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ORIGINAL ARTICLE

Influence of three microbial feed additives of *Megasphaera elsdenii*, *Saccharomyces cerevisiae* and *Lactobacillus* sp. on ruminal methane and carbon dioxide production, and biofermentation kinetics

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biogases, lactic acid bacteria, microbial, rumen biofermentation, yeast.

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Abstract

Aims: This study was performed to investigate the effects of *Megasphaera elsdenii* (Me), *Saccharomyces cerevisiae* (SC) and lactic acid bacteria (FP—*Lactobacillus fermentum* plus *Lactobacillus plantarum*) alone or in combination on biogas production and ruminal biofermentation parameter in a heterofermenter system.
Methods and Results: Eight treatments were evaluated; (i) control (without additive; CON); (ii) Me; (iii) SC; (iv) FP; (v) Me plus SC (MSC); (vi) Me plus FP (MFP); (vii) SC plus FP (SCFP) and (viii) Me plus SC plus FP (MSCFP). Doses of FP, Me and SC were $1{\cdot}5 \times 10^8$ (CFU per ml), $1{\cdot}5 \times 10^8$ (CFU per ml) and $1{\cdot}4 \times 10^7$ (CFU $0{\cdot}002^{-1}$ g), respectively. Biogas production in all time increased ($P < 0{\cdot}05$) by MSCFP than CON additive. The proportional methane (CH_4) decreased ($P < 0{\cdot}05$) in MSCFP and FP, while carbon dioxide (CO_2) was decreased ($P < 0{\cdot}05$) by SC compared MSCFP and MSC. The proportional CO_2 decreased ($P < 0{\cdot}05$) by MSCFP and FP additive. The mean concentration of NH_3-N was not affected by treatments. Concentration of total volatile fatty acids and the percent of acetate and propionate was not affected by treatments. The highest ($P < 0{\cdot}05$) percent of butyrate and valerate were observed in MSCFP additive. The experiment showed that microbial additives of FP, SCFP and MSCFP reduced proportional CH_4 and CO_2.
Conclusions: Microbial additives of MFP and MSCFP had a sustainable positive efficiency on pH and volatile fatty acids and mitigate CH_4 and CO_2.
Significance and Impact of the Study: The use of microbial additives control on the ruminal pH (MFP) and improve VFA such as butyrate (MSC, MSCFP) and valerate (MSCFP) and reduce the greenhouse gases production showed a reduced risk of ruminal acidosis.

Introduction

In years to come, the need for livestock products (meat and milk) will increase due to the increase in population and urban life. Although ruminants will play an important role in the production of livestock products, but they are a source of greenhouse gas emissions. Methane (CH_4) and carbon dioxide (CO_2) are produced as a result of microbial fermentation in the ruminants digestive tract (Elghandour *et al.* 2016a). CH_4 production not only causes losses of energy (2 to 12% of gross energy ingested) for ruminants, but also is a greenhouse gas. The emission of CH_4, CO_2 and other harmful gases in the environment is the main cause of global warming (Bunthoeun *et al.* 2007). Hence, many strategies have been considered to improve rumen biofermintation and reduce CH_4 production such as the use of exogenous enzymes (Kholif *et al.* 2017), essential oils (Hernandez *et al.* 2017), plant extracts (Elghandour *et al.* 2018) and probiotics or microbial additives (Elghandour *et al.* 2017).

The use of microbial additives is more and more considered and the results show that the use of microbial additives to manipulate rumen biofermintation in addition to improving livestock performance can be useful in reducing greenhouse gas emissions (Pedraza-Hernández *et al*. 2019). In fact, microbial additives have been used as the main goal to improve feed efficiency, performance, modify ruminal biofermintation, prevent nutritional disorders (Direkvandi *et al*. 2020b) and also reduce the incidence of diarrhoea in calves (Nagashima *et al*. 2010), but their impact on greenhouse gas emissions can be interesting. We can point out that bacterial additives especially lactic acid utilizing bacteria (LUB; *Megasphaera elsdenii* and *Selenomonas ruminantium*) lead to improve ruminal pH during acidosis (Goto *et al*. 2016) and causes ruminal biofermintation shift to produce propionate and butyrate. Hydrogen (H_2) is an important substrate for CH_4 production and propionate production is considered as a route for its utilization (Jeyanathan *et al*. 2014). On the other hand, lactic acid bacteria (LAB; *Streptococcus bovis* and *Lactobacillus* sp.) can be beneficial by stimulating the growth of LUB (Seo *et al*. 2010). Also, Nollet *et al*. (1998) reported that CH_4 production was significantly reduced by *L. plantarum*.

Yeast additives such as *Saccharomyces cerevisiae* provide the nutrient requirement of rumen micro-organisms through the nutrients in their cell walls such as vitamins B (Callaway and Martin 1997), amino acids (Chaucheyras *et al*. 1996) and malic acid (Dawson and Girard 1997) and lead to an increase in the concentration of rumen bacteria, especially cellulolytic bacteria, through the equilibrium in rumen pH (Beauchemin *et al*. 2006). Yeast additives also has a beneficial effect on growth and H_2-utilisation of acetogenic bacteria (Chaucheyras *et al*. 1995) and in several studies, yeast products have been investigated to reduce CH_4 production (Hernandez *et al*. 2017; Adegbeye *et al*. 2019). With these interpretations, there are still few studies on the use of bacterial additives (LAB and LUB) and yeast on greenhouse gas production and ruminal biofermintation in sheep. It was hypothesized that microbial feed additives (LAB, LUB and *S. cerevisiae*) can positively affect alone or in combination on biogas production and ruminal biofermintation parameters in high concentrate diet. However, this study was performed to investigate the effect of microbial feed additives (LAB, LUB and *S. cerevisiae*) on biogas production and ruminal biofermintation parameters.

