Acta Veterinaria-Beograd 2023, 73 (1), 71-86
UDK: 636.2.09:616-008.9:547.211
DOI: 10.2478/acve-2023-0006

Research article

METHANE EMISSION AND METABOLIC STATUS IN PEAK LACTATING DAIRY COWS AND THEIR ASSESSMENT VIA METHANE CONCENTRATION PROFILE

Dušan BOŠNJAKOVIĆ[1], Danijela KIROVSKI[1], Radiša PRODANOVIĆ[2], Ivan VUJANAC[2], Sveta ARSIĆ[2], Milica STOJKOVIĆ[1], Slavica DRAŽIĆ[1], Sreten NEDIĆ[2]*, Ljubomir JOVANOVIĆ[1]

[1]University of Belgrade, Faculty of Veterinary Medicine, Department of Physiology and Biochemistry, Bul. Oslobodjenja 18, Belgrade, Serbia; [2]University of Belgrade, Faculty of Veterinary Medicine, Department of Ruminants and Swine Diseases, Bul. Oslobodjenja 18, Belgrade, Serbia

(Received 22 October, Accepted 21 December 2022)

Ruminant husbandry contributes to global methane (CH_4) emissions and beside its negative impact on the environment, enteric CH_4 emissions cause a loss of gross energy intake in cows. The study is aimed to estimate CH_4 emission and metabolic status in dairy cows via the methane concentration profile as a tool for analyzing the CH_4 production pattern. The study included eighteen cows whose enteric CH_4 emission was measured during three consecutive days in three periods: 2 hours before (P1), 2–4 hours (P2) and 6–8 hours (P3) after the morning feeding. Based on CH_4 enteric emissions, cows were divided into two groups (n=6, respectively): HM (average CH_4 concentration: 5430.08 ± 365.92 ppm) and LM (average CH_4 concentration: 1351.85 ± 205.20 ppm). Following CH_4 measurement, on day 3, venous blood was sampled to determine the indicators of the metabolic status. HM cows had significantly higher average CH_4 concentrations, maximum and average CH_4 peak amplitude than LM cows in all measuring periods (P1-P3), while the number of CH_4 peaks tended to be higher in HM than in LM cows in P2. There were no differences in the maximum and average CH_4 peak width and average distance among two CH_4 peaks between examined groups of cows. HM cows had significantly higher total protein concentrations and significantly lower total bilirubin and NEFA concentrations than LM cows. In conclusion, HM cows have a greater number of eructations and release more CH_4 per eructation than LM cows, hence the differences in metabolic status are most likely related to the differences in their liver function.

Keywords: dairy cows, methane emission, metabolic status

*Corresponding author: e-mail: sreten.nedic@vet.bg.ac.rs

INTRODUCTION

Methane (CH_4) is one of the most potent greenhouse gases (GHG), with a warming potential of 25 to 28 times greater compared to carbon dioxide (CO_2) [1]. Ruminant husbandry releases approximately 80 million tons of CH_4 into the Earth's atmosphere annually, representing around 28% of the total anthropogenic CH_4 emissions [2,3]. Enteric CH_4 is predominantly generated in the rumen (87-90%) and, to a lesser extent, in the large intestine (10-13%) by the metabolic activity of methanogenic microorganisms such as archaea [1,4]. The rumen represents an anaerobic environment made up of microorganisms that participate in the fermentation processes by degrading the feed organic matter such as plant structural carbohydrates, proteins and other organic polymers to volatile fatty acids (VFAs) and gases, including CO_2 and molecular hydrogen (H_2) [5]. Released VFAs such as acetate, propionate, and butyrate are absorbed by the rumen and are utilized as an energy source for milk production in dairy cows. On the other hand, methanogenic archaea use H_2 to reduce CO_2 and thereby produce CH_4, as one of the final products of ruminal fermentation, in a process known as methanogenesis [6,7]. Enteric CH_4 produced in the rumen is almost completely eliminated by eructation (95-99%) providing a possibility that methanogenesis could also be seen as a loss of gross energy intake of 2% to 12% [8]. Considering the environmental effects of enteric CH_4 emissions, as well as the loss of gross energy intake, it is important to examine all the factors that influence methanogenesis. The large proportion of the variation in CH_4 emissions can be explained by diet composition and feed intake but there is additional variation among animal phenotypes. Although methanogenesis and energy metabolism are closely related, to the best of our knowledge no research on dairy cows has been performed to examine the differences in the blood metabolic parameters in cows with different levels of enteric CH_4 emissions. There is only one research conducted by Kim et al. [9] on Japanese Black steers which showed that these cattle have different metabolic statuses based on the enteric CH_4 emissions. Since lactating cows are exposed to metabolic challenges [10], it is reasonable to expect some differences in their metabolic status in relation to the enteric CH_4 emissions level.

Despite the numerous studies related to CH_4 emissions, the methodology of determining CH_4 emissions from individual animals is very diverse. Methods could be classified into long-term, intended for measuring CH_4 emissions during a 24-hours period, and short-term methods, based on measurements of enteric CH_4 emissions in exhaled or eructated gas in short (minute) intervals over a few consecutive days [3,11-15]. The respiration chamber as a long-term method of measuring CH_4 emissions is used as the „gold standard". However, this method is accompanied by disruption of the animal's natural behavior and needs great technical and personnel requirements, limiting its widespread use for practical and research purposes [12,13]. Consequently, numerous short-term methods for measuring enteric CH_4 emissions have been established, such as GreenFeed and methane hood system, sniffer method and laser

methane detection, which are applicable to a larger number of animals at lower costs and enable the accurate measurement of enteric CH_4 emissions in the natural environment of animals [11-18]. Bearing in mind that a large number of factors influence methane emission, the average CH_4 emission values are not sufficient to define the phenotype of cows according to CH_4 production. Therefore, it is necessary to examine additional parameters such as variables of CH_4 concentration profile, including the average CH_4 concentrations and CH_4 peak-related variables (number of CH_4 peaks, maximum CH_4 peak amplitude, average CH_4 peak amplitude, maximum CH_4 peak width, average CH_4 peak width and distance among two CH_4 peaks). These variables would clearly indicate how increased CH_4 production interacts with physiological processes such as gas elimination through eructation, but also CH_4 production interactions with ration composition and management.

Since some countries, like New Zealand, provide financial support for farmers who implement protocols for the mitigation of CH_4 emissions on dairy farms [19], the use of methods that provide enteric CH_4 concentration profiles may contribute to understand the cause of high CH_4 emissions from dairy cows and thus enable the development of protocols with CH_4 emissions mitigation as the outcome.

This study aimed to examine differences in CH_4 concentration profiles (examined through average CH_4 concentration, number of CH_4 peaks, maximum CH_4 peak amplitude, average CH_4 peak amplitude, maximum CH_4 peak width, average CH_4 peak width and distance among two CH_4 peaks) and metabolic status in peak lactating dairy cows regarding the level of enteric CH_4 emissions estimated by the short-term method using a gas analyser that operates on the principle of infrared spectroscopy.

MATERIAL AND METHODS

Ethics statement

The experiment was performed during June 2022 on a commercial dairy farm (Lepušnica, AlDahra Corporation) in the vicinity of Belgrade, Serbia (44°56'08.6" N, 20°28'44.5" E). The experimental protocol was evaluated and approved by the Veterinary Directorate, Ministry of Agriculture, Forestry and Water Economy of the Republic of Serbia (approval number 323-07-11720/2020-05/4) in accordance with the National Regulation on Animal Welfare.

Animals and experimental design

Eighteen clinically healthy, multiparous and peak lactating Holstein-Friesian dairy cows were involved in the study. At the beginning of the study the cows were averaging (mean ± standard error of the mean – SEM) 600 ± 13.35 kg body weight (BW), 2.72 ± 0.06 body condition score (BCS), 2.77 ± 0.26 parity, 49.33 ± 2.58 days in milk (DIM), 37.88 ± 2.96 L/day milk production and 21.67 ± 0.44 kg dry matter intake (DMI). The experimental cows were housed under equal husbandry conditions (tie-

stall barn) and fed with the same total mixed ration (TMR; Table 1) to meet or exceed National Research Council (NRC, 2001) requirements [20]. The daily amount of TMR was divided into three meals and offered to the animals three times a day at 7:00 a.m. (35%), 12:00 a.m. (20%) and 7:00 p.m. (45%), respectively. Water was freely available via automated water bowls. The experimental cows were milked individually three times a day at 6.00 a.m., 12.00 a.m. and 6.00 p.m. Cow health status was monitored on a daily basis and the cows did not show any symptoms of illness during the entire study.

Table 1. Composition and nutritional value of the TMRs for lactating cows.

Ingredients, kg DM/day	HM and LM groups
Corn silage	6.8
Alfalfa haylage	1.9
Brewers grain (wet)	1.9
Molasses	1.5
Cottonseed meal	2.3
Corn grain	5.1
Barley	0.3
Rye grain	0.2
Wheat grain	0.3
Sunflower meal (34%CP)	4.1
Sodium bicarbonate	0.1
Calcium carbonate	0.2
NaCl	0.1
Monocalcium phosphate	0.1
Vitamin/Mineral Mix	0.1
Nutritional value	
Dry matter (%)	50.13
Ash % of DM	6.04
Fat % of DM	4.90
Cellulose % of DM	20.47
Starch % of DM	12.84
Sugar % of DM	2.69
Protein CP % of DM	17.85
RDP (%)	11.84
RUP (%)	5.96
MP (g/kg)	110.75
NDF (%)	35.07
ADF (%)	20.32
Ca (%)	0.68
P (%)	0.48
Energy value – Metabolic energy (MJ/kg DM)	15.0
pH	4.63

RDP – rumen degradable protein; RUP – rumen undegradable protein; MP – metabolizable protein; NDF – neutral detergent fiber; ADF – acid detergent fiber.

Measurement of enteric CH_4 emissions

Enteric CH_4 emissions were measured for each cow in three periods of three consecutive days: 2 hours before the morning feeding (P1), 2 – 4 hours (P2) and 6 – 8 hours (P3) after the morning feeding resulting in a total of 9 measurements per cow. After the initial measurement of enteric CH_4 emissions, twelve of the initial eighteen Holstein-Friesian cows were selected and divided into two numerically equal groups (6 cows per group) in a way that was described by Kim et al. [9]. The first group included six Holstein-Friesian cows with a high enteric CH_4 emission (HM group; average CH_4 concentration: 5430.08 ± 365.92 ppm), and the second group included six Holstein-Friesian cows with a low enteric CH_4 emission (LM group; average CH_4 concentration: 1351.85 ± 205.20 ppm).

A portable gas analyser (BIOGAS 5000 ATEX, IECEx, Geotech, UK) equipped with an integrated gas sensor, pump (sampling rate of 550 mL/min), sampling tube with filter and exhaust tube was used to measure enteric CH_4 emissions in the eructated gas of experimental cows. Namely, a standard (orogastric) flexible stainless steel stomach probe with polished bulb end having open end (180 cm length; 1.5 cm internal diameter) was placed in the caudal third of the esophagus before each measurement. Immediately, the sampling tube of the gas analyser was placed into the stomach probe lumen to collect the eructated gas, enabling the gas analyser to measure CH_4 concentration (ppm) using the dual beam infrared absorption spectroscopy method. Afterward, the probe was checked for clogging and flushed with water. The gas analyser was set to draw gas continuously for 3 minutes and read the CH_4 concentration at 5-second intervals resulting in a total of 36 readings per measurement. The obtained values were

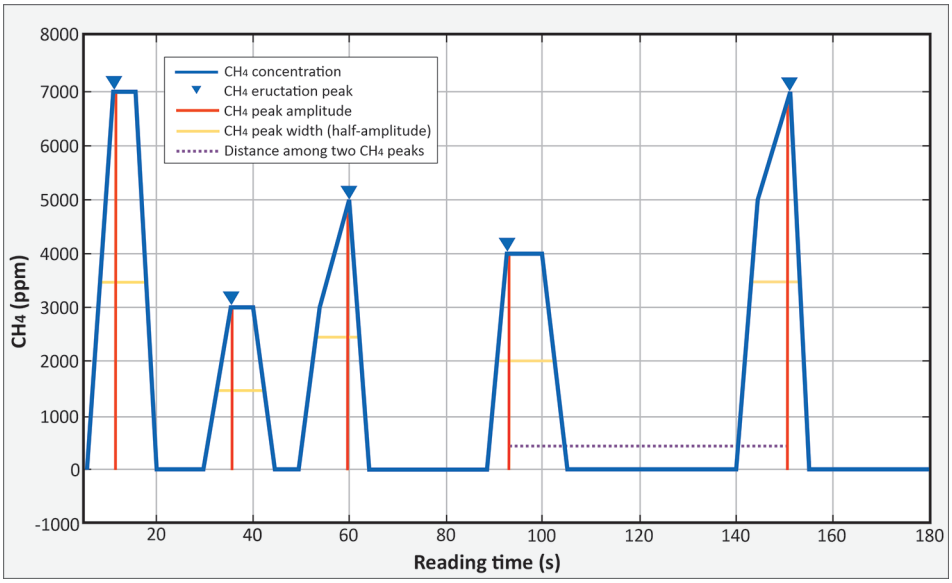

Figure 1. The CH_4 concentration profile described by CH_4 concentrations (blue line), CH_4 peaks (solid blue triangle), CH_4 peak amplitude (orange line), CH_4 peak width (yellow line)

downloaded to the computer using Gas Analyser Manager software (Geotech, UK) and used for the estimation of enteric CH_4 emissions and CH_4 concentration profiles based on CH_4 eructation peaks analysis. These analyses were performed in the MatLab Signal Processing Toolbox (version R2022b; The MathWorks, Inc., Natick, USA) [21] as recommended by Bell et al. [22]. Accordingly, for each experimental cow the following variables of CH_4 concentration were calculated: average CH_4 concentration, number of CH_4 peaks, maximum CH_4 peak amplitude, average CH_4 peak amplitude, maximum CH_4 peak width, average CH_4 peak width and distance among two CH_4 peaks (Figure 1). Finally, in the presented study, a profile was defined as a recording of the CH_4 concentration in the eructated gas of the cows examined for over 3 minutes. These profiles were used to calculate and compare different phenotypes for the CH_4 emissions following the suggestions presented by Sorg et al. [23].

Blood sampling and biochemical analyses

Blood sampling was performed one day after enteric CH_4 emission measurement was completed. Blood samples were collected by jugular venipuncture from each cow approximately one hour before the morning feeding using a sterile 18-gauge needle (BD Vacutainer, Plymouth, Devon, UK) into 10.0-mL vacutainer tubes (BD Vacutainer, Plymouth, Devon, UK), containing a clot activator. The samples were placed in an icebox immediately and transferred to a laboratory within 1 hour, where they were kept on ice for 2 hours to clot spontaneously. After clotting, samples were centrifuged at 1500 x *g* for 10 minutes to harvest the serum which was transferred into 1.5-mL polypropylene tubes (Eppendorf AG, Hamburg, Germany) and then stored at −20 °C until biochemical analyses. Each blood sample was analysed for total protein (g/L), albumin (g/L), blood urea nitrogen (BUN; mmol/L), total bilirubin (μmol/L), aspartate aminotransferase (AST; U/L), triacylglycerols (TAG; mmol/L), total cholesterol (mmol/L), HDL-cholesterol (HDL-C; mmol/L), glucose (mmol/L), beta-hydroxybutyrate (BHB; mmol/L), and non-esterified fatty acids (NEFA; mmol/L). Biochemical metabolites were analyzed using the respective methods/kits: total protein (biuret reaction); albumin (bromcresol green method), BUN (urease/glutamate dehydrogenase method); total bilirubin (diazotized sulphanilic acid method); AST (IFCC method); TAG (glycerol phosphate oxidase/peroxidase); total cholesterol (cholesterol oxidase/peroxidase method), HDL-C (direct detergent method) and BHB (enzymatic method) by BioSystems S.A. (Barcelona, Spain); and NEFA (colorimetric method) by Randox Laboratories Ltd. (Crumlin, UK). Analyses were performed automatically with spectrophotometer (A15; BioSystems S.A., Barcelona, Spain). Glucose was measured immediately after blood collection in a drop of whole blood from the tip of a vacutainer needle using commercial test strips (Abbott Diabetes Care Ltd., Oxon, UK) based on enzymatic method (glucose dehydrogenase, GDH-NAD method).

Data analysis

The data were statistically processed using the software STATISTICA, v. 8.0 (StatSoft, Inc., Tulsa, OK, USA). The results are presented as means ± SEM. The normality of data distribution was tested using Shapiro-Wilk test. All data were within the normal distribution ($p>0.05$). The significance of differences in the observed parameters between the different experimental groups was determined using the independent Student's t-test. In addition, the significance of differences in the observed parameters between measurement periods within the same experimental group was estimated using dependent Student's t-test. Correlations between enteric CH_4 emissions and indicators of metabolic status of observed cows were calculated using Pearson's test. Significance was declared at $p<0.05$ and $p<0.01$, and a tendency was acknowledged at $0.05\leq p<0.10$.

RESULTS

Variables of CH_4 concentration profile

Average values (±SEM) of CH_4 concentration profile variables in peak lactating dairy cows with different levels of enteric CH_4 emissions are shown in Table 2. It is noticeable that average CH_4 concentrations were statistically significantly higher in the HM group compared to the LM group in all three measurement periods ($p<0.05$ for P1, $p<0.01$ for both P2 and P3). Additionally, the average CH_4 concentrations within the HM group were statistically significantly higher in P3 than in P1 and P2 ($p=0.01$ and $p<0.05$, respectively). On the contrary, no statistically significant differences ($p>0.05$) were found in average CH_4 concentrations between the three measurement periods in LM cows; however, average CH_4 concentrations in P3 tended to be higher compared to P1 ($p=0.06$) in these cows. By analysing the numerical values, it can be seen that the average CH_4 concentration in both groups of cows increased from P1 to P3. Although the number of CH_4 peaks was numerically higher in HM cows than in LM cows in all three measurement periods, it was determined that this variable tended to be higher ($p=0.09$) only in P2 in HM cows compared to LM cows. It was also found that the number of CH_4 peaks tended to be higher in P3 than in P1 ($p=0.08$) within HM cows, while it was significantly higher in P3 than in P1 ($p=0.01$) and tended to be higher in P3 than in P2 ($p=0.07$) within LM cows. The maximum CH_4 peak amplitude was significantly higher in HM cows than in LM cows in all three measurement periods ($p<0.05$ for P1, $p<0.01$ for both P2 and P3). Furthermore, within HM cows, the maximum CH_4 peak amplitude was significantly higher in P2 than in P1 ($p<0.05$) and tended to be higher in P3 than in P1 ($p=0.09$). In contrast, no statistically significant differences were recorded in the values of this variable between the three periods of measurement within the LM cows. The average CH_4 peak amplitude was statistically significantly higher in HM than in LM cows in all three measurement periods ($p<0.05$ for P1, $p<0.01$ for both P2 and P3). Additionally, the average CH_4 peak amplitude

in HM cows tended to be higher in P3 than in P2 (p=0.08), while in LM cows, no significant differences between three measurement periods in the average CH_4 peak amplitude were determined. Finally, no statistically significant differences were noticed in maximum CH_4 peak width, average CH_4 peak width and average distance among two peaks, neither between HM and LM cows, nor between three measurement periods within respective groups of cows.

Table 2. Variables of CH_4 concentration profile (mean ± SEM) in peak lactating dairy cows with different level of enteric CH_4 emissions.

Variables	Units	Groups	Periods of measurement		
			P1	P2	P3
Average CH_4 concentrations	ppm	HM	4170.5 ± 1038.8^{Aa}	4520.8 ± 849.6^{Aa}	9032.4 ± 1932.3^{Ab}
		LM	908.2 ± 330.4^{Ba}	1433.6 ± 429.2^{Ba}	1641.9 ± 458.9^{Ba}
Number of CH_4 peaks	N	HM	2.4 ± 0.2^{Aa}	2.7 ± 0.4^{Aa}	3.1 ± 0.4^{Aa}
		LM	1.8 ± 0.4^{Aa}	1.9 ± 0.2^{Aab}	2.4 ± 0.4^{Ab}
Maximum CH_4 peak amplitude	ppm	HM	33305.6 ± 9043.9^{Aa}	48583.3 ± 5186.8^{Ab}	$91777.8 \pm 21644.7^{Aab}$
		LM	10250.0 ± 4612.9^{Ba}	11555.0 ± 5426.1^{Ba}	19277.8 ± 6753.1^{Ba}
Average CH_4 peak amplitude	ppm	HM	19079.0 ± 5028.3^{Aa}	23981.0 ± 2990.3^{Aa}	53848.0 ± 13749.4^{Aa}
		LM	4166.7 ± 1730.1^{Ba}	5309.3 ± 2560.0^{Ba}	5951.4 ± 1311.3^{Ba}
Maximum CH_4 peak width	s	HM	13.0 ± 1.1^{Aa}	12.3 ± 1.2^{Aa}	11.7 ± 0.6^{Aa}
		LM	12.1 ± 1.8^{Aa}	10.5 ± 0.9^{Aa}	12.5 ± 1.2^{Aa}
Average CH_4 peak width	s	HM	11.1 ± 1.6^{Aa}	9.0 ± 0.8^{Aa}	9.8 ± 0.5^{Aa}
		LM	6.5 ± 2.01^{Aa}	9.7 ± 1.1^{Aa}	8.9 ± 1.2^{Aa}
Average distance between two peaks	s	HM	46.4 ± 7.8^{Aa}	44.1 ± 8.5^{Aa}	50.4 ± 3.1^{Aa}
		LM	68.8 ± 20.4^{Aa}	45.3 ± 7.1^{Aa}	44.3 ± 7.6^{Aa}

HM – high enteric CH_4 emissions group; LM – low enteric CH_4 emissions group.
P1 – period of 2 hours before the morning feeding; P2 – period of 2 – 4 hours after the morning feeding; P3 – period of 6 – 8 hours after the morning feeding.
^{A,B} – different uppercase letters in superscript indicate statistically significant differences ($p<0.05$) between HM and LM groups of cows in the same period; ^{a,b,c} – different lowercase letters in superscript indicate statistically significant differences ($p<0.05$) between measurement periods in the same group of cows.

Indicators of metabolic status

Results obtained for blood metabolic indicators are summarized in Table 3. The results show that HM cows had a statistically significantly higher concentrations of total protein ($p<0.05$), and a statistically significant lower concentrations of total bilirubin ($p<0.05$) and NEFA ($p<0.01$). On the other hand, no statistically significant differences ($p>0.05$) were found in the concentrations of albumin, BUN, AST, TAG, total cholesterol, HDL-C, glucose and BHB between HM and LM cows. Interestingly, the results of the Pearson's correlation test indicate that there is a significant moderately negative correlation between enteric CH_4 emission and total bilirubin concentration

(r=-0.61; p<0.05) and a significantly strong negative correlation between enteric CH_4 emission and NEFA concentrations (r=-0.82; p<0.01). No correlation was found between the enteric CH_4 emission and other indicators of metabolic status of the observed cows.

Table 3. Comparison of metabolic status indicators (mean ± SEM) between cows with different level of methane emission.

Indicator (unit)	HM	LM
Total protein (g/L)	81.83 ± 2.0^{A}	71.58 ± 4.1^{B}
Albumin (g/L)	38.22 ± 1.8^{A}	37.23 ± 1.7^{A}
BUN (mmol/L)	4.70 ± 0.41^{A}	5.00 ± 0.3^{A}
Total bilirubin (μmol/L)	2.04 ± 0.1^{A}	3.33 ± 0.5^{B}
AST (U/L)	108.63 ± 17.1^{A}	92.50 ± 10.2^{A}
TAG (mmol/L)	0.17 ± 0.01^{A}	0.17 ± 0.04^{A}
Total cholesterol (mmol/L)	6.04 ± 0.3^{A}	5.04 ± 0.6^{A}
HDL-C (mmol/L)	159.80 ± 7.4^{A}	141.64 ± 15.1^{A}
Glucose (mmol/L)	2.77 ± 0.1^{A}	2.72 ± 0.1^{A}
BHB (mmol/L)	0.46 ± 0.04^{A}	0.45 ± 0.1^{A}
NEFA (mmol/L)	0.27 ± 0.02^{A}	0.42 ± 0.03^{B}

HM – high enteric CH_4 emissions group; LM - low enteric CH_4 emissions group; ^{A,B} – different uppercase letters in superscript indicate statistically significant differences (p<0.05) between HM and LM groups of cows.

DISCUSSION

The results of the presented study reveal significant differences in the variables of CH_4 concentration profiles and some indicators of metabolic status in peak lactating dairy cows with different levels of enteric CH_4 production.

In this study, a novel approach of signal processing was used to determine the variables of the CH_4 concentration profile of peak lactating dairy cows. It is known that this approach is widely applied in medical science, but it is also considered appropriate for processing potentially noisy and dense data such as those obtained by measuring the concentration of CH_4 in the exhaled or eructated gas of animals [22,24,25]. Using this approach, the obtained results for the average CH_4 concentrations indicate that HM cows had significantly higher values of this variable compared to LM cows in all measurement periods. These results suggest that HM and LM cows truly belong to different phenotypes for CH_4 production (high and low), which is needed for comparisons of the observed variables between HM and LM cows to be justified and relevant. At the same time, the average CH_4 concentrations showed an increasing trend from P1 to P3 in both groups of cows, which agrees with the results of Crompton et al. [26], who examined fluctuations in enteric CH_4 emissions associated with feeding patterns in Holstein-Friesian dairy cows in mid-lactation. Additionally,

similar results were obtained by Jonker et al. [27] and Rooke et al. [28], who observed enteric CH_4 emission in relation to time after feeding and feed allowance in beef cattle. In the mentioned studies [26-28], enteric CH_4 emission was determined in open-circuit respiration chambers and it was shown that CH_4 emission was the lowest before the morning feeding. Furthermore, these studies have also documented that CH_4 emissions rise gradually and reach a peak between 40 (for beef cattle) and 140 (for dairy cows) minutes after each feeding, and then gradually decline until the next feeding. Although, based on these findings, a decrease in enteric CH_4 emissions in P3 could be expected in our study because this measurement was performed 6 to 8 hours after the morning feeding, this was not the case. This can be explained by the fact that the cows in our study had one more meal between P2 and P3 measurements, which constitutes approximately 20% of the total daily amount of TMR offered, and could have contributed to CH_4 concentrations in P3 being higher than in P2. Finally, our results for the average CH_4 concentrations suggest that, despite the different levels of CH_4 emissions, HM and LM cows have a comparable daily pattern of CH_4 emissions. The lowest average CH_4 concentrations could describe this pattern before the morning feeding (P1) and those rises 2-4 hours after the morning feeding (P2) and further rise 6-8 hours after the morning feeding (P3).

The number of CH_4 peaks in the presented study did not statistically differ between HM and LM cows in different measurement periods. However, the CH_4 number of peaks tended to be higher in P2 ($p=0.09$) and was numerically higher in the remaining two measurement periods in HM cows compared to LM cows. Moreover, similar to the average CH_4 concentration, the obtained values for the CH_4 number of peaks indicates an increasing trend from P1 to P3 in both groups of cows. Given that the methodology for measuring CH_4 emissions used in this study is based on determining its concentration in the eructated gas, it is important to explain that the appearance of each CH_4 peak could be attributed to an eructation event in the examined cow. A similar interpretation of the appearance of CH_4 peaks was given by Bell et al. [22] and Hardan et al. [25], who used infrared gas analysers to measure enteric CH_4 emissions in the exhaled air of dairy cows and applied the same mathematical approach for the CH_4 concentration profile analysis. Unfortunately, there is no similar study in the available literature that examined the CH_4 number of peaks or eructation rate in cows with different levels of CH_4 emissions, with which our results could be compared. Nevertheless, the higher number of eructations in HM cows than in LM cows and its increasing trend from P1 to P3 in both groups of cows could be associated with the levels of enteric CH_4 production. It is obvious that a higher number of eructation events followed higher average CH_4 concentrations in this study. Therefore, it can be hypothesized that there is a stronger stimulation of the eructation reflex through the activation of the vagal afferents of the dorsal rumen by produced gas [29] in cows with high CH_4 production, as well as in the periods after feeding that were coupled with increased CH_4 production in both groups of cows. This hypothesis is additionally supported by our results obtained for the maximum CH_4 peak amplitude, average CH_4

peak amplitude, maximum CH_4 peak width and average CH_4 peak width. Namely, HM cows had a significantly higher maximum CH_4 peak amplitude and average CH_4 peak amplitude than LM cows in all three measurement periods, whereby in both groups of cows, there was an increasing trend in both parameters from P1 to P3. Also, there were no significant differences in maximum CH_4 peak width and average CH_4 peak width between HM and LM groups in different measurement periods or between measurement periods within the respective groups of cows. Accordingly, the described results suggest that higher production of enteric CH_4 more powerfully stimulates the eructation reflex and leads to a greater number of eructations (CH_4 peaks) and a greater release of CH_4 per eructation event (CH_4 amplitude variables) of approximately equal duration (CH_4 width variables). This is clearly noticeable both in HM cows compared to LM cows, as well as in later measurement periods after the morning feeding compared to the previous ones (P3 vs. P2) or those before the morning feeding (P2 vs. P1 and P3 vs. P1). However, additional studies are needed in the physiology of the eructation reflex and its connection with the level of enteric CH_4 production to confirm the presented hypothesis. Finally, it should be noted that the average number (±SEM) of eructation events in both groups of cows in all measurement periods was in the general range of 0.7 to 1.5 eructations per minute [30,31]. Furthermore, our results for the average distance among two CH_4 peaks (eructations) did not differ significantly between HM and LM cows or between different measurement periods within the same group of cows. They were also in the general range that predicts the appearance of one eructation event every 40 to 90 seconds in cattle [32].

To the best of our knowledge, this is the first study on the metabolic status of dairy cows with different levels of enteric CH_4 emissions. Nevertheless, the results obtained for the indicators of the metabolic status in our study could be compared with the results reported by Kim et al. [9], who observed differences in metabolic status in various fattening phases of Japanese Black steers with high (HME) and low (LME) methane emissions. In the mentioned study, it was found that HME steers have only significantly higher concentrations of BHB compared to LM steers in all fattening phases, while differences between these groups of steers in the concentration of total protein, total cholesterol, NEFA and glucose and AST activity were not recorded. These findings are not in agreement with ours as we observed significantly higher total protein concentrations and significantly lower total bilirubin and NEFA concentrations in HM cows compared to LM cows. The possible explanation for the described disagreements can be addressed to the cattle breed and production category used in the compared studies. Kim et al. [9] included Black Japanese steers in their study, while Holstein-Friesian dairy cows in peak lactation were used in our study. As these two categories of cattle have different production purposes and different metabolomics and proteomics signatures [33], it is reasonable to expect differences in the metabolic response between these cattle in relation to enteric CH_4 production. Additionally, the mentioned authors performed the study in three phases of fattening, which differ in their metabolic challenge for the steers, while our study included cows

averaging (±SEM) 49.33 ± 2.58 DIM, avoiding the most pronounced negative energy balance and its influence on the monitored indicators of the metabolic status [34]. The values obtained for NEFA concentrations in our study indicate that HM cows have a more favorable energy balance compared to LM cows. Namely, NEFA concentrations reflect the magnitude of peripheral fat breakdown and energy balance of cows, while BHB concentrations reflect the ability of the liver to manipulate inflowing NEFA [35]. Although there are no significant differences in BHB concentration between HM and LM cows, attention should be paid to other parameters of liver function observed in this study, such as concentrations of total protein and total bilirubin. In this regard, significantly lower total protein concentrations and significantly higher total bilirubin concentrations in LM cows compared to HM cows indicate that the energy balance of LM cows affects their functional liver capacity [36,37]. Moreover, the significant negative correlation between enteric CH_4 emissions and total bilirubin and NEFA concentrations, respectively, found in the presented study additionally supports these considerations. On the other hand, the absence of differences in the concentration of BHB between HM and LM cows can be explained by the fact that blood BHB concentration may be affected by the production of butyrate in the rumen [38]. In other words, higher production of butyrate in the rumen of HM cows could have caused an increase in BHB blood concentrations in these cows and masked any differences in the values of this parameter between HM and LM cows. Moreover, the higher production of butyrate in HM cows would not be surprising because there are indications that butyrate synthesis is a metabolic pathway in the rumen that may favor higher CH_4 production because it is associated with the release of H_2 [7,9]. However, additional studies are needed in the ruminal microbiome and metabolome and liver metabolome and transcriptome fields that will elucidate the nature of the differences found in the metabolic status between HM and LM cows in the present study. Finally, it should be highlighted that all observed indicators of the metabolic status of peak lactating dairy cows in this study were within the reference ranges proposed by Cozzi et al. [39] and Moretti et al. [40].

CONCLUSION

In conclusion, this study showed that peak lactating dairy cows with different phenotypes for CH_4 emissions (high and low) have similar daily pattern of CH_4 emissions, since the lowest CH_4 emission, detected before the morning feeding, gradually increased after each feeding. This finding is in accordance with other studies in which different methods of CH_4 emissions measurement were used. However, our study revealed that dairy cows with a high CH_4 emission phenotype have more eructations and release more CH_4 per eructation. Additionally, the metabolic status of peak lactating dairy cows is affected by the level of CH_4 emission. This is most noticeable in the parameters that depict different aspects of liver function since in cows with high CH_4 production, higher concentrations of total proteins and lower

concentrations of total bilirubin and NEFA were found. Finally, further research in the field of liver transcriptome and metabolome is needed in order to determine the origin and significance of these differences.

Acknowledgments

The research was conducted with the support of the Science Fund of the Republic of Serbia, project number 7750295, "Mitigation of methane production from dairy cattle farm by nutritive modulation of cow`s metabolism-MitiMetCattle".

Authors' contributions

DB, DK, SN and LJ contributed to the design and conception of the study. RP, IV, SA and SN conducted the field experiment. RP and SA performed biochemical analyses of blood serum samples. DB, MS and SD participated in data analyses. DB, DK, and LJ were involved in draft manuscript preparation. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Hook SE, Wright ADG, McBride BW: Methanogens: methane producers of the rumen and mitigation strategies. Archaea 2010, 1-11.
2. Lassen J, Løvendahl P: Heritability estimates for enteric methane emissions from Holstein cattle measured using noninvasive methods. J Dairy Sci 2016, 99:1959-1967.
3. Zhao Y, Nan X, Yang L, Zheng S, Jiang L, Xiong B: A review of enteric methane emission measurement techniques in ruminants. Animals 2020, 10:1004.
4. Broucek J: Production of methane emissions from ruminant husbandry: a review. Journal of Environmental Protection 2014, 5: 1482.
5. Loor JJ, Elolimy AA, McCann JC: Dietary impacts on rumen microbiota in beef and dairy production. Anim Fron 2016, 6:22-29.
6. Knapp JR, Laur GL, Vadas PA, Weiss WP, Tricarico JM: Invited review: Enteric methane in dairy cattle production: Quantifying the opportunities and impact of reducing emissions. J Dairy Sci 2014, 97:3231-3261.
7. Morgavi DP, Forano E, Martin C, Newbold CJ: Microbial ecosystem and methanogenesis in ruminants. Animal 2010, 4:1024-1036.
8. Johnson KA, Johnson DE: Methane emissions from cattle. J Anim Sci 1995, 73: 2483-2492.
9. Kim M, Masaki T, Ikuta K, Iwamoto E, Nishihara K, Hirai M, Uemoto Y, Terada F, Roh S: Physiological responses and adaptations to high methane production in Japanese Black cattle. Sci Rep 2022, 12(1):1-14.

10. Gross JJ, Bruckmaier RM: Metabolic challenges in lactating dairy cows and their assessment via established and novel indicators in milk. Animal 2019, 13:75-81.
11. Hegarty RS: Applicability of short-term emission measurements for on-farm quantification of enteric methane. Animal 2013, 7:401-408.
12. Hammond KJ, Crompton LA, Bannink A, Dijkstra J, Yáñez-Ruiz DR, O'Kiely P, Kebreabf E, Eugèneg MA, Yuh Z, Shingfieldij KJ, Schwarmk A, Hristov AN, Reynolds CK: Review of current in vivo measurement techniques for quantifying enteric methane emission from ruminants. Anim Feed Sci Tech 2016, 219:13-30.
13. Patra AK: Recent advances in measurement and dietary mitigation of enteric methane emissions in ruminants. Front Vet Sci 2016, 3:39.
14. Castelán Ortega OA, Pedraza Beltrán PE, Hernández Pineda GS, Benaouda M, González Ronquillo M, Molina L, Vázquez Carrillo MF: Construction and operation of a respiration chamber of the head-box type for methane measurement from cattle. Animals 2020, 10(2):227.
15. Sorg D: Measuring Livestock CH4 Emissions with the Laser Methane Detector: A Review. Methane 2022, 1:38-57.
16. Chagunda MGG, Yan T. Do methane measurements from a laser detector and an indirect open-circuit respiration calorimetric chamber agree sufficiently closely? Anim Feed Sci Tech 2011, 165:8-14.
17. Garnsworthy PC, Craigon J, Hernandez-Medrano JH, Saunders N: On-farm methane measurements during milking correlate with total methane production by individual dairy cows. J Dairy Sci 2012, 95:3166-3180.
18. Troy SM, Duthie CA, Ross DW, Hyslop JJ, Roehe R, Waterhouse A, Rooke JA: A comparison of methane emissions from beef cattle measured using meth ane hoods with those measured using respiration chambers. Anim Feed Sci Tech 2016, 211:227–240.
19. Ministry for the Environment and Ministry for Primary Industries: Pricing agricultural emissions: Consultation document. Ministry for the Environment 2022, Wellington, New Zealand (https://environment.govt.nz/assets/publications/Pricing-agricultural-emissions-consultation-document.pdf).
20. National Research Council: Nutrient requirements of dairy cattle, Seventh Revised Edition 2011, Washington, D.C.
21. MathWorks. Peak Analysis. Available online: https://uk.mathworks.com/help/signal/examples/peak-analysis.html.
22. Bell MJ, Garnsworthy P, Mallis D, Eckard R, Moate P, Yan T: Modified approach to estimating daily methane emissions of dairy cows by measuring filtered eructations during milking. J Sustainable Organic Agric Syst 2020, 69:47-56.
23. Sorg D, Difford GF, Mühlbach S, Kuhla B, Swalve HH, Lassen J, Strabel T, Pszczola M: Comparison of a laser methane detector with the GreenFeed and two breath analysers for on-farm measurements of methane emissions from dairy cows. Comput Electron Agr 2018, 153:285-294.
24. Grešáková Ľ, Holodová M, Szumacher-Strabel M, Huang H, Ślósarz P, Wojtczak J, Sowińska N, Cieślak A: Mineral status and enteric methane production in dairy cows during different stages of lactation. BMC Vet Res 2021, 17:1-9.
25. Hardan A, Garnsworthy PC, Bell MJ: Detection of Methane Eructation Peaks in Dairy Cows at a Robotic Milking Station Using Signal Processing. Animals 2021, 12:26.

26. Crompton LA, Mills JA, Reynolds CK, France J: Fluctuations in methane emission in response to feeding pattern in lactating dairy cows. In *Modelling nutrient digestion and utilisation in farm animals*. Wageningen Academic Publishers, Wageningen; 2011, 176-180.
27. Jonker A, Molano G, Antwi C, Waghorn G: Feeding lucerne silage to beef cattle at three allowances and four feeding frequencies affects circadian patterns of methane emissions, but not emissions per unit of intake. Anim Prod Sci 2014, 54:1350-1353.
28. Rooke JA, Wallace RJ, Duthie CA, McKain N, de Souza SM, Hyslop JJ, Ross DW, Waterhouse T, Roehe R: Hydrogen and methane emissions from beef cattle and their rumen microbial community vary with diet, time after feeding and genotype. Brit J Nutr 2014, 112:398-407.
29. Goff JP: Ruminant Digestive Physiology and Intestinal Microbiology. In: *Dukes' Physiology of Domestic Animals*. John Wiley & Sons; 2015, 522-531.
30. Pickering NK, Oddy VH, Basarab J, Cammack K, Hayes B, Hegarty RS, Lassen J, McEwan JC, Miller S, Pinares-Patiño CS, De Haas Y: Animal board invited review: genetic possibilities to reduce enteric methane emissions from ruminants. Animal 2015, 9:1431-1440.
31. Suzuki T, Kamiya Y, Oikawa K, Nonaka I, Shinkai T, Terada F, Obitsu T: Prediction of enteric methane emissions from lactating cows using methane to carbon dioxide ratio in the breath. Anim Sci J 2021, 92, e13637.
32. Mortola JP, Lanthier C: Breathing frequency in ruminants: a comparative analysis with non-ruminant mammals. Resp Physiol Neurobi 2005, 145:265-277.
33. Elolimy AA, Zeineldin M, Abdelmegeid M, Abdelatty AM, Alharthi AS, Bakr MH, Elghandour MMMY, Salem AZM, Loor JJ: Metabolomics and Proteomics Signatures in Feed-Efficient Beef and Dairy Cattle. In *Sustainable Agriculture Reviews*. Springer, Cham; 2021,153-165.
34. Nigussie T: A review on the role of energy balance on reproduction of dairy cow. J Dairy Res Technol 2018, 1:1-8.
35. LeBlanc S: Monitoring metabolic health of dairy cattle in the transition period. J Reprod Develop 2010, 56:29-35.
36. González FD, Muiño R, Pereira V, Campos R, Benedito JL: Relationship among blood indicators of lipomobilization and hepatic function during early lactation in high-yielding dairy cows. J Vet Sci 2011, 12:251-255.
37. Kalaitzakis E, Panousis N, Roubies N, Giadinis N, Kaldrymidou E, Georgiadis M, Karatzias H: Clinicopathological evaluation of downer dairy cows with fatty liver. Can Vet J 2010, 51:615-622.
38. Yanibada B, Hohenester U, Pétéra M, Canlet C, Durand S, Jourdan F, Boccard J, Martin C, Eugène M, Morgavi DP, Boudra H: Inhibition of enteric methanogenesis in dairy cows induces changes in plasma metabolome highlighting metabolic shifts and potential markers of emission. Sci Rep 2020, 10:1-14.
39. Cozzi G, Ravarotto L, Gottardo F, Stefani AL, Contiero B, Moro L, Brscic M, Dalvit P: Reference values for blood parameters in Holstein dairy cows: Effects of parity, stage of lactation, and season of production. J Dairy Sci 2011, 94:3895-3901.
40. Moretti P, Paltrinieri S, Trevisi E, Probo M, Ferrari A, Minuti A, Giordano A: Reference intervals for hematological and biochemical parameters, acute phase proteins and markers of oxidation in Holstein dairy cows around 3 and 30 days after calving. Res Vet Sci 2017, 114:322-331.

EMISIJA METANA I METABOLIČKI STATUS MLEČNIH KRAVA U PIKU LAKTACIJE I NJIHOVA PROCENA PUTEM PROFILA KONCENTRACIJE METANA

Dušan BOŠNJAKOVIĆ, Danijela KIROVSKI, Radiša PRODANOVIĆ, Ivan VUJANAC, Sveