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SHORT COMMUNICATION

Dairy propionibacteria as direct-fed microbials: in vitro effect on acid metabolism of *Streptococcus bovis* and *Megasphaera elsdenii*

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Abstract
Ruminal acidosis caused by accumulation of lactic acid, a decrease of pH in the rumen and subsequent imbalance of the rumen fermentation process, affects the health and productivity of dairy cows and beef cattle. Direct-fed microbials have potential for use in the control and prevention of ruminal acidosis. This study investigated the interaction between five strains of dairy propionibacteria, *Megasphaera elsdenii* and *Streptococcus bovis* in various co-culture combinations in a simulated rumen environment comprising unmodified rumen digesta supplemented with excess glucose. While suppression of lactic acid accumulation by both the dairy propionibacteria and *M. elsdenii* in the presence of *S. bovis* in the simulated rumen conditions was evident, propionibacteria were found to be more effective than *M. elsdenii* in controlling lactic acid levels.

Keywords Probiotics · Propionibacteria · Rumen acidosis · Rumen microbes

Findings

Propionibacteria are characterized by utilization of lactic acid as the favored carbon source, with propionic acid produced as a by-product (Luo et al. 2017a). Dairy propionibacteria have been proposed as potential probiotic candidates for the treatment and prevention of ruminal acidosis—a prevalent disorder in ruminants (Luo et al. 2017b). Ruminal acidosis is caused primarily by the inclusion of a high percentage of readily fermentable dietary carbohydrates. This disorder presents as an accumulation of lactic acid with a decrease of pH in the rumen and subsequent imbalance of the rumen flora and fermentation processes, resulting in impaired health and productivity of dairy cows and feedlot beef cattle (Enemark 2008; Luo et al. 2017a, b). The rumen is a complex environment haboring a variety of microorganisms, some of which have been shown to influence the development of acidosis. *Streptococcus bovis* is an inhabitant of the rumen environment, but is usually found in relatively low numbers in the healthy rumen. However, *S. bovis* is relatively acid tolerant (Russell and Hino 1985; Miwa et al. 2000) and has been identified as the major lactic acid producer responsible for the development of acidosis (Maroune and Bartos 1987; Owens et al. 1998; Enemark 2008). Although antibiotic treatment has proven to be effective in treating this condition, potential facilitation of the spread of antibiotic-resistant bacteria makes this an unattractive option for routine use in preventing acidosis (Millet and Maertens 2011). Therefore, alternative approaches such as use of direct-fed microbials (DFM) to control the growth of *S. bovis* are becoming increasingly popular (Luo et al. 2017b).

Megasphaera elsdenii, the major lactic acid utilizer in the rumen, is able to use lactate, fructose and glucose as carbon sources, and produces propionate, acetate and butyrate as major metabolic products (Holt et al. 1994). In the healthy rumen, *M. elsdenii* mainly utilizes the maltose hydrolyzed from starch and the lactate produced by *S.bovis* as carbon sources (Russell et al. 1981; Hino et al. 1994), and, therefore, maintains the lactic acid level. However, this balance is often disrupted when cattle are fed concentrates that include a higher percentage of starch, because it stimulates the growth of amylolytic bacteria to produce more volatile fatty acids (VFAs) and glucose. Since

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S. bovis can use both starch and glucose as carbon sources, this increasing availability of substrate stimulates the rapid growth of *S. bovis* and leads to accumulation of lactic acid causing acidification in the rumen (Hino et al. 1994). The effects are further exacerbated by the vulnerability of *M. elsdenii* to acidic conditions (Russell and Dombrowski 1980), which reduces their capacity for lactic acid consumption.

Although propionibacteria are common inhabitants in the rumen, they are normally present in low numbers (Oshio et al. 1987) and therefore exert no significant influence in controlling the development of acidosis. However, it has been demonstrated that the introduction of dairy propionibacteria to a nutrient broth medium containing co-cultures of *S. bovis* and *M. elsdenii* was able to influence the fermentation process as the lactic acid was efficiently converted into acetic acid and propionic acid (Luo et al. 2017b). These VFAs produced by dairy propionibacteria can then be absorbed through the rumen wall and serve as an energy source for cattle (Dieho et al. 2016). Although these findings indicated that the dairy propionibacteria may have potential in the alleviation of ruminal acidosis, given the complexity and the diversity of healthy ruminal micro-flora, the influence of other indigenous micro-flora on these interactions in the same growth environment must also be considered. In relation to this, the interaction between dairy propionibacteria, *S. bovis*, and *M. elsdenii* in the rumen environment has not been well documented. Hence, this study was designed to examine the metabolic interactions between dairy propionibacteria, *S. bovis* and *M. elsdenii* in unmodified rumen fluid. In this context, the introduction of glucose and *S. bovis* to the rumen content samples was carried out to create in vitro conditions similar to those of ruminal acidosis. The hypothesis was that, under the simulated ruminal acidosis conditions, the inoculation of either propionibacteria, *M. elsdenii*, or their combination, would prevent the accumulation of lactic acid via consumption and conversion to acetic and propionic acids. Moreover, that the extent of the effect would vary dependent upon the specific propionibacteria strains involved.

Whole rumens (beef cattle) were obtained post-mortem from a local abattoir (Kurri Meats, Newcastle, Australia) as part of the waste by-product of normal abattoir operations. In accordance with Australian government guidelines for the use of animals for scientific purposes, the study was exempt from ethical approval requirements on the basis that no live animals were handled, euthanized or subject to any variation from the operators licensed processing procedures, for the purposes of the study (NHMRC 2013). The handling and preparation of the rumen content was the same as previously described (Luo et al. 2017a). In addition, glucose (1%, by weight) was added to rumen content samples in all preparations to simulate the effect of a high concentrate carbohydrate diet in stimulating the growth and lactic acid production of *S. bovis*. The preparation of the bacteria followed the same procedure as described previously (Luo et al. 2017b). Maximum grown (~10^9 cfu/ml) and saline-washed bacterial preparations of propionibacteria, *S. bovis* and *M. elsdenii* were used as inoculants. The five strains of dairy propionibacteria used in the study, along with the abbreviated names in brackets used throughout this paper, were *Propionibacterium jensenii* 702 (PJ702), *P. acidopropionici* ATCC 25562 (PA25562), *P. acidopropionici* 341 (PA341), *P. freudenreichii* CSCC 2206 (PF2206) and *P. freudenreichii* CSCC 2207 (PF2207). There were ten preparations assigned in this study, both as two-strain and three-strain co-cultures. The details of the inoculation for each preparation are listed in Table 1. Both two- and three-strain co-culture studies were performed. No additional bacterium was inoculated to the control. For the treatment SB, only one strain of bacteria (*S. bovis*) was introduced. Two strains of bacteria were inoculated to treatments PJ702 + SB, PA25562 + SB, PA341 + SB, PF2206 + SB, PF2207 + SB and ME+SB, which contain *S. bovis* and either one strain of *Propionibacterium* or *M. elsdenii*. Three different strains of bacteria *S. bovis*, *P. jensenii* 702 and *M. elsdenii* were inoculated together for the treatment PJ702 + ME+SB. All inoculated rumen samples were incubated in a CO_2 incubator (Thermo Electrone, Thermo Scientific, Waltham, MA) with 10% CO_2 under 37 °C for 48 h. At 0 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h post inoculation, 5 ml sample was taken for high pressure liquid chromatography (HPLC) analysis for the lactic, propionic, and acetic acid profiles as described previously (Luo et al. (2017a) using a HPLC system (Hewlett-Packard 1100 DAD, Santa Clara, CA) fitted with a Pyrospher RP-18 (125 mm × 4 mm, 5 μm) column (Hewlett-Packard). Bacterial cell abundances were not quantified during analysis. Comparisons of the difference between the acid concentration curves against time in different preparations in rumen contents were performed using the General Linear Model Repeated Measurement in SPSS (PASW statistic 18), to measure the difference between the trajectories of each individual acid concentration curve across the whole experimental period.

In this study, the acid concentration profiles were altered significantly by the introduction of different strains of *Propionibacterium* and *M. elsdenii* in the glucose-fortified rumen content samples inoculated with *S. bovis* (Fig. 1). Among the tested propionibacteria, PA341 and PJ702 were the strains found to be associated with the lowest levels of lactic acid accumulation and highest production of acetic and propionic acid. By comparison, the PA25562, PF2206, and PF2207 treatments were found to be generally less effective in limiting lactic acid levels and generating acetic and propionic acid. As such, for the purposes of visual clarity the results for these treatments have been omitted from Fig. 1. The introduction of *S. bovis* alone had little effect ($P > 0.05$) on the acid profile relative to that observed for the control; however, the levels of lactic acid accumulation were markedly less ($P > 0.05$) in the preparations containing either propionibacteria, *M. elsdenii*, or both. The acetic and propionic acid concentrations were both

Table 1 Sample sets prepared for analysis of the effects of inoculation of different bacterial combinations on acid metabolism in the rumen content

Group	Inoculants[a]
Control	2 ml saline
SB	1 ml *Streptococcus bovis* + 1 ml saline
Two-strain-inoculation	
PJ702 + SB	1 ml *S.bovis* + 1 ml *Propionibacterium jensenii* 702
PA25562 + SB	1 ml *S.bovis* + 1 ml *Propionibacterium acidopropionici* ATCC 25562
PA341 + SB	1 ml *S.bovis* + 1 ml *P. acidopropionici* 341
PF2206 + SB	1 ml *S.bovis* + 1 ml *P. freudenreichii* CSCC 2206
PF2207 + SB	1 ml *S.bovis* + 1 ml *P. freudenreichii* CSCC 2207
ME+SB	1 ml *S.bovis* + 1 ml *Megashaera elsdenii*
Three-strain-inoculation	
PJ702 + ME+SB	1 ml *S.bovis* + 1 ml *P. jensenii* 702 + 1 ml *M.elsdenii*

[a] Inoculate environment for each group listed above was 300 g rumen sample with 1% glucose (w/w)

higher in the treatments than in the control preparation. The results of the three-strain inoculation relative to the two-strain preparations suggest that strain PJ702 was more influential on the acid profile than *M. elsdenii*, and that the effects were not diminished by their co-cultivation.

Significant differences were apparent for the lactic acid concentration curves between different treatments in the rumen samples during the incubation period ($P < 0.01$) (Fig. 1). Lactic acid was not detected in any of the preparations over the first 4 h of incubation. In all treatments, significant increases in lactic acid concentration were observed with peaks in concentration observed after either 8 h (for ME, PA 341) or 12 h (for control, SB, PJ702 and PJ702 + ME). Peak lactic acid concentrations for the control and SB treatments (52.60 mM and 55.3 mM, respectively) were significantly ($P < 0.01$) higher than those observed for the ME treatment (35.6 mM) or the propionibacteria treatments (PA341, 20.70 mM; PJ702, 21.60 mM; PJ702 + ME, 20.90 mM). Lactic acid levels returned to below detection limits after 24 h in treatments ME, PA341, PJ702 and PJ702 + ME. This 'end point' was not reached in the control and SB treatments, even at the end of the 48 h incubation (Fig. 1a).

Marked increases in acetic acid levels were apparent in all preparations ($P = 0.008$) (Fig. 1b). The initial average acetic acid concentration was 12.92 mM across all treatments, steadily increasing to ~ 45 mM by the end of the incubation. Significant differences were evident between treatments for the acetic acid concentration curves ($P = 0.04$). Treatment PJ 702 recorded the highest acetic acid level at both 24 h and 48 h with a final concentration of 48.99 mM, with similar final concentrations observed for both the PJ702 + ME and SB treatments. The lowest acetic acid levels at both 24 h and 48 h (29.83 mM and 30.48 mM respectively) were observed in the ME treatment.

The initial propionic acid concentration in rumen samples was zero across all preparations, but was detectable after a 2-h incubation. The average final propionic acid concentration across all treatments was 26.94 mM. Significant differences between treatments were evident for the propionic acid concentration curves during the incubation ($P < 0.001$) (Fig. 1c). For treatments PJ702, PA341 and PJ702 + ME, the propionic acid concentration rapidly increased to above 30 mM at 24 h, remaining relatively constant after this time point. Treatments ME, SB and control all exhibited lower propionic acid concentration levels across the incubation period. At 24 h, their propionic acid concentrations (11.21 mM, 11.45 mM and 12.40 mM, respectively), less than one-half the levels recorded for the other preparations. The final concentrations for treatment SB and control were 24.98 mM and 22.81 mM respectively, while further increases were not observed in the ME treatment.

The rumen samples used in this study were directly transferred from whole rumen content, which contains a large amount of semi-digested feed (digesta) and indigenous rumen micro-flora. The handling of the rumen content was carried out carefully under aseptic procedure to preserve the indigenous microorganisms as much as possible, and avoid the introduction of any contamination to the rumen system, providing a rumen fermentation environment as close as possible to the normal in vivo conditions. Interactions between propionibacteria strains, *M. elsdenii* and *S. bovis* were subsequently investigated, and significant differences in acid profiles were observed between treatments and control. The key finding was that, in comparison with *M. elsdenii*, dairy propionibacteria exhibited greater capacity to limit the accumulation of lactic acid produced by *S. bovis* in rumen content samples, under conditions conducive to the development of acidosis.

In accordance with the negligible levels normally observed in the rumen of healthy animals (Owens et al. 1998; Russell and Rychlik 2001), lactic acid was not detected in these rumen samples prior to inoculation and incubation. The addition of excess glucose (1% w/v) clearly appeared to promote the production and accumulation of lactic acid, indicating successful

