EVALUATION OF LACTICIN ADDITION ON GAS PRODUCTION KINETICS AND RUMEN METHANOGENESIS *in Vitro*

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ABSTRAK

Tujuan penelitian ini adalah untuk mengamati efek sebuah bakteriosin yang relatif baru yakni laktisin 3147 terhadap kinetika produksi gas dan emisi metana dalam lingkungan rumen secara in vitro. Penelitian terdiri dari dua buah eksperimen. Eksperimen 1 menggunakan rancangan acak lengkap (tiga ulangan) untuk mengamati efek penambahan laktisin (0 dan 10 μM) pada substrat berupa rumput kering dan campuran rumput:konsentrat (1:1) terhadap kinetika produksi gas dan emisi metana. Pada eksperimen 2, level penambahan laktisin ditingkatkan menjadi 0, 10, 25 dan 50 μM. Substrat, laktisin dan campuran cairan rumen-buffer diinkubasi pada suhu 39ºC selama 24 jam. Produksi total gas dan emisi metana diamati selama dan setelah periode inkubasi. Hasil menunjukkan bahwa secara umum penambahan laktisin tidak berpengaruh secara nyata terhadap peubah produksi gas dibandingkan dengan kontrol, baik ketika ditambahkan pada substrat berupa rumput maupun rumput:konsentrat. Penambahan laktisin hingga konsentrasi 50 μM masih belum dapat menurunkan emisi metana, meskipun pada penambahan 25 μM terdapat kecenderungan menurunkan emisi metana. Substrat berupa rumput menghasilkan metana yang lebih rendah secara signifikan dibandingkan dengan rumput:konsentrat baik pada eksperimen 1 maupun 2 (P<0,05). Dapat disimpulkan bahwa penambahan laktisin hingga 50 μM masih belum dapat menurunkan emisi metana sehingga perlu diuji lebih lanjut pada konsentrasi yang lebih tinggi.

Kata kunci: laktisin, bakteriosin, metana, rumen, in vitro

ABSTRACT

The present study was aimed to investigate the effect of a novel bacteriocin, i.e. lacticin 3147, on gas production kinetics and methane emission under *in vitro* rumen environment in two consecutive experiments. In experiment 1, either no or 10 μM of lacticin was added to hay or hay:concentrate (1:1, w/w) substrate. In experiment 2, the levels of lacticin additions were extended to 0, 10, 25 and 50 μM. Samples were incubated in three replicates in both experiments at 39ºC for 24 h. Total gas production and methane emission were measured during and after the incubation, respectively. Results revealed that, in general, addition of lacticin had limited significant effects on gas production parameters compared to control (without lacticin addition). Lacticin addition up to 50 μM did not significantly decrease CH_4 emission, although a tendency of methane reduction existed when lacticin was added at 25 μM. Hay diet produced significantly less methane emission than that of hay:concentrate diet both in experiment 1 and experiment 2 (P<0.05). It can be concluded that lacticin addition up to 50 μM was unable to decrease CH_4 emission *in vitro* and therefore need to be tested further at higher concentrations.

Keywords: lacticin, bacteriocin, methane, rumen, in vitro

INTRODUCTION

Concern on global warming problem has received a lot of attention during the past decades. A steady increase of earth surface temperature is considered to be due to the accumulation of some major green-house gases (GHG) such as carbon dioxide (CO_2), methane (CH_4), nitrogen oxide

(N_2O) and chloro fluoro carbon (CFC) through anthropogenic activities. Ruminant production system is a source of CH_4 emission that contributes significantly by approximately 80 million tonnes of annual CH_4 (Beauchemin *et al*., 2008). The gas itself is produced in the digestive tract of ruminants, particularly in the rumen, by methanogenic archaea via utilization of CO_2 and H_2 as their main substrates to form CH_4 (Morgavi *et al*., 2010). Although such reaction is a way for eliminating the toxicity of H_2 when being accumulated (McAllister and Newbold, 2008), the process can be optimized without confronting a normal rumen function. Further, in addition to its contribution to global warming, CH_4 emission is a form of energy losses from ruminants which may account up to 14% of their digestible energy intake (Cottle *et al*., 2011).

