 300 g maltose was administered by rumen cannula 1 h before drenching. The maize meal not yet consumed by the time of drenching was administered through the rumen cannula at 10h00 on day 0, immediately prior to drenching. Rumen pH was measured 1 h before drenching and thereafter hourly until +12 h. Rumen samples to determine lactic acid concentration were taken at −1 h and then at 2-h intervals until +12 h. The samples were collected on ice and then frozen at −20 °C. At day +1 *ad lib* feeding of the forage diet was resumed.

D- and L-lactic acid was determined by gas chromatography with a Carlo Erba GC4200 with flame ionization detector and a Tupelo 1-1825 column (Supelco Inc., Bellefonte, PA, USA).

In the MIXED model of SAS (Genstat, 2000) provision was made for period, treatment and time and the respective two-way and three-way interactions. As in Phase 1, LSD was accepted at the 5% level of probability.

3. Results

3.1. Phase 1

3.1.1. pH

Fig. 1 shows trends of rumen pH with time for batch culture isolates A–K.

The effect of treatment (*T*), time (Ti) and the interaction ($T \times$ Ti) was highly significant ($p<0.001$). The trend in most batch cultures was for pH to decline rapidly from +2 h to +5 h but then to stabilize between pH levels 4.6 and 5.3. The exception was the Control, where pH consistently declined from 6.4 at 0 h to 4.4 at +12 h. The pH of treatment E declined rapidly between +1 h and +5 h, but then recovered to level off at 5.2, comparable to most other treatments. Treatment F (type strain ATCC 25940), apart from Control, showed the sharpest pH decline to 4.6 at +6 h with recovery to above pH = 5 only at +11 h. The two treatments which apparently were the most successful in preventing a severe drop in pH were G (CH3) and I (CH7), although their trends were not significantly different from those of J (CH4).

Although the trend of pH decline with time post-feeding is valuable to observe, the actual time that the pH remains below a critical level is more informative in relation to preventing lactic acidosis or minimizing its effect. To that effect the length of time as well as the extent to which pH remained below particular critical levels (in this case pH = 6.0 and pH = 5.0) were calculated using the following model:

$$\text{time} \times \text{pH} < 6.0 = \sum_{i=1}^{n} (6 - \text{pH}_i)$$

$$\text{time} \times \text{pH} < 5.0 = \sum_{i=1}^{n} (5 - \text{pH}_i)$$

where *i* = each hour where pH < 6 or pH < 5 respectively

The results in Table 2 are consequently compiled to depict the length of time pH remained below respectively 6 and 5 for batch culture isolates A–K.

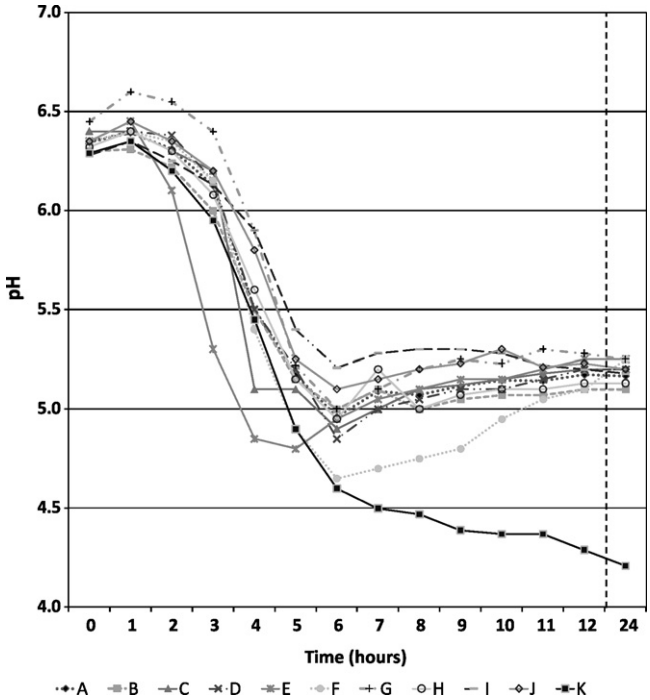

Fig. 1. Trends of pH with time of batch culture isolates A–K. The SE was 0.03 when comparing means at a specific time and 0.04 when comparing means at different times within the same treatment.