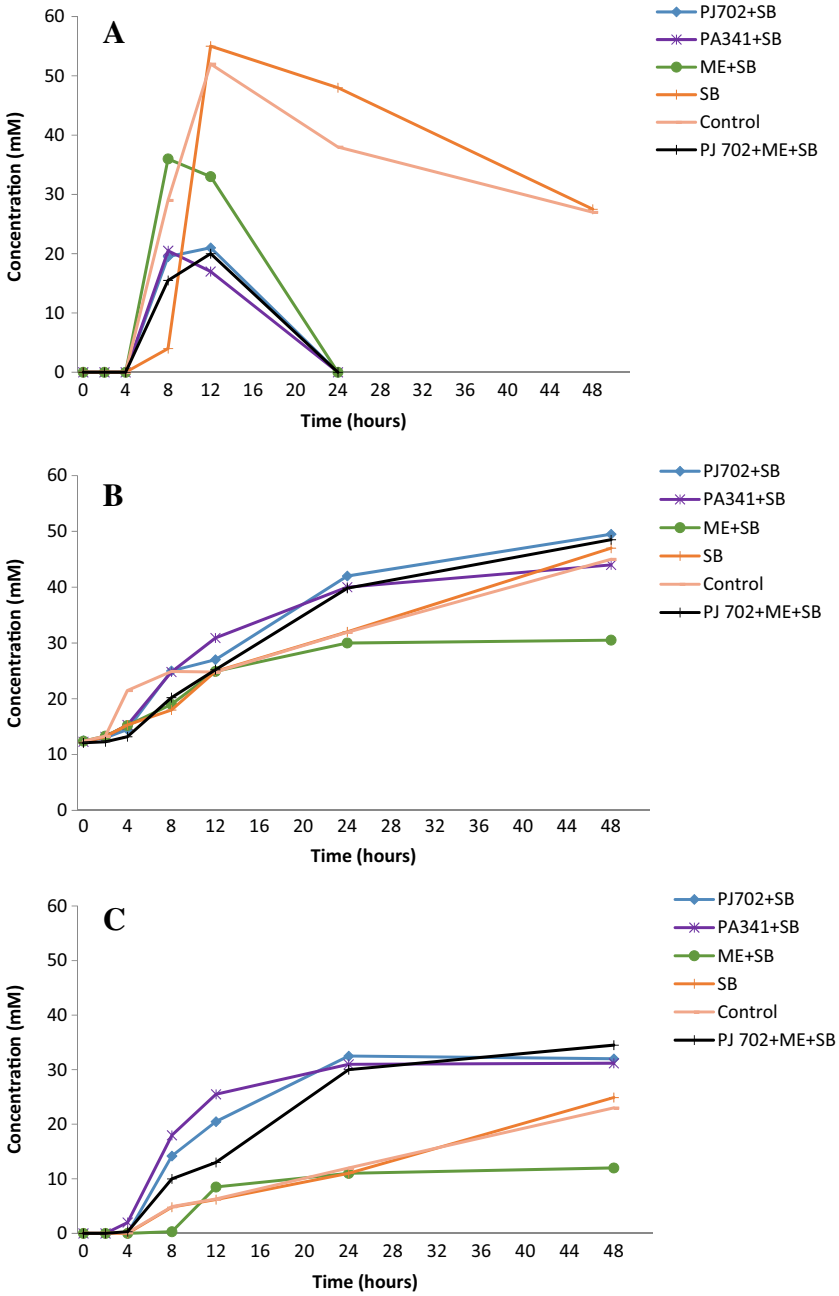

Fig. 1 Change in **a** lactic, **b** acetic and **c** propionic acid concentrations in the various treatments in two-strain-inoculation rumen cultures. Details of each treatment are as listed in Table 1. Each *point* represents the mean value of replicate measurements ($n = 3$)

establishment of simulated ruminal acidosis conditions. The extra glucose introduced into the rumen would have stimulated the growth of indigenous lactic acid producing bacteria, enabling them to generate large amounts of lactic acid in a short period of time, as evidenced by the exponential increase of lactic acid in the control samples to 52.65 mM during the first 12 h. Such a reaction reflects the progression of lactic acidosis in the rumen, with glucose serving as the easily fermentable carbohydrate. The fact that the SB treatment, containing inoculation of *S. bovis* only, produced the highest level of lactic acid production of any of the treatments appeared to confirm its role as a major lactic acid producer in the rumen. While this peak level was only 5% greater than that produced by the control samples, it should be recognized that all rumen samples received a fixed supply of glucose, thus limiting the maximum amount of lactic acid that could be produced. That is, despite the elevated presence of *S. bovis*, production of lactic acid was ultimately limited by the finite availability of substrate.

The most significant finding was the reduction in lactic acid accumulation in rumen cultures inoculated with either strains of *Propionibacterium*, *M. elsdenii*, or both. The peak lactic acid concentrations in those treatments were substantially lower compared with the control and the SB treatment. Moreover, after reaching peak levels, the lactic acid in the rumen samples was reduced and eventually eliminated in the

treatments inoculated with propionibacteria or *M. elsdenii* (Fig. 1). This result has successfully demonstrated that the application of dairy propionibacteria, *M. elsdenii*, or their combination in the rumen, was able to prevent the accumulation of large amounts of lactic acid and remove them from the rumen content samples completely.

Similarly, reduction of lactic acid by inoculation of *M. elsdenii* in a simulated rumen acidosis environment was reported by Kung and Hession (1995), where elevation of lactic acid to a concentration of 50 mM was observed in the glucose- and maltose-enriched rumen fluid medium in the first 12 h. The inoculation of *M. elsdenii* was able to prevent the accumulation of lactic acid. This finding is in agreement with the results of the present study, where similar levels of lactic acid reduction were also observed in the treatments containing *Propionibacterium* spp. It is important to note however, the differences in the composition of the growth environment between the present and previous studies. In the Kung and Hession (1995) study, the medium comprised filtered rumen fluid with several additives, including cysteine HCL and maltose, to enhance the growth of *M. elsdenii*. In the present study, the medium was whole rumen content comprising unmodified digesta and indigenous micro-flora, and no extra enhancement other than the glucose. The same level of lactic acid elevation in the control and reduction of lactic acid accumulation in the treatment in both studies confirm that the application of lactic acid utilizing bacteria has potential for the treatment and prevention of ruminal acidosis.

Among the tested dairy propionibacteria, strains PJ702 and PA341 have shown greater capacity for lactic acid consumption than other strains as well as their lactic aid consumption in previous studies in SLB medium (Luo et al. 2017b) and rumen samples (Luo et al. 2017a). In the study of Luo et al. (2017a), no extra *S. bovis* was introduced to the rumen content samples, and the lactic acid in the rumen was provided before the inoculation of bacteria, while in the present study the lactic acid in the rumen samples was produced by the *S. bovis*, which grew on the available glucose in the environment. This change in the rumen culture environment appeared to provide different impacts on the metabolism of these strains of *Propionibacterium*.

Compared with the previous study (Luo et al. 2017b), the production of propionic acid had similar profiles between different preparations. In relation to the acetic acid profile, the control produced a higher final concentration (45.55 mM) in the present study than the previous study (27.96 mM). This may reflect the addition of extra glucose in the present study rather than lactic acid in the previous study. Many indigenous bacteria in the rumen are able to use glucose to produce acetic and propionic acid. *S. bovis* itself and other common cellulolytic bacteria in the rumen such as *Fibrobacter succinogenes* (Weimer 1993), *Ruminococcus flavefaciens* (Shi and Weimer 1992) and *Ruminococcus albus* (Pavlostathis et al. 1988) all have this capacity. In contrast, very few bacteria in the rumen are able to utilize lactic acid as a carbon source, and this could explain why the introduction of lactic acid in rumen samples in the previous study (Luo et al. 2017b) had limited effect on elevation of acetic acid concentration levels.

In the three-strain-bacterial inoculation, *Propionibacterium* strain PJ702 appeared to have a stronger influence on acid metabolism than *M. elsdenii* where both existed in the rumen sample. In terms of lactic acid levels, no synergistic effect was evident when using two lactate utilisers together. Although the lactic acid concentration was lowest at 8 h in the three strain preparation (PJ702 + ME+SB) than in the two-strain treatments, the peak level of lactic acid in treatment PJ702 + ME+SB was similar to that in treatment PJ702 + SB, which appeared in both cases at 12 h. The acetic and propionic acid concentration was significantly higher in the treatment PJ702 + ME+SB than the ME+SB and comparable to those in the PJ702 + SB and PA341 treatments. These results indicate that the majority of the carbon source was converted to acetic and propionic acid during the incubation. This implies that the metabolic activity of strain PJ702 was largely overpowering the metabolic activity of *M. elsdenii* in the rumen sample during the incubation.

It would appear that the characteristic physiological properties of *M. elsdenii* may restrict its potential for application in treating ruminal acidosis. Firstly, *M. elsdenii* is sensitive to a low pH environment, and exposure to pH values lower than 5 can have a severe impact on the metabolism and survival of this bacterium (Russell et al. 1981). Secondly, *M. elsdenii* is sensitive to oxygen. Van Dijk et al. (1980) reported that the dehydrogenase enzyme isolated from *M. elsdenii* was highly sensitive to oxygen, and that partial inactivation occurs even before oxygen can be detected in the bacterial broth. Therefore, the sensitivity of *M. elsdenii* to the surrounding environment may severely impair its capacity for lactic acid consumption, which appeared evident in the present study. On the other hand, dairy propionibacteria, seemingly more resilient bacteria with higher tolerance to low pH and oxygen (dairy propionibacteria are facultative bacteria) and demonstrated capacity for lactic acid consumption, may be a more feasible option for application in the treatment and prevention of ruminal acidosis.

In this study, it was hypothesized that the acid profile would vary significantly based on the inoculation of different combinations of bacteria, and this was clearly confirmed. The most significant finding was the suppression of lactic acid accumulation by the dairy propionibacteria such as PJ702 and PA341 during the growth of *S. bovis* in the simulated rumen environment. Moreover, these dairy propionibacteria demonstrated superior lactic acid consumption capacity over that of *M. elsdenii*. Although the synergistic effect of applying both dairy propionibacteria and *M. elsdenii* together was not shown to be strong, in the actual rumen environment the removal of excessive lactic acid by the application of these

propionibacteria may create more favorable conditions for the recovery of *M. elsdenii* numbers, thereby helping to restore the fermentation process in the rumen. Under such circumstances, the cooperation of suitable strains of propionibacteria and *M. elsdenii* may be beneficial in preventing the occurrence of ruminal acidosis more effectively.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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