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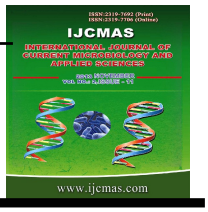

Original Research Article

Influence of pediocin and enterocinon *in-vitro* methane, gas production and digestibility

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Keywords

Bacteriocin; pediocin; enterocin; rumen; ruminants; methane; digestibility.

A B S T R A C T

Methane emitted from ruminant's digestive system is one of the major greenhouse gases attributed to animal agriculture and represents an energy loss of 2 to 12% of dietary energy. Therefore, abatement of enteric methane production is necessary not only from an economical viewpoint of livestock production but also from an environment perspective. Therefore, the objective of this study was to evaluate the effects of bacteriocins (pediocin and enterocin) on methane production and *in vitro* dry matter digestibility. The Bacteriocin from the *Pediococcus pentosaceus* 34 and *Enterococcus faecium* 99 strain was produced optimally in MRS broth by incubating at 37ºC for 18-24 h. Gas production test was performed *in vitro*, using 200 mg of substrate containing wheat straw and concentrate mixture in equal proportion (50:50). Treatments contains pediocin with different activity units (P1: 1, 20,000AU/2mL; P2: 90,000AU/2mL; P3: 60,000AU/ 2mL), enterocin (E1: 1, 20,000AU/2mL) as well different combination of pediocin and enterocin (EP1: 0.75: 0.25%and EP2: 0.5:0.5%, each having AU/2mL of 90,000) along with control having no bacteriocin. Methane content in fermentation gas was determined by gas chromatography. Pediocin (P1) resulted in significantly lower methane production (4.81%) followed byP2 (5.08%), compared to control (9.47%). P3 andE1 showed similar reduction in methane emission i.e., 6.18% and 6.08%.Whereas, EP1 and EP2 showed 8.25% and 7.10%methane emission, respectively. *In vitro* gas production test was increased significantly in all the treatments compared to control. Increased, *in vitro* dry matter and organic matter digestibility was also observed with pediocin, enterocin and combinations of both, but differences were not significant. The study concluded that pediocin (P1) has a potential for reducing enteric methane emission from ruminants.

Introduction

Global warming due to increasing atmospheric concentration of greenhouse gases (GHG) has received attention in developed and developing nations.

Methane is one of the most potent greenhouse gases contributing to farm level emissions, due to its warming potential (Beauchemin *et al*., 2010; Veysset *et al*., 2010). Rumen methanogenesis results in the loss of 6–10% of gross energy intake (GEI), or 8–14% of the digestible energy intake of ruminants (Kumar *et al*., 2009). Reducing emissions, particularly methane production, helps improves feed energy use and system efficiency.

Different strategies to reduce methane production in ruminants have been applied and investigated such as dietary manipulations, vaccines (Boadi *et al*., 2004; Anderson *et al*., 2008,), ionophores, plant extracts (Beauchemin *et al*., 2008, Martin *et al*., 2010), halogenated methane analogues, probiotics etc.(Kumar *et al*., 2009; Patra *et al*., 2011). However, to date, very few of these strategies have been adopted on –farm due to their effect on animal performance and its improvement.

Currently there is an increasing interest in use of prebiotics and probiotics as natural feed additives improve livestock production as alternatives to the antibiotics due to concerns about incidences of resistant bacteria and environmental pollution by the excreted active-antibacterial substances (Mwenya *et al*., 2006). Particular interest has been in bacteriocins which are produced by lactic acid bacteria. Bacteriocins are antimicrobial proteinaceous polymeric substances that are ubiquitous in nature and produced by a variety of Gram-negative and Gram-positive bacteria. They are typically narrow spectrum antibacterial substances under the control of plasmid and play a role in competition among microbial species for niches within the rumen system.

The most well-known and understood bacteriocin is nisin, which is effective not only against food borne bacteria but also against rumen staphylococci and enterococci (Laukova 1995). Nisin obtained from *Lactobaccilus lactis* ssp. *lactis*, has also been shown to decrease methane) production *in vitro*. Although the mechanism is still unclear, nisin has been shown to reduce rumen methanogenesis by 36% (Callaway *et al*., 1997). A combination of nisin & nitrate, an alternative electron receptor, has been reported to reduce methane emissions in sheep (Sar *et al*., 2005).

Bovicin HC5, another bacteriocin produced by *Streptococcus bovis* from the rumen, has been reported to suppress methane production by 50% (Lee *et al*., 2002). *In vitro* studies of the class I lantibiotic bovicin HC5, produced by *S.bovis* HC5 revealed that it may be equally as useful as monensin in limiting methane production and amino acid degradation in the rumen (Lee *et al*., 2002: Lima *et al*., 2009). The studies on the effects of bacteriocins *in vivo* are limited and only few specific bacteriocins have been examined, and found effective in rumen environment. First is the enterocin CCM 4231, produced by the rumen isolate *Enterococcus faecium* CCM 4231 (Laukova and Marekova 1998).

McAllister and Newbold (2008) reported that bacteriocins could prove effective in directly inhibiting methanogens and redirecting H_2 to other reductive bacteria, such as propionate producers or acetogens. Since many lactic acid bacteria produce bacteriocins, reduced methane production observed at very low pH (Russel, 1998) may be due to bacteriocins effects on methanogens may not be through direct pH effect. The potential for bacteriocins

produced in rumen to suppress methanogenesis is unknown, but their potential as a new generation of rumen modifiers is already being exploited (Teather and Forster 1998). Altogether, the use of bacteriocins may be prospective for inhibiting methanogen populations in the rumen system.

The objectives of this experiment were to determine the effect of different bacteriocins (pediocin and enterocin) as well as their combination on methane, *in vitro* gas production and *in vitro* dry matter digestibility using wheat straw based medium fibre diet.

Materials and Methods

Bacteriocin Preparation

The bacteriocins (pediocin and enterocin) by the *Pediococcus pentosaceus* 34 strain and *Enterococcus faecium*99 were produced optimally in MRS broth by inoculating 1 liter of the medium by the active culture and incubating at 37^{o}C for 18-24 h. The cell free supernatant (CFS) of the culture growth was prepared by centrifugation at 10,000 *g* for 10 min and was heat treated at 95^{o}C for 5 min. The antibacterial spectrum of bacteriocin (pediocin and enterocin) was checked against different organisms and bacteriocin activity was determined against *Pediococcus acidilactici* LB 42 (a sensitive strain used for detection of bacteriocin producers) according to Gupta *et al*., 2010

Determination of Activity unit of Bacteriocins

The activity of bacteriocin was determined by agar spot assay. The bacteriocin produced by *Pediococcus pentosaceus* 34 strain and *Enterococcus faecium* 99 showed bacteriocin activity of 60,000 AU/mL, respectively. The bacteriocins used for the *in vitro* gaseous quantification trials are shown in Table 1.

Preparation of Diet and Experimental Design

To evaluate the effect of bacteriocins, diets were prepared by taking 50:50 roughage and concentrate ratio. The physical and chemical compositions of diet are shown in Table 2. *In vitro* Hohenheim gas test apparatus (Menke and Steingass, 1988) was used according to Kumar *et al* (2013). Six sets of syringes and control (no bacteriocin) were prepared using different bacteriocins as well as their combinations (Table 1). All the treatments were arranged in three replicates. The syringes were kept in an incubator at 39^{o}C until incubation. Additional set in triplicate was also incubated, devoid of substrate and treatment which served as blanks for particular treatment and values were corrected for different parameters with these blanks.

Inoculums preparation and *in vitro* gas production and methane emission

Rumen liquor from fistulated non-lactating Murrah buffaloes (≈12 months of age, fed on standard diet of concentrate: roughage ratio; 40: 60) was collected before morning feed by squeezing the collected feed mass into pre-warmed (39±0.5ºC) thermos flasks and strained through 100 mm nylon net before being used as inoculums.

For estimating the total gas and methane production, 30 mL of the buffered medium (Menke and Steingass,1988) containing rumen microbes was dispensed into

syringes and incubated at 39 ºC for 24 h. The bacteriocins were injected about 1h before incubation.

The syringes were shaken every one hour from the start of the incubation up to 10 hour of incubation. After 24 h incubation, total gas production was calculated by subtracting gas produced in blank syringe (containing no substrate, but only the inoculums and buffer) from total gas produced in the syringe containing substrate, inoculum and buffer.

After 24 hour of incubation, volume of gas was withdrawn from the tip of the incubation syringe using Hamilton gas tight syringe and analyzed for methane with the help of gas chromatograph (Nucon 5700, India) according to Kumar *et al*., (2012a).

In vitro dry matter digestibilty (IVDMD) and *in vitro* organic matter digestibility (IVOMD)

The true DM digestibility of each syringe containing residues after incubation was estimated as per (Van Soest *et al*., 1991; Kumar *et al.*, 2012b). The DM degradability was calculated as weight of DM incubated minus weight of DM residue.

IVDMD (%) = (Weight of dried sample- Weight of residue/ Weight of Dried sample) x 100

The true organic matter digestibility was estimated as per (Van Soest *et al*., 1991) was calculated by estimating the ash content in the residual NDF as well in the original sample.