A lower value is considered more beneficial since pH stays for less hours below critical levels. Treatment I showed the lowest value (5.73) at pH < 6.0, followed by treatment G (6.29). The control treatment (K) had the highest value (13.11). The trend at pH < 5.0 was similar, with the value of treatment I (CH7) being 1.64, treatment G (CH3) 2.19 and Control 8.59. *Me* isolates CH7 and CH3 therefore appeared to be superior in this regard, although the difference with treatment J (CH4) was not significant.

3.1.2. Lactic acid concentration

Fig. 2 shows trends in mean lactic acid concentration with time for batch culture isolates A–K.

Treatment differences, time and the ($T \times$ Ti) interaction were all highly significant ($p<0.001$). The mean lactic acid concentration of most treatments was less than 10 mmol/l across the measuring period, the exception being treatments E, F, G and K (Control). At +6 h the lactic acid concentration of treatment F was 24 mmol/l and the corresponding pH = 4.76. At +12 h the value was 1.4 mmol/l and the corresponding pH = 5.06. The initial lactic acid concentration of treatment K was 4.1 mmol/l with pH = 6.30, which increased to 79 mmol/l at +24 h and the corresponding pH = 4.25, suggesting as expected, a comparatively well-defined inverse relationship between lactic acid concentration and pH.

Table 2
Length of time pH remained below respectively pH = 6.0 and pH = 5.0 in batch culture isolates A–K.

Treatment	Hours	
	pH < 6.0[a]	pH < 5.0[a]
A (170692A9)	7.34^{bc}	2.97^{bc}
B (220792A4)	8.17^{c}	3.71^{cd}
C (110892A8)	7.61^{c}	3.26^{c}
D (110892A13)	8.16^{c}	3.72^{cd}
E (110992C13)	9.41^{d}	4.43^{de}
F (ATCC 25940)	9.69^{d}	5.22^{e}
G (CH3)	6.29^{a}	2.19^{ab}
H (CH6)	7.90^{c}	3.37^{c}
I (CH7)	5.73^{a}	1.64^{a}
J (CH4)	6.56^{ab}	2.35^{ab}
K (Control)	13.11^{e}	8.59^{f}
SEM	0.44	0.39

[a] Means in a column with different superscripts differ ($p<0.001$).

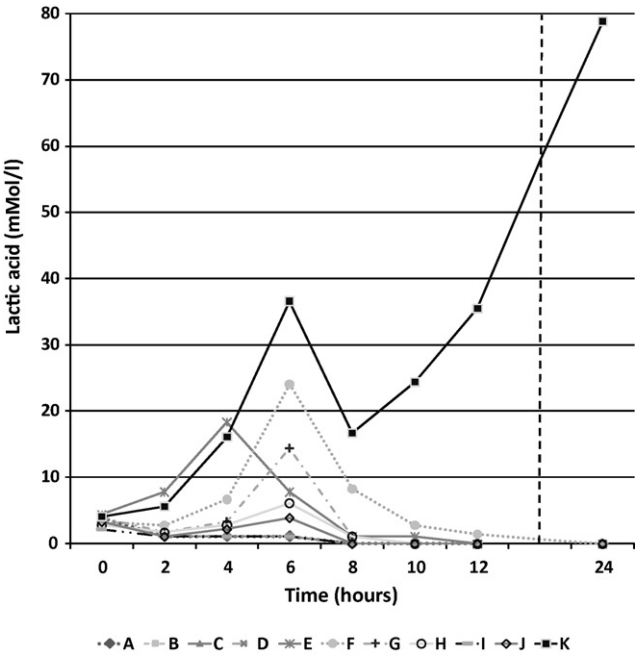

Fig. 2. Trends in mean lactic acid concentration with time of batch culture isolates A–K. The SE was 0.09 when comparing treatment means at a specific time and 0.03 when comparing means at different times within the same treatment.

Since *Me* isolate CH3 (Treatment G), in contrast to the pH result, was disappointing with regard to lactic acid utilization, *Me* isolates CH4 and CH7 were the cultures of choice to take through to Phase 2 of the study. The choice of *Me* isolate CH4 was further supported by its exceptional high growth rate, high biomass production and satisfactory growth rate at pH < 5 (Horn et al., 2009).

3.2. Phase 2

3.2.1. Rumen pH

During both periods treatment differences, time and the ($T \times$ Ti) interaction were highly significant ($p<0.001$). The trends of rumen pH post-drenching in sheep of *Me* isolates CH4, CH7 and the Control (CON) during periods 1 and 2 are displayed in Fig. 3.

Rumen pH in both CH4 and CH7 remained appreciably higher than in CON for most of the measuring period. Of the two *Me* isolates, CH4 was apparently superior since pH remained above 5.5 at all hours and it recovered to above 6.0 at +24 h, whereas with CH7 pH was still at 5.5 by +24 h.

3.2.2. Lactic acid concentration

Fig. 4 shows trends in mean post-drenching lactic acid concentrations in the rumen of sheep of *Me* isolates CH4 and CH7 and the Control (CON) during periods 1 and 2. Lactic acid results reflect a similar trend as pH and correspondingly, treatment differences, time and the ($T \times$ Ti) interaction were highly significant ($p<0.001$).

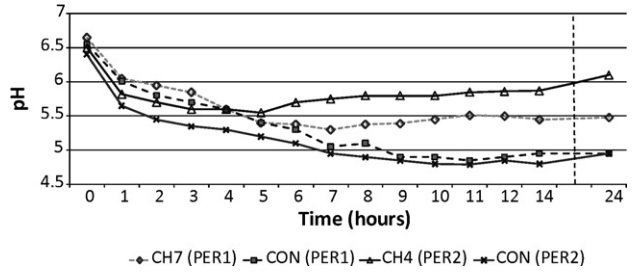

Fig. 3. Trends of rumen pH in *Me* isolate treatments CH4 and CH7 and Control (CON) during periods 1 and 2. The SE was respectively 0.15 and 0.12 when comparing treatment means at specific times and 0.04 and 0.03 when comparing means at different times within the same treatment.

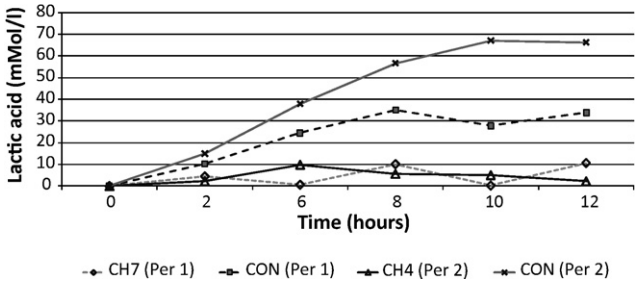

Fig. 4. Trends in mean rumen lactic acid concentrations of *Me* isolate treatments CH4 and CH7 and Control (CON) during periods 1 and 2. The SE was respectively 0.62 and 0.66 when comparing treatment means at specific times and 0.23 and 0.25 when comparing means at different times within the same treatment.

Table 3
Pre- and post-drenching dry matter (DM) intake of the forage diet by sheep of the *Me* isolates CH4 and CH7 and control treatments during periods 1 and 2.

Treatment	DM intake, g/day		
	Day −4 to −1	Day +1 to +4	Day −4 to +4
Period 1			
CH7	865	842 (0.97)[a]	863
Control	930	765 (0.82)	848
Period 2			
CH4	1122	1017 (0.91)	1070a
Control	831	701 (0.84)	766b

Means with different letters differ ($p<0.05$).
[a] Post-drenching intake as a proportion of pre-drenching intake.

The lactic acid concentrations of both *Me* isolates CH4 and CH7 remained below that of CON for the entire measuring period of +2 h to +12 h, measuring on average less than 10 mmol/l. In contrast, CON in both periods showed a sharp increase in lactic acid concentration which peaked above 55 mmol/l 12 h post-drenching.

3.2.3. *Feed intake*

Table 3 shows dry matter intake of the forage diet in sheep pre- and post-drenching of *Me* isolates CH4 and CH7 and the Control during periods 1 and 2.

In period 1 pre-drenching forage intake of the CH7 treatment declined with only 2% after concentrate administration, whereas the decline for Control was 18% (Table 3). In period 2 pre-drenching forage intake of the CH4 treatment declined with 9% following concentrate administration and Control 16%, suggesting similar trends for the two isolates in supporting intake. A confounding factor, however, was the much higher intake of treatment CH4 than Control even before drenching and concentrate administration.

4. Discussion

In Phase 1 the results suggest that the selected lactate-utilizing isolates were successful in metabolizing lactic acid, to the extent that lactic acid accumulation and the associated decline in pH could be prevented. The results of *Me* isolates CH4 and CH7 in the Phase 2 *in vivo* trial apparently support this contention. Noteworthy in this regard is the reduction in time, compared to Control and the *Me* type strain (F), that pH stayed below critical rumen functional levels for particular isolates (Phase 1) (Table 2), and the well-pronounced pH difference between *Me* isolates CH4 and CH7 *vs* Control in Phase 2 (Fig. 3). Compared to the batch culture results (Fig. 2), the suppression of lactic acid accumulation *in vivo* by *Me* isolates CH4 and CH7 (Fig. 4) was even more pronounced; the trends nevertheless were similar. The results on the prevention of the accumulation of lactic acid supported the investigation of Kung and Hessian (1995) for strain *Me* B 159 and maintenance of rumen pH that of Wiryawan and Brooker (1995) for strain *Me* JDB301.

Nagaraja and Titgemeyer (2007) suggested that lactic acid concentrations of 0–5 mmol/l in the grain-fed rumen should be considered normal whereas levels of 50–150 mmol/l will result in acute acidosis. In the *in vitro* study (Phase 1), lactic acid levels were mostly lower than 50 mmol/l (Fig. 2) but for Control in the *in vivo* (Phase 2) trial, lactic acid concentration did increase to 50–60 mmol/l. The majority of promising *Me* CH isolates (Fig. 2) in Phase 1 and CH4 and CH7 (Fig. 4) in Phase 2 successfully reduced lactic acid concentration to below 10 mmol/l and even to within the normal range of 0–5 mmol/l.

The choice of critical rumen pH levels was based on the well-recognized inhibition of cellulolitic activity below pH = 6 and the onset of acute acidosis at pH = 5 or below (Owens et al., 1998; Nocek, 1997). Whereas this is the critical cut-off point for the acute form, ruminal acidosis is also defined in the more often observed chronic or sub-acute form (known as “sub-acute

ruminal acidosis" or SARA), described by Nagaraja and Titgemeyer (2007) as being when VFA concentration in the rumen is 150–225 mmol/l and the pH is 5–5.5. In concert with the contention that it is more important to define acidosis in terms of the time rumen pH stays below the critical level, Plaizier et al. (2008) defined SARA as being when rumen pH remains below 5.6 for more than 3 h/24 h. If this definition is accepted as critical in the management of concentrate fed systems such as feedlots and TMR-fed dairy cows, Phase 1 results (Table 2) suggest that *Me* isolates G (CH3), I (CH7) and J (CH4) probably met the criteria. In Phase 2, rumen pH in the case of *Me* isolate CH7 remained in the SARA range of 5–5.5 for most of the day (Fig. 3), whereas with *Me* isolate CH4 rumen pH never declined below 5.5. This probably indicates an advantage for *Me* isolate CH4 compared to *Me* isolate CH7 in maintaining rumen stability.

The effect of maintaining feed intake by drenching with *Me* isolates CH4 and CH7 was confounded by the vast difference in pre-drenching forage intake of treatment CH4 compared to Control (Table 3). The reason for this is unclear, since the variation in sheep weight was comparatively small and the time difference between periods was only 14 days. The short measuring period was obviously also a stumbling block. Nevertheless, the numeric reduction in intake from pre- to post-drenching was of the order of 16–18% for Control and only 3–9% in the case of the *Me* isolates. In support, in the experiments of Henning et al. (2010) intake measurements of 40–50 days showed significant and sustained higher intakes of about 10–20% for *Me* strain CH4 (patent: *Me* NCIMB 41125) compared to Control, whereas Robinson et al. (1992) reported a 24% increase with strain *Me* (407A).

5. Conclusions

The study was designed to evaluate *M. elsdenii* strains that were isolated according to stringent criteria, as to their ability to prevent lactic acid acidosis or minimizing its effect. Indicator parameters of measurement were pH and lactic acid concentration in batch culture and *in vivo* rumen pH, lactic acid levels and trends in the rumen, and voluntary feed intake of sheep. *Me* strains CH3, CH4 and CH7 were apparently successful in maintaining pH above SARA levels, whereas *Me* strains CH3, CH4, CH6 and CH7 in batch culture and CH4 and CH7 *in vivo* depressed lactic acid concentration within 8 h post-administration to <10 mmol/l, which is considered normal (non-acidic). *In vivo*, forage intake after abrupt administration of 1 kg maize meal and 300 g maltose was depressed by 16–18% in Control compared to only 3–9% in the CH4–CH7 treatments. It is concluded that the evaluated strains have potential in controlling ruminal acidosis.

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