Materials and methods

Microbial preparation and treatments

Three microbial additives used were (i) LAB (*L. fermentum* and *L. plantarum* (in the ratio of 50 : 50)), (ii) LUB (*M. elsdenii*) and (iii) *S. cerevisiae*. The LAB was cultured under anaerobic conditions and in a sterile environment at de Man, Rogosa and Sharpe (MRS) agar medium and MRS broth (Biolife, Milano, Italy) and then used at a concentration of 0·5 McFarland (0·5 McFarland standard corresponds to $1{\cdot}5 \times 10^8$ CFU per ml). LUB was similarly cultured in LH medium (Mackie and Heath 1979) under anaerobic and sterile conditions and then used at a concentration of 0·5 McFarland. *Saccharomyces cerevisiae* was used from Iran Mollasses Company (Mashhad, Iran), that each gram of this yeast contains 7×10^9 CFU per gram. In this research, we used 0·002 g of *S. cerevisiae* for treatments containing *S. cerevisiae* (which contains $1{\cdot}4 \times 10^7$ CFU $0{\cdot}002^{-1}$ g). According to the microbial additive used, the treatments were: (i) control diet (without additive; CON), (ii) *M. elsdenii* (Me), (iii) *S. cerevisiae* (SC), (iv) *L. fermentum* and *L. plantarum* (FP), (v) Me+SC (MSC), (vi) Me+FP (MFP), (vii) SC+FP (SCFP) and (viii) Me+SC+FP (MSCFP).

Rumen fluid preparation and biogas production

Rumen fluid (RF) was collected from three fistulated adult male Arabi sheep before morning feeding and pooled. Animals were fed on *ad libitum* a ration based on 60% forage (25% wheat straw, 10% alfalfa hay, 25% corn silage) and 40% concentrate (8% corn, 7% barely, 20% wheat bran, 2% soybean meal, 1% salt, 2% mineral vitamin supplement). Animals were subject to the diet for a period of 2 months before collecting the rumen liquor samples. The ration was formulated to contain a crude protein (CP) and metabolizable energy (ME) contents of 105 g kg^{-1} dry matter (DM) and 2·3 Mcal kg^{-1} DM, respectively (NRC 2007). The RF was filtered by 4-layer cheesecloth and after that, it was kept in a warm bath at 39°C. Then, remaining digesta after filtering was rinsed with a buffer solution (McDougall) to isolate particle-bound micro-organism and placed in a warm bath at 39°C (BS) (Kung and Hession 1995).

For *in vitro* biogas technique, the basal diet was formulated based on 30% forage (alfalfa hay and wheat straw) and 70% concentrate (barley grain, corn grain, soybean meal, wheat bran) (Table 1) (NRC 2007). A 1000 mg (based on DM) of the basal diet was placed into 100 ml vials (three replicates for each treatment, eight treatments and six incubation time; 144 vials for all time with the six blanks for each time). Then, 20 ml of RF plus 20 ml of BS was added pre-flushed with CO_2 to each vial then incubated for 48 h in a warm bath at 39°C. The microbial feed additives of each treatment were injected into the vials. Distilled water (3 ml) was added to the CON instead of microbial feed additive. To determine the effect of the microbial feed additives on *in vitro* ruminal

Table 1 Ingredients (g kg^{-1} DM), chemical composition (g kg^{-1} DM) and metabolizable energy (Mcal kg^{-1} DM) of basal diet used in the experimental

Ingredients	
Alfalfa	201
Wheat straw	99
Barley grain	300
Corn grain	210
Soybean meal	123·5
Wheat bran	55
Calcium carbonate	4
NaCl	2·5
Vitamin and mineral supplements*	5
Chemical composition	
Dry matter	903
Organic matter	948
Crude protein	161
Ether extract	27
NDF	290
ADF	165
Metabolizable energy†	2·65
Non–fiber carbohydrates‡	472

NDF, neutral detergent fiber; ADF, acid detergent fiber.
*Premix contained (kg^{-1}): Vitamin A, 500 000 IU mg^{-1}; vitamin D_3, 100 000 IU mg^{-1}; vitamin E, 100 mg kg^{-1}; Ca, 180 g kg^{-1}; P, 60 000 mg kg^{-1}; Na, 60 000 mg kg^{-1}; Mg, 19 000 mg kg^{-1}; Zn, 3000 mg kg^{-1}; Fe, 3000 mg kg^{-1}; Mn, 19 000 mg kg^{-1}; Cu, 300 mg kg^{-1}; Co, 100 mg kg^{-1}; Se, 1 mg kg^{-1}; I, 100 mg kg^{-1}; antioxidant, 400 mg kg^{-1}; carrier, up to 1000 g.
†Calculated from each feed composition.
‡Calculated as: 1000 – (NDF g kg^{-1} DM+CP g kg^{-1} DM+EE g kg^{-1} DM+ash g kg^{-1} DM).

volatile fatty acids (VFA), NH_3-N and pH and biogas production the sampling carried out at 0, 2, 4, 8, 24 and 48 h after incubation.

Chemical analyses

Following the AOAC International procedure basal diet were analysed for CP (Number. 988.05), ash (Number. 924.05), ether extract (EE) (Number. 920.39) and acid detergent fibre (ADF) (Number. 973.18) (AOAC 1998). Neutral detergent fibre (NDF) was analysed by using the method of Van Soest *et al.* (1991). After incubation time vial contents were obtained from each vials and pH was determined immediately by a portable pH meter (Metrohm model, Swiss). The *in vitro* ruminal NH_3-N concentration was measured by phenol-hypochlorite assay (Broderick and Kang 1980). *In vitro* ruminal VFA concentration was determined by gas chromatography (GC; Chrompack, Model CP-9002, Chrompack, EA Middelburg, Netherlands) that equipped with a 50-m (0·32 mm ID) silica-fused column (CP-Wax Chrompack Capillary Column, Varian, Palo Alto, CA.). 2-ethyl-butyric acid was used as internal standard. The helium was used as a carrier and oven initial and final temperatures were 55 and 195°C, respectively, and detector and injector temperatures were set at 250°C. Both of CH_4 and CO_2 productions were calculated at 0, 2, 4, 8, 24 and 48 h of incubation according Moss *et al.* (2000).

Statistical analyses

The data obtained were analysed as factorial experiment based on a completely randomized design using the PROC MIXED procedures of SAS (2008, ver. 9.2), based on the statistical model: $y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \alpha\beta_{ij} + \alpha\gamma_{ik} + \beta\gamma_{jk} + \alpha\beta\gamma_{ijk} + e_{ijkl}$. Where y_{ijkl} is observation, μ is the general mean, α_i is the effect of first factor (Me), β_j is the effect of second factor (SC), γ_k is the effect of third factor effect (FP), $\alpha\beta_{ij}$ is the interaction between treatment (Me × SC), $\alpha\gamma_{ik}$ is the interaction between treatment (Me × FP), $\beta\gamma_{jk}$ is the interaction between treatment (SC × FP), $\alpha\beta\gamma_{ijk}$ is the interaction between treatment (Me × SC × FP) and e_{ijkl} is the standard error of term. Also, graphs of CH_4, CO_2, pH, NH_3-N and VFA were plotted using GraphPad Prism software (ver. 8.4.3, La Jolla, CA) and analysed as repeated measurements using the PROC MIXED procedures of SAS (2008), based on the statistical model: $Y_{ijk} = \mu + T_i + H_j + (TH)_{ij} + e_{ijk}$. Where Y_{ijk} is observation (CH_4, CO_2, pH, NH_3-N, VFA), μ is the general mean, T_i is the effect of microbial additives, H_j is the effect of sampling hours, $(TH)_{ij}$ is interactions between the effect of treatment and sampling hours and e_{ijk} is the standard error of term. To determine the difference between treatments we used Duncan multiple comparison tests at $P < 0{\cdot}05$.

Results

Biogas production

Gas production (GP) along incubation time increased ($P < 0{\cdot}05$) by MSCFP than CON. The volume of CH_4 produced was affected by microbial additives only at 4, 8 and 24 h after incubation, but in general, the volume of CH_4 produced was not affected ($P = 0{\cdot}426$) by the treatments. The proportional CH_4 decreased ($P < 0{\cdot}05$) in MSCFP and FP additives. The volume of CO_2 produced was affected by microbial additives only at 2, 4, 8 and 24 h after incubation, and in general, the CO_2 produced was affected ($P = 0{\cdot}001$) by treatments (Fig. 1). The proportional CO_2 decreased ($P < 0{\cdot}05$) in MSCFP and FP additives (Table 2).

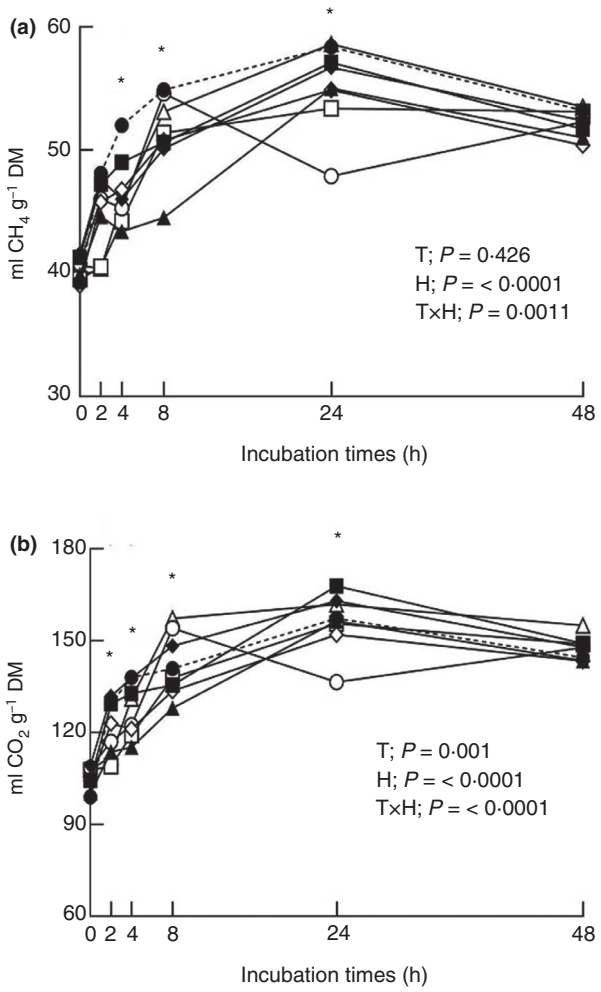

Figure 1 Changes in methane (CH_4) (a) and carbon dioxide (CO_2) (b) after 48 h ruminal fermentation incubated with different microbial additives. ● and dotted line (CON), without microbial additive (control); ■ (Me), *Megasphaera elsdenii*; ▲ (SC), *S. cerevisiae*; ◊ (FP), *L. fermentum and L. plantarum*; ♦ (MSC), Me plus SC; ○ (MFP), Me plus FP; □ (SCFP), SC plus FP; Δ (MSCFP), Me plus SC plus FP. * Indicates a differ significantly ($P < 0·05$) (treatment effect), T, effect of treatments; H, effect of sampling hours; T × H, interaction effect of treatments and sampling hours.

Ruminal pH, NH_3-N and VFA

After incubation, pH decreased for all treatments and reached the lowest value at 4 h after incubation. However, the pH at 48 h was affected by experimental treatments and the lowest and highest levels of pH were observed in the CON and MFP additives (5·87 and 5·99, respectively) ($P < 0·05$, Table 3). The highest ($P < 0·05$) value and maximum of pH were observed in MFP. Concentration of NH_3-N was not affected by experimental treatments. The NH_3-N reached to the maximum value at 4 h (SCFP, 38·70 mg dl^{-1}), and after that have a downward trend and reached to the minimum value (MSC, 4·02 mg dl^{-1}) during incubation. Ruminal concentration of NH_3-N differed ($P < 0·05$, treatment effect) only at 4 and 8 h after incubation (Fig. 2).

Concentration of total VFA (TVFA) and the percent of acetate and propionate were not affected ($P > 0·05$) by experimental treatments. The highest ($P < 0·05$) percent of ruminal butyrate were observed by MSCFP and MSC (23·6% of TVFA) additives, but there was no difference ($P > 0·05$) among Me, MFP and SCFP additives. Ruminal valerate percent increased ($P < 0·05$) in MSCFP additive (Table 4). Acetate concentration was lower ($P < 0·05$) than CON only at 4 h (MSC and MSCFP), 8 h (SC, MSC, MFP and MSCFP) and 48 h. Propionate increased ($P < 0·05$) for experimental treatments at 4 h (MSC) and 8 h (SC) than CON additive. Butyrate concentration at 4, 8, 24 and 48 h showed differences ($P < 0·05$) among treatments, but the difference of butyrate at 4 h was only among treatments containing microbial feed additives. Valerate concentration was affected ($P < 0·05$) by microbial feed additives at all times except 8 h. Concentration of total VFA (TVFA) was meaningful only at 8 h ($P < 0·05$). However, at this time the higher ($P < 0·05$) concentration of TVFA was observed in MSCFP additive (Fig. 3).

Discussion

Biogas production

The information about the effect of Me on *in vitro* biogas production is limited. As can be seen in Table 2, biogas production was not affected by Me in treatment Me, MSC and MFP except MSCFP. Due to the lack of differences between treatments Me, MSC and MFP with CON, it is possible that a difference between MSCFP and CON, resulted from the presence of SC and FP in this treatment. The volume of biogas production was increased in treatments containing FP (except MFP). Similarly, in another experiment, the use of 14 strains of *L. plantarum* lead to an increase in the biogas production compared to control (Astuti *et al.* 2018). However, the volume of biogas produced by FP can vary depending on the strain and substrate used, may be due to being homofermenters and heterofermenters, LAB produce lactic acid, acetic acid, CO_2 and ethanol (Leahy *et al.* 2019). The positive effect of yeast on biogas production compared to CON was seen only in treatments MSCFP and SCFP. It seems that SC provides the conditions for the optimal activity of other ruminal organisms by consuming oxygen (O_2) in the rumen. Hernandez *et al.* (2017) stated that yeast has a positive effect on increasing gas production, but it was not always.

Table 2 Effect of different source of microbial additives on *in vitro* gas production, methane (CH_4) and carbon dioxide (CO_2)

	Gas production			CH_4 production		CO_2 production	
	ml g^{-1} of DM incubated						
Treatment*	4 h	24 h	48 h	ml g^{-1} of DM incubated	Proportional CH_4 production	ml g^{-1} of DM incubated	Proportional CO_2 production
CON	23.9^{c}	172^{d}	215^{c}	51·0	25.1^{a}	135^{ab}	68.2^{a}
Me	20.2^{cd}	172^{d}	219^{c}	49·5	23.7^{a}	137^{ab}	68.5^{a}
SC	20.2^{cd}	172^{d}	222^{c}	46·4	23.0^{a}	126^{b}	64.8^{a}
FP	60.3^{b}	264^{b}	312^{b}	47·9	16.3^{c}	130^{ab}	46.4^{c}
MSC	18.0^{d}	167^{d}	213^{c}	49·1	24.6^{a}	140^{a}	69.4^{a}
MFP	23.9^{c}	180^{d}	217^{c}	47·8	24.1^{a}	131^{ab}	68.1^{a}
SCFP	67.0^{a}	239^{c}	283^{b}	47·0	18.8^{b}	130^{ab}	52.9^{b}
MSCFP	70.0^{a}	296^{a}	347^{a}	48·7	15.4^{c}	138^{a}	44.6^{c}
SEM	1·93	10·4	12·0	1·71	0·91	5·33	2·52
P-value							
Interaction							
Me	0·101	0·269	0·346	0·617	0·169	0·101	0·051
SC	<0·001	0·009	0·012	0·585	0·036	0·350	0·040
FP	<0·001	<0·001	<0·001	0·847	<0·001	0·388	<0·001
Me × SC	0·001	0·001	0·001	0·740	0·020	0·833	0·119
Me × FP	0·002	0·473	0·476	0·596	0·220	0·880	0·339
SC × FP	<0·001	0·005	0·014	0·219	0·152	0·020	0·010
Me × SC×FP	0·003	0·001	0·001	0·299	0·001	0·033	0·001

SEM, standard error of means.

[a–d]Means in the same column with different superscript letters are different ($P < 0·05$).

*CON, without microbial additive (control); Me, *Megasphaera elsdenii* ($1·5 \times 10^8$ CFU per ml); SC, *Saccharomyces cerevisiae* ($1·4 \times 10^7$ CFU $0·002^{-1}$ g); FP, *Lactobacillus fermentum* and *Lactobacillus plantarum* ($1·5 \times 10^8$ CFU per ml); MSC, Me plus SC; MFP, Me plus FP; SCFP, SC plus FP; MSCFP, Me plus SC plus FP.

Preventing the formation of H_2 in the rumen or consuming it is a way to prevent it from entering the CH_4 production cycle. In the rumen, production propionate and butyrate produce less H_2 than acetate production. In fact, this action will be possible through the growth and stimulation of LUB (Ungerfeld 2020). Use of Me as LUB alone or in combination with other additives numerically reduced CH_4 production. Interactions also showed that the effect of Me alone was not significant on CH_4 production and proportional. This indicates that achieving a reduction in CH_4 production through the simultaneous use of additives would be beneficial. In an *in vivo* study, the simultaneous use of all three microbial additives (MSCFP) reduced methanogens (Direkvandi *et al.* 2020a). The effect of LAB on the reduction of CH_4 production may be due to its beneficial effect on LUB. LAB stimulates the growth of LUB through the continuous production of low concentrations of lactic acid (Seo *et al.* 2010). In dairy lactating cow using a combination of LUB (*Propionibacterium jensenii*) and LAB (*Lactobacillus* sp.) lead to reducing the emission of CH_4 (Lettat *et al.* 2012). Another positive effect of LAB may be due to the production of bacteriocin. Lee *et al.* (2002) showed that the bacteriocin (Bovicin HC5) produced by *Streptococcus equinus* reduced the amount of CH_4 by 53%.

The addition of SC reduces the H_2 availability for methanogens and shift it to biofermintation towards butyrate or propionate (Erasmus *et al.* 2005). Hristov *et al.* (2013) reported that yeast culture reduced ruminal CH_4 emissions by stimulating the acetogens to compete with methanogenic bacteria. The growth of *Fibrobacter succinogenes* and also decreases lag time for the growth of *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens* were stimulated by SC in the *in vitro* study (Girard and Dawson 1995). Direkvandi *et al.* (2020a) reported that the use of yeast-containing microbial additives (SCFP and MSCFP) increased the population of fibrobacteria compared to the control. Increasing the population of *F. succinogenes* can be a factor in reducing the population of methanogens. Because *F. succinogenes* is a non-H_2 producing bacteria, and H_2 is a substrate for methanogens, therefore, less substrate will be accessible for methanogens (Mamuad *et al.* 2019). Waldrip and Martin (1993) reported that the fungal additive stimulates the LUB to uptake lactate but does not change the biofermentation profile. In agreement with the current study, Opsi *et al.* (2011) also reported that CH_4 production was not affected by yeast. Similar to the current study, McGinn *et al.* (2004) reported a decrease in CH_4 production by yeast, but the difference was not

Table 3 Effect of different source of microbial feed additives on *in vitro* ruminal pH

	pH				
Treatment*	Initial	Final	Mean	Min	Max
CON	5·91	5·87^{d}	5·74^{e}	5·45^{c}	5·91^{c}
Me	5·98	5·98^{a}	5·80^{c}	5·64^{b}	5·98^{b}
SC	5·87	5·96^{b}	5·82^{c}	5·70^{a}	5·96^{b}
FP	5·91	5·93^{c}	5·78^{d}	5·69^{a}	5·93^{c}
MSC	5·94	5·96^{b}	5·85^{b}	5·72^{a}	5·99^{b}
MFP	5·99	5·99^{a}	5·88^{a}	5·71^{a}	6·05^{a}
SCFP	5·95	5·96^{b}	5·85^{b}	5·70^{a}	5·96^{b}
MSCFP	5·87	5·97^{a}	5·83^{b}	5·64^{b}	5·97^{b}
SEM	0·062	0·008	0·010	0·018	0·009
P-value					
Interaction					
Me	0·439	0·001	<0·001	0·022	0·001
SC	0·342	0·033	<0·001	0·001	0·798
FP	0·167	0·033	0·001	0·007	0·139
Me × SC	0·342	0·001	0·001	0·003	0·009
Me × FP	0·439	0·297	0·666	0·002	0·524
SC × FP	0·226	0·125	0·002	<0·001	0·054
Me × SC×FP	0·053	0·170	0·018	0·239	0·171

SEM, standard error of means.

[a–e]Means in the same column with different superscript letters are different ($P < 0·05$).

*CON, without microbial additive (control); Me, *Megasphaera elsdenii* ($1·5 \times 10^8$ CFU per ml); SC, *Saccharomyces cerevisiae* ($1·4 \times 10^7$ CFU $0·002^{-1}$ g); FP, *Lactobacillus fermentum* and *Lactobacillus plantarum* ($1·5 \times 10^8$ CFU per ml); MSC, Me plus SC; MFP, Me plus FP; SCFP, SC plus FP; MSCFP, Me plus SC plus FP.

significant. Contrary to our results, Elghandour *et al.* (2016b) reported an increase in CH_4 production by the yeast. Patra (2012) reported that the effect of SC cultures on the increase or decrease in CH_4 production depends on the strain of yeast and nature of diet.

Yeast is effective in creating anaerobic conditions in the rumen by consuming O_2 in the rumen and producing CO_2. Yeast also produces more CO_2 and acetate by increasing fibre degradation. The volume of CO_2 produced in MSC and MSCFP additives was numerically higher than the CON, which agreed with the effect of yeast on CO_2 production, but in SC and SCFP, the volume of CO_2 was numerically lower than the CON. According to the present experiment results, doses of 2 and 4 mg of SC produced less CO_2 than controls (Hernandez *et al.* 2017). They suggested that yeast may have produced antimicrobial metabolites that affected ruminal digestion. In the current study, two strain of *L. plantarum* (facultative homofermenter) and *L. fermentatum* (obligate heterofermenter) were used as a LAB, that given the volume of CO_2 probably *L. plantarum* played a predominant role, otherwise, more CO_2 would be expected as follow (Leahy *et al.* 2019):

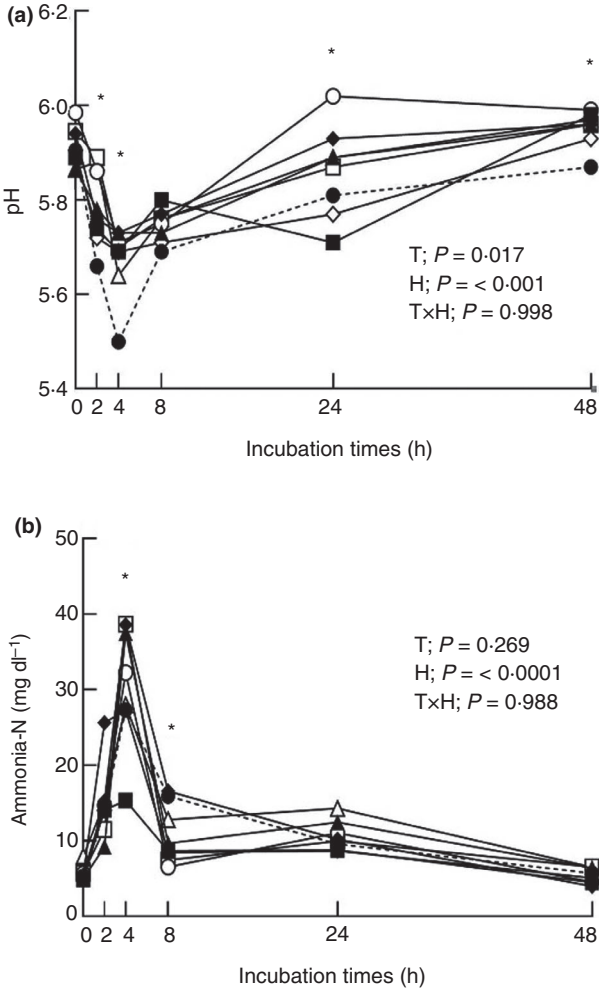

Figure 2 Changes in pH (a) and NH_3-N (b) after 48 h ruminal fermentation incubated with different microbial additives. ● and dotted line (CON), without microbial additive (control); ■ (Me), *M. elsdenii*; ▲ (SC), *S. cerevisiae*; ◊ (FP), *L. fermentum and L. plantarum*; ◆ (MSC), Me plus SC; ○ (MFP), Me plus FP; □ (SCFP), SC plus FP; Δ (MSCFP), Me plus SC plus FP. * Indicates a differ significantly ($P < 0·05$) (treatment effect), T, effect of treatments; H, effect of sampling hours; T × H, interaction effect of treatments and sampling hours.

$$\text{Heterolactic fermentation}: \text{Glucose} \rightarrow \text{lactic acid} + \text{ethanol} + CO_2$$

Ruminal pH, NH_3-N and VFA

The pH in the range of 5–5·6 is commonly known as subacute acidosis (SARA), and a pH of less than 5 is considered acute acidosis (Krause and Oetzel 2006). However, in all treatments, pH was not less than 5 and also using microbial feed additives, pH was not less than 5·5, which appears that dosed microbial feed additives

Table 4 Effect of different source of microbial feed additives on *in vitro* ruminal VFA concentration after 48 h incubation

Treatment*	TVFA, mM	Individual VFA, % of TVFA			
		Acetate	Propionate	Butyrate	Valerate
CON	167	43·7	20·6	22.4^{c}	5·69^{e}
Me	167	42·0	21·3	23.3^{ab}	6·16^{cd}
SC	160	43·0	21·9	21.4^{d}	5·92^{d}
FP	161	42·8	21·2	22.6^{bc}	6·16^{cd}
MSC	171	40·4	22·1	23.6^{a}	6·55^{b}
MFP	161	41·9	21·0	23.2^{abc}	6·44^{b}
SCFP	161	41·6	21·4	22.9^{abc}	6·38^{bc}
MSCFP	169	40·4	21·7	23.6^{a}	6·86^{a}
SEM	10·9	1·51	0·75	0·38	0·104
P-value					
Interaction					
Me	0·271	0·696	0·679	0·014	0·003
SC	0·112	0·868	0·657	0·045	<0·001
FP	0·271	0·610	0·584	0·062	<0·001
Me × SC	0·410	0·685	0·946	0·712	0·052
Me × FP	0·381	0·976	0·862	0·946	0·248
SC × FP	0·169	0·320	0·862	0·958	0·001
Me × SC×FP	0·906	0·845	0·827	0·584	0·039

SEM, standard error of means; TVFA, total concentration VFA.

[a–e]Means in the same column with different superscript letters are different ($P < 0·05$).

*CON, without microbial additive (control); Me, *Megasphaera elsdenii* ($1·5 \times 10^8$ CFU per ml); SC, *Saccharomyces cerevisiae* ($1·4 \times 10^7$ CFU $0·002^{-1}$ g); FP, *Lactobacillus fermentum* and *Lactobacillus plantarum* ($1·5 \times 10^8$ CFU per ml); MSC, Me plus SC; MFP, Me plus FP; SCFP, SC plus FP; MSCFP, Me plus SC plus FP.

increase lactic acid metabolism and pH adjustment in the rumen (Qadis *et al.* 2014). Similarly, Kung and Hession (1995) found that pH was higher in the Me additive than control. *In vitro* ruminal pH was higher in treatments containing SC than CON, which was consistent with previous studies (Thrune *et al.* 2009; Pinloche *et al.* 2013). Different mechanisms have been identified for the effect of microbial feed additives on pH, such as competition with *S. bovis* and other species of lactobacillus for glucose utilization (Chaucheyras *et al.* 1996), stimulates LUB (Nagaraja 2014) and protozoa modification in the rumen (Galip 2006) that compete with LAB for glucose uptake (Nagaraja 2014).

The NH_3-N concentration was in the optimum range 8·5–30 mg dl described by McDonald *et al.* (2002). In a continuous culture study, ruminal concentration of ammonia-N was not affected by using *Propionibacterium*, *Enterococcus faecium*, *E. faecium* + yeast (Yang *et al.* 2004), which was in agreement with the results of the present study. Contrary with the current results, in an *in vitro* experiment, the combination of yeast extract and *Bacillus licheniformis* as microbial feed additives reduced the concentration of ammonia-N (Doto and Liu 2011). But they reported that reducing ammonia-N concentration due to the inclusion of more ammonia-N in the microbial protein (Doto and Liu 2011).

The concentration of TVFA in the present experiment was between 140 and 190 mmol l^{-1} at 2–48 h after incubation. In this case Nagaraja and Lechtenberg (2007) reported that during subacute acidosis, the concentration of TVFA increased between 150 and 225 mmol l^{-1}. On the other hand, they stated that in more severe cases of acidosis, the concentration of TVFA is reduced (Nagaraja and Titgemeyer 2007). However, in agreement with the current results in several *in vitro* experiments microbial feed additives did not have any effect on TVFA (Jeyanathan *et al.* 2016; Ellis *et al.* 2016).

The concentration of acetate decreased, and the concentration of propionate increased as a result of the activity of sugar utilization bacteria, which is inevitable for a concentrate-based diet. However, due to the high concentrate and low fibre diet, it is expected that the final biofermintation product will contain high concentrations of propionate (Sutton *et al.* 2003). It also leads to decreased acetate and acetate to propionate ratio. The current result was similar to other *in vitro* experiments that reported that microbial feed additives had no significant effect on the concentration of acetate and propionate compared to controls (Ellis *et al.* 2016). Philippeau *et al.* (2017) attributed the increase in propionate concentration as a result of the use of microbial feed additives to the increased amylase activity of the ruminal organisms and Me. It has been shown that Me converts lactate to propionate and butyrate (Drouillard *et al.* 2012). Me produces propionate and butyrate relative to acetate (Horn *et al.* 2009) and the growth of Me in pure culture causes butyrate accumulation (Slyter *et al.* 1992). Numerically increasing the butyrate concentration in treatments containing microbial mixture observed due to the presence of Me. However, in the case of ruminal acidosis or high cereal grain rations, has been reported the increase (Khafipour *et al.* 2009) and reduction (Kennelly *et al.* 1999) of butyrate concentrations. The increase of ruminal concentration of valerate in experimental treatments (especially in treatments containing Me) indicates the reduction of risk ruminal acidosis. However, valerate is a safe sink for utilization H_2 and lactate removal (Bramley *et al.* 2008).

The results of the current study showed that, although the effect of microbial additives on CH_4 production was not significant, but the proportional CH_4 production reduced by FP, SCFP and MSCFP. The proportional CO_2 production was reduced under the influence of these treatments, which showed the positive effect of these treatments on changing the pattern of ruminal biogas production. Using microbial additives is one of the strategies to prevent ruminal acidosis. Indeed, the use of microbial additives had a positive effect on ruminal pH

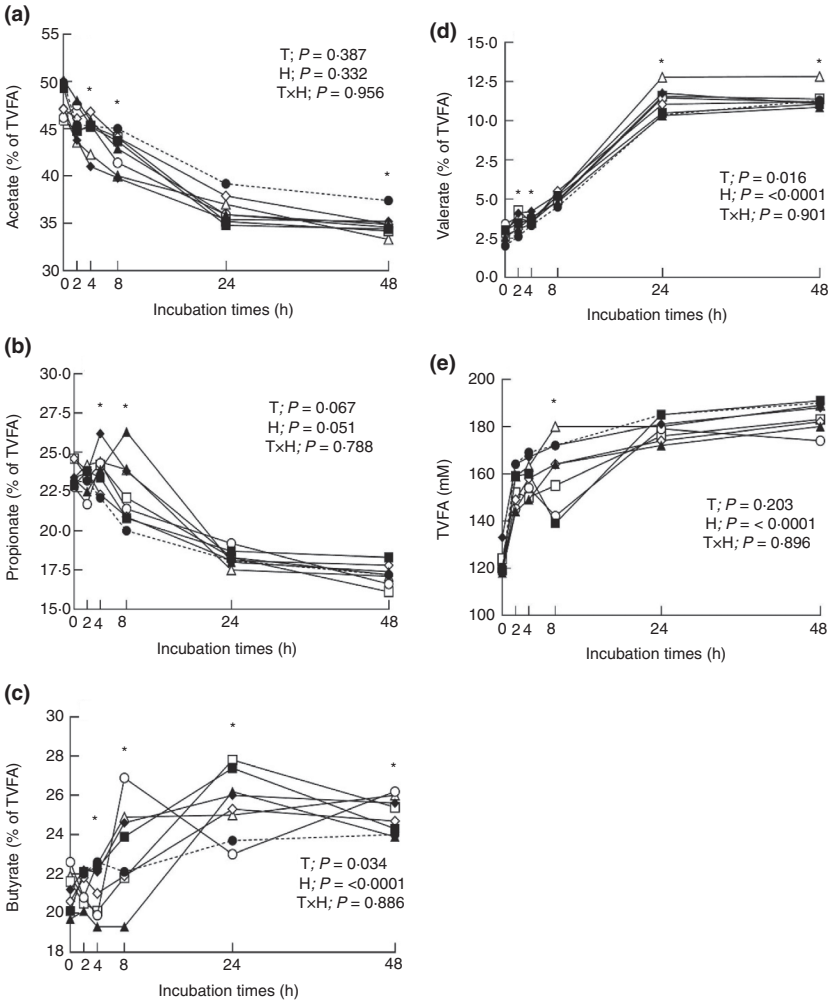

Figure 3 Changes in ruminal VFA (a, acetate; b, propionate; c, butyrate; d, valetare and e, TVFA (total VFA)) after 48 h ruminal fermentation incubated with different microbial additives. ● and dotted line (CON), without microbial additive (control); ■ (Me), *M. elsdenii*; ▲ (SC), *S. cerevisiae*; ◊ (FP), *L. fermentum and L. plantarum*; ♦ (MSC), Me plus SC; ○ (MFP), Me plus FP; □ (SCFP), SC plus FP; Δ (MSCFP), Me plus SC plus FP. * Indicates a differ significantly ($P < 0·05$) (treatment effect), T, effect of treatments; H, effect of sampling hours; T × H, interaction effect of treatments and sampling hours.

(MFP) and caused a shift in ruminal fermentation pattern towards the production of butyrate and valerate (MSC, MSCFP), which indicates a reduced risk of ruminal acidosis.

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Conflict of Interest

Authors declare that there is no conflict of interest exists.

Animal welfare statement

All animal management and sampling procedures conducted according to The Care and Use of Agricultural Animals in Research and Teaching guidelines (FASS 2010). All procedures and guidelines involving animals were approved by the Animal Experiment Committee at Agricultural Sciences and Natural Resources University of Khuzestan, Mollasani, Iran.

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