IVOMD (%) = (OM taken for incubation – residual OM / (OM taken for incubation) x 100

Partitioning factor and microbial biomass yield

The partitioning factor (PF) was calculated as the ratio of substrate (truly degraded dry matter) *in vitro* (mg) to the volume of gas (mL) produced by it. The Microbial Biomass (MBM) yield was calculated by using the degradability of substrate and gas volume and stoichiometrical factor (Blummel *et al*., 1997).

Microbial mass (mg) = Substrate truly degraded - (Gas volume × Stoichiometrical factor).

Where, the stoichiometrical factor used was 2.25.

Proximate analysis and Cell wall constituents

The proximate analysis (organic matter, crude protein, ether extract and total ash) of substrate was carried out as per the methods of AOAC (1995). The Neutral detergent fiber of substrates was determined according to the method of Van Soest *et al*., (1991) and other cell wall components such as acid detergent fiber (ADF) and hemicellulose (HC) as per the method of Robertson and Van Soest (1981).

Statistical Analysis

All experiments used a completely randomized design. The data were analyzed statistically using one way analysis of variance to compare the means as per the procedure of statistical analysis system (SAS/SPSS 2012 version 21.0 for windows). Significant differences ($p<0.05$) among treatment mean values were determined by the Duncan's multiple range test according to the principles of Steel and Torrie (1980).

Results and Discussion

Effect of pediocin and enterocin on IVGPT

Increased gas production in relation to control, was observed for wheat straw based diet inoculated with pediocin, enterocin and combinations of both after 24 h of incubation and results are shown in table 3.Increase in gas production was significant in comparison to control, but the differences were not significant between the treatments. This is due to the increase in amount of gas (carbon dioxide and methane) released when feeds are incubated in vitro with rumen liquor, its digestibility and to the energetic feed value of diets for ruminants. (Tilley and Terry 1963) These effects could also occur due to independent effects of bacteriocin (pediocin and enterocin) in vitro culture conditions and an increase in gas production are likely to occur as has been observed by Asa *et al*., (2010).

Effect of bacteriocin on methane production

The methane content was observed to decline with pediocin, enterocin and combinations of both after 24 h incubation. Pediocin P1 and P2 resulted in low level of methane (4.81% and 5.08%) and the results were significant ($P<0.05$) in comparison to control and combinations of bacteriocin.

The P3 and E also significantly reduced methane levels (by 6.08% and 6.18%) in comparison to control (9.47%). However, with different combinations of bacteriocin, EP2 showed methane reduction (7.10%) which was higher than EP1 (8.25%). EP2showed significant difference as compared to control. But the results were not significant between EP1 and EP2 (Table 3).

This can be due to broad inhibitory spectrum of pediocin in comparison to enterocin and stable activity units. The pediocin and enterocin used in this study were previously screened for their antimicrobial activity and found to exhibit inhibitory spectra against different organism (Gupta *et al*., 2010). That's why pediocin with highest activity showed the low methane production in comparison to enterocin with same activity level. Similarly EP2in combination, reduced methane level production as compared to EP1 due to higher concentration of pediocin which indicated pediocin reduce more methane percentage in comparison to enterocin with same activity levels. These finding are in agreement with Asa *et al.*, (2010), who reported the effect of PRA-1 produced by *Lactobacillus plantarum* TUA1490L on rumen methanogenesis.

Some bacteriocins produced by lactic acid bacteria have been identified as an alternative group of antimicrobials for manipulation of the rumen microbial ecosystem and characterized biochemically and genetically (Kalmokoff *et al*., 1996; Ennahar *et al*., 1999; Chen and Hoocver, 2003). Lee *et al*. (2002) and Sang *et al*. (2002) reported that bovicin HC5, produced from *Streptococcus bovis* HC5 decreased the methanogens*in vitro* by 50%. Mantovani and Russell (2002) suggested that this bacteriocin inhibited a variety of Gram- positive bacteria and the spectrum of activity was similar to monensin.

Table.1 Bacteriocins produced by *Pediococcus pentosaceus* 34 (pediocin) and *Enterococcus faecium* 99 (enterocin) with different activity unit dilution

Bacteriocin	**Activity unit (AU/2mL)**
Pediocin (P_1)	1, 20,000
Pediocin (P_2)	90,000
Pediocin (P_3)	60,000
Enterocin (E_1)	1, 20,000
Enterocin (0.75%) + Pediocin (0.25%) EP1	90,000
Enterocin (0.50%) + Pediocin (0.50%) EP2	90,000

Table.2 Physical and Chemical composition of diet used as substrate in *in vitro* incubations**.**

Ingredient of diets		**g/Kg on DM Basis**	
Diet	**Wheat straw**	**Concentrate**	
Medium fibre diet (50R: 50C)	500	500	
Ingredients of concentrate (on DM basis)	**g/Kg**	**Constituents of diet [g/kg]**	
Maize	330	Crude protein	178.1
Ground nut cake	210	Ether extract	22.6
Mustard cake	120	Total Ash	100
Wheat bran	200	Organic matter	900
Deoiled rice bran	110	Neutral detergent fiber	422.6
Mineral mixture	20	Acid detergent fiber	290.1
Salt	10	Hemicelluloses	132.5

Table.3 Effect of different Bacteriocin (Pediocin and Enterocin) concentration on methane production and IVGPT

Treatment	IVGPT(mL/200mg)DM	Methane %
Control	30.00^{b}±0.00	9.47^{a}±1.32
P1	40.00^{a}±2.00	4.81^{d}±0.20
P2	41.00^{a}±1.53	5.08^{d}±0.38
P3	44.33^{a}±2.19	6.18^{cd}±0.05
E1	43.33^{a}±1.76	6.08^{cd}±0.37
EP1	44.33^{a}±0.88	8.25^{ab}±0.35
EP2	41.00^{a}±1.00	7.10^{bc}±0.12

Pediocin: P_1; Pediocin: P_2; Pediocin: P_3; Enterocin: E_1; Enterocin (0.75%) + Pediocin (0.25%): EP1; Enterocin (0.50%) + Pediocin (0.50%): EP2.

Table.4 Effect of different bacteriocin concentration on rumen fermentation parameters

Treatment	IVDMD	IVOMD	PF	MBM	SCFA
Control	67.83^{a}±3.90	68.33^{a} ±3.03	3.02^{a}±0.09	59.40^{a}±4.02	0.24^{b}±0.00
P1	75.17^{a} ±2.67	77.83^{a} ±2.83	2.45^{a}±0.41	59.99^{a}±9.32	0.46^{a}±0.04
P2	75.75^{a} ±0.43	74.50^{a} ±2.57	2.72^{a}±0.16	51.82^{a}±8.00	0.48^{a}±0.03
P3	74.00^{a}±1.00	75.17^{a} ±0.83	2.84^{a}±0.24	59.67^{a}±12.61	0.55^{a}±0.04
E1	71.67^{a}±2.89	73.17^{a} ±2.59	2.55^{a}±0.04	43.65^{a}±2.50	0.53^{a}±0.39
EP1	70.00^{a} ±4.51	72.83^{a} ±5.18	2.49^{a}±0.23	40.67^{a}±13.1	0.55^{a}±0.01
EP2	71.17^{a} ±2.67	69.16^{a} ±1.64	2.50^{a}±0.09	40.13^{a}±4.70	0.48^{a}±0.02

For details see table 3; IVDMD: In vitro dry matter digestibility; IVOMD: In vitro organic matter digestibility; PF: Partition factor; MBP: Microbial biomass; SCFA: Small chain fatty acids

Nisin is widely utilized in the food industry for its highly effective and very strong antibacterial activity against many Gram-positive bacteria (Yuan *et al*., 2004); it is nearly as potent a methane inhibitor as monensin and it was just as effective in decreasing the acetate to propionate ratio (Callaway, 1997) and has been reported that the 36% methanogenesis was reduced by the use of bacteriocin nisin. A combination of nisin & nitrate, an alternative electron receptor, has also been reported to reduce methaneemissions in sheep (Sar *et al*., 2005). Furthermore, Alazzeh *et. al*., (2012) reported the use of some strains of propionibacteria have the potential to lower methane production from mixed rumen cultures and this reduction is not always associated with an increase in propionate production.

Effect of bacteriocin on rumen fermentation parameters

The IVDMD (*in vitro* dry matter digestibility) and IVOMD (*in vitro* organic matter digestibility) was correlated most highly with *in vivo* digestibility (Marten and Branes 1979); many factors may influence IVDMD and IVOMD, including the source and activity of inoculums. The IVDMD and IVOMD increased with increasing level of activity unit with pediocin, enterocin and different combinations of both in comparison to control but the differences were not significant (Table 4).

The results of partitioning factor (PF) and microbial biomass (MBM) were non–significantly affected due to the addition of pediocin, enterocin and combination of both. SCFA of all the treatments were significantly higher in comparison to control (Table 4. It can be due to the increase in gas production by bacteriocin treatments. As, there is an inverse relationship between gas volume or SCFA and microbial mass production particularly, when both were expressed per unit of substrate truly degraded given by Blummel *et al.,* (1997).

The present study concludes that inoculating bacteriocin (pediocin and enterocin) into *in vitro* rumen incubations noticeably reduced methane production. However, the degree of methane mitigation depends on the type and activity of bacteriocins used. Pediocin (P1) with activity unit level of 1,20,000 AU/2mL with increased gas production showed higher decrease in methane emission. Therefore, pediocin (P1) has a potential for mitigation of enteric methane production.

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