A number of nutritional strategies have been attempted to mitigate ruminal CH_4 emission. Bacteriocin, a proteinaceous substance produced by certain bacterial species to inhibit the growth of other species, is considered as a promising option in term of mitigating CH_4. Nisin, a bacteriocin produced by certain strains of *Lactococcus lactis*, and bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5, have been tested for their effects in inhibiting ruminal methanogenesis; both substances were demonstrated to mitigate ruminal CH_4 emission under *in vitro* incubation system and the magnitudes of the effects were dose-dependent (Callaway *et al*., 1997; Lee *et al*., 2002). Further investigation on other bacteriocins that are effective in mitigating ruminal CH_4 emissions has to be continued in order to obtain the most promising substance with regard to the objective while maintaining an optimum condition of general rumen fermentation.

Lacticin 3147 is a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* DPC3147, a strain isolated from an Irish kefir grain that has a broad spectrum of inhibition and heat stable especially at low pH (Ryan *et al*., 1996). Due to its anti-microbial properties particularly by inhibiting a wide range of Gram-positive bacteria, lacticin has been used in a number of food and biomedical applications such as in cheese making, for the treatment and/or prevention of mastitis in cattle, and for treating some antibiotic resistant human pathogens (Twomey *et al*., 2002). Despite such beneficial effects of lacticin, the substance has not been assessed for its influence on rumen fermentation yet. Based on the anti-methanogenic effect of other bacteriocins, i.e. nisin and bovicin, therefore, lacticin may possess such property as well. The present study was therefore aimed to investigate the effect of lacticin addition on gas production kinetics and methane emission under *in vitro* rumen environment. The addition was done into two different substrates, i.e. hay or hay:concentrate (1:1, w/w) and at various levels of application.

MATERIALS AND METHODS

Experimental Design

Lacticin 3147 was obtained from Dairy Products Research Centre, Teagasc, Moorepark, Republic of Ireland. Evaluation of the respective bacteriocin as an anti-methanogenic agent was performed in two consecutive experiments. In Experiment 1, a factorial completely randomized design was applied to examine the effects of different levels of lacticin (0 and 10 µM) added to hay or hay:concentrate (1:1, w/w) substrates on gas production kinetics and CH_4 emission. Experiment 2 was conducted as a continuation of Experiment 1 in order to confirm the results obtained and to increase the levels of lacticin used. The experimental set up of Experiment 2 was almost similar to Experiment 1, except that the levels of lacticin were extended to 0, 10, 25 and 50 µM. In both experiments, samples were incubated in three replicates.

In Vitro Gas Production

Amounts of 380 mg hay or hay:concentrate substrates (containing approximately 93% dry matter) as basal diets were weighed and transferred into 100 ml calibrated glass syringes (Haeberle Labortechnik GmbH & Co. KG, Lonsee, Germany). The substrates were then added with 30 ml of incubation medium (consisted of 10 ml rumen liquor and 20 ml buffer) by following the method of Menke and Steingass (1988). The rumen fluid and particulate matter were collected before the morning feeding from two rumen fistulated cows fed on roughage and concentrate based diets, mixed, homogenized, strained and filtered through 100 µm nylon net. The glassware used was kept at approximately 39^oC and flushed with CO_2 before use. Lacticin was prepared by solubilizing it in sodium phosphate buffer at pH 6.8. Subsequently, the substance was injected (≤1 ml) into 100 ml calibrated syringe from the syringe nozzle before dispensing buffered rumen liquor. The 30 ml

buffered medium containing rumen microbes was thereafter dispensed into the syringes, and the incubation was carried out at 39 ^{o}C for 24 h.

Chemical Analysis

Hay and concentrate used as substrates in the present experiments were subjected to proximate (AOAC, 1990) and Van Soest analyses (Van Soest *et al*., 1991). Dry matter (DM) was determined by drying the samples at 105^{o}C for 16 h. Crude protein (CP) was analysed using Kjeldahl method by considering CP = N × 6.25, whereas ether extract (EE) was analysed based on Soxhlet extraction system using petroleum ether as the solvent. For the cell wall fractions, neutral detergent fiber (NDF) and acid detergent fiber (ADF) values are expressed inclusive of residual ash and without addition of amylase. Non-fiber carbohydrate was obtained by subtracting CP, EE and NDF from organic matter. Chemical composition of the substrates is presented in Table 1.

Table 1. Chemical Composition of Hay (H), Concentrate (C) and Hay:Concentrate (HC; 1:1, w/w) Used as Substrates in the *in vitro* Incubation (in % Dry Matter)

Component	H	C	HC
	….....%.....		
Dry matter	93.6	93.0	93.3
Organic matter	89.3	95.6	92.5
Crude ash	10.7	4.4	7.5
Crude protein	11.2	19.3	15.3
Ether extract	2.0	1.6	1.8
Neutral detergent fiber	52.8	17.3	35.1
Acid detergent fiber	32.6	5.8	19.2
Non-fiber carbohydrate*	23.3	57.4	40.3

* Defined as: organic matter – (crude protein + ether extract + neutral detergent fiber)

Gas Reading and Methane Determination

During 24 h incubation period, the total gas was recorded at various time point interval from the calibrated scale on the syringe, i.e. at 0, 4, 8 and 24 h. At the end of incubation, methane production was measured using an infrared (0–30% range) methane analyser (Pronova Analysentechnik GmbH & Co. KG, Berlin, Germany) calibrated against 10.6% methane (Jayanegara *et al*., 2009a). Technically, after measuring the total gas volume, the tubing of the syringe outlet was inserted into the inlet of the methane analyser. The display on the methane analyser gives methane as percent of the total gas and this value was used for calculation of methane in total gas volume.

Data Analysis

Kinetics of gas production during incubation period was fitted into an exponential equation proposed by Orskov and McDonald (1979) as follow:

$$p = a + b\ (1 - e^{-ct})$$

Dependent variable p is the cumulative gas production (ml) at t hour incubation, whereas a, b and c are the constants of the equation. The constants are interpreted as gas production from soluble feed fraction (a), gas production from insoluble but fermentable fraction (b), and rate of gas production (c). Thus a+b is the theoretical maximum gas production during incubation at infinite t hour (the asymptotic value).

Data obtained were analysed using factorial analysis of variance (ANOVA). The factors were substrate (hay or hay:concentrate, 1:1 w/w) and level of lacticin addition. Allocation of factors or treatments into incubation units were based on a completely randomized design. When at least a factor showed significantly different at $P<0.05$, the analysis was continued using Tukey's test for a more specific treatment comparison. All statistical analyses were performed using STATISTICA software version 6.0.

RESULTS AND DISCUSSION

Gas Production Kinetics

In general, addition of lacticin had limited significant effects on gas production parameters compared to control (without lacticin addition). This was true for hay and hay:concentrate substrates, and was consistently observed both in Experiment 1 (Table 2) and Experiment 2 (Table 3). However, there were few exceptions: addition of 10 µM lacticin to hay substrate decreased gas production at 4 and 8 h ($P<0.05$; Experiment 1), and addition of 50 µM lacticin to hay:concentrate decreased gas production at 24 h ($P<0.05$; Experiment 2). The respective results suggest that

lacticin addition up to 50 µM does not influence fermentation and digestion of carbohydrate (both fiber and starch). Since lacticin is basically a polypeptide, hence, addition of lacticin is theoretically increases protein content in the *in vitro* system. Apart from the fact that gas production from protein fermentation is relatively small as compared to that of carbohydrate (Getachew *et al*., 1998), addition a proteinacous substance up to 50 µM is considered to be too small to influence the change of total gas production.

The main source of variation that led to differences in gas production parameters was the different incubated substrates, i.e. between hay and hay:concentrate. It had to be noted that the differences did not appear during early fermentation period (<8 h), but the differences were significants at 24 h (P<0.05); higher gas production in the incubation of hay:concentrate compared to hay was observed both in experiment 1 and experiment 2 at 24 h fermentation period (P<0.05). For the gas production kinetics parameters, a+b was higher in the incubation of hay:concentrate compared to that of hay in experiment 1 and 2 (P<0.05). Interaction between substrate and lacticin addition was found to be significantly different in experiment 2; lower a+b was observed at higher levels of lacticin addition (P<0.05). Conversely to the previous parameter, c was significantly higher in the incubation of hay compared to hay:concentrate both in experiment 1 and experiment 2 (P<0.05). No interaction between substrate and lacticin addition was observed for the rate of gas production parameter.

Higher gas production in the incubation of hay:concentrate substrate compared to hay was due to higher proportion of non-fiber carbohydrate and lower proportion of fiber fraction (both NDF and ADF) in hay:concentrate. It has been well-established that fiber, i.e. structural components of plant such as NDF, ADF, cellulose and lignin, is negatively influenced the feed quality and digestibility (Jayanegara *et al*., 2009b). On the contrary, non-structural carbohydrate like starch, which is presumably high in concentrate, is considered to be positively correlated with feed quality. It is therefore not surprising that incubation of hay:concentrate substrate produced higher gas production over the hay only substrate. In agreement with the results, Jayanegara *et al*. (2009c) observed that substrates with higher ADF contents produced less total gas production and lower organic matter digestibility as shown by the correlation coefficients between the respective parameters. Further, addition of concentrate may stimulate rumen microbial activity and population and, in turn, contributes to the overall fermentation and digestion process.

Table 2. Influence of Addition of Lacticin 3147 to Different Substrates on Gas Production Kinetics (Experiment 1)

Substrate (S)	Lacticin (L; µM)	Gas Production (mL)			a+b (mL)	c (mL/h)
		4 h	8 h	24 h		
H	0	23.5^{b}	42.0^{b}	76.6^{a}	91.7^{a}	0.076^{b}
	10	21.7^{a}	39.8^{a}	74.0^{a}	90.5^{a}	0.071^{b}
HC	0	22.8^{b}	42.2^{b}	89.1^{b}	128.2^{b}	0.050^{a}
	10	22.7^{b}	41.3^{b}	85.6^{b}	118.3^{b}	0.054^{a}
SEM		0.216	0.310	1.985	5.314	0.004
P-value						
S		0.049	ns	<0.001	<0.001	<0.001
L		0.001	0.002	ns	ns	ns
S*L		0.004	ns	ns	ns	ns

H, hay; HC, hay:concentrate (1:1, w/w); SEM, standard error of the mean
Superscripts with different letters are significantly different at P<0.05

Table 3. Influence of Addition of Lacticin 3147 to Different Substrates on Gas Production Kinetics (Experiment 2)

Substrate (S)	Lacticin (L; µM)	Gas production (mL)			a+b (mL)	c (mL/h)
		4 h	8 h	24 h		
H	0	25.3	45.0	77.2^{ab}	88.0^{a}	0.088^{bc}
	10	26.3	46.0	77.3^{ab}	86.7^{a}	0.093^{bc}
	25	26.0	45.8	75.0^{a}	83.1^{a}	0.098^{c}
	50	24.8	44.3	77.8^{b}	90.2^{a}	0.083^{b}
HC	0	25.0	45.3	90.8^{d}	119.9^{c}	0.059^{a}
	10	25.5	45.8	90.0^{cd}	115.7^{bc}	0.063^{a}
	25	26.3	46.8	91.8^{d}	117.5^{bc}	0.064^{a}
	50	25.5	45.5	87.5^{c}	109.7^{b}	0.067^{a}
SEM		0.148	0.181	1.646	3.63	0.004
P-value						
S		ns	ns	<0.001	<0.001	<0.001
L		ns	ns	ns	ns	ns
S*L		ns	ns	ns	0.013	ns

H, hay; HC, hay:concentrate (1:1, w/w); SEM, standard error of the mean
Superscripts with different letters are significantly different at P<0.05

Ruminal Methane Emission

Lacticin up to 50 µM did not significantly influence CH_4 emission when added to hay or hay:concentrate substrate. This was consistently observed in experiment 1 (10 µM lacticin; Figure 1) and experiment 2 (10-50 µM lacticin; Figure 2). However, a tendency of methane reduction existed when lacticin was added at 25 µM (experiment 2) especially in the hay substrate. It appears that lacticin up to 50 µM is still ineffective to be used for modifying rumen fermentation, including for mitigating CH_4 emission. As for other bacteriocins like nisin and bovicin in which their effects are dose-dependent (Callaway *et al*., 1997; Lee *et al*., 2002), it seems that lacticin at such concentration is still below a threshold level to significantly decrease CH_4 emission. This certainly opens the opportunity for further subsequent studies to test lacticin at higher levels of addition. Apart from that, Kalmokoff *et al.* (1996) stated that the effectifity of a certain bacteriocin in the rumen depends on its stability against ruminal proteolytic. Therefore it is probable that lacticin may loss part of its activity due to proteolytic mechanism occurring intensively in rumen environment.

As observed for gas production parameter, variation of CH_4 emission due to substrate different was also existed. In this study, hay:concentrate diet produced higher CH_4 than that of hay as consistently observed both in experiment 1 and experiment 2 (P<0.05). Such a result apparently contrasts to a commonly known concept that increasing proportion of concentrate instead of forage in diet would reduce methane emission from ruminants (Beauchemin *et al*., 2008). The concept is based on a theory that feeding of concentrate favors propionate production in the rumen over the acetate so that more hydrogen is being utilized rather than being produced. Furthermore, the concentrate creates unconducive rumen environment for the archaea methanogens by increasing rate of passage, lowering pH and eliminating some population of protozoa where part of the methanogens are symbiotically living together with the protozoa (Moss *et al*., 2000).

It has to be noted, however, that methane decrease by increasing level of concentrate in the diet is usually expressed in a relative unit, such as relative to gross energy intake or digestible energy intake or relative to unit of animal product (such

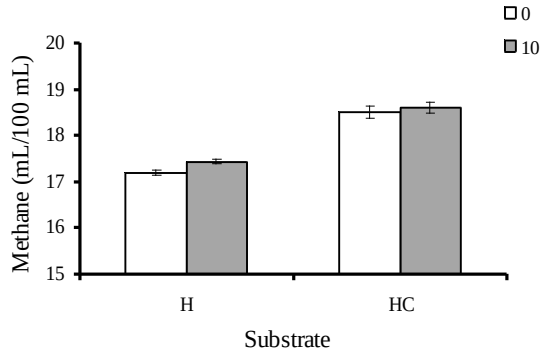

Figure 1. Ruminal Methane Emission on the Addition of Lacticin 3147 (0 and 10 µM) to Different Substrates (Experiment 1). H, hay; HC, hay:concentrate (1:1, w/w). P-value: substrate, <0.001; lacticin, ns; substrate*lacticin, n

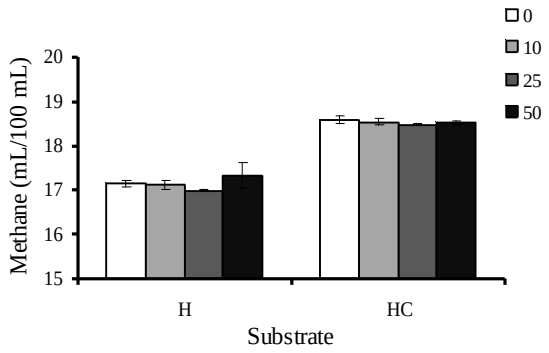

Figure 2. Ruminal Methane Emission on the Addition of Lacticin 3147 (0, 10, 25 and 50 µM) to Different Substrates (Experiment 2). H, hay; HC, hay:concentrate (1:1, w/w). P-value: substrate, <0.001; lacticin, ns; substrate*lacticin, ns

as per unit of milk produced or body weight). When methane emission is expressed in an absolute unit, such l/day or mol/day, interestingly, the emission is higher at higher level of concentrate in the diet. For instance, in an *in vitro* study, Garcia-Martinez *et al*. (2005) showed that methane productions of high, medium and low forage diets were 701, 754 and 812 µmol, which indicated that higher proportion of concentrate led to a higher absolute methane emissions. In agreement with the study, Lovett *et al*. (2003) found that absolute methane emissions (in L/day) were higher when finishing beef heifers were fed with a diet containing 60% concentrate as compared to those of fed with a diet containing 35% concentrate. But, methane emission per unit of animal product (per kg of live weight gain and carcass gain; in a relative unit) was reduced by lower forage to concentrate ratio. A plausible explanation to such contrasting results is that, although there is a shift towards more propionate by increasing proportion of concentrate in the diet, the concentrate is clearly more digestible than that of forage. As a consequence, on the whole, hydrogen is produced more as well as the methane emission.

CONCLUSION

Lacticin addition up to 50 µM was unable to significantly decrease CH_4 emission *in vitro*. It seems that lacticin at such concentration is still below a threshold level to create such an effect. Despite the fact, a tendency of methane reduction existed when lacticin was added at 25 µM. Further studies are therefore required to test lacticin at higher concentrations in order to decrease methanogenesis at simultaneously improving rumen fermentation activity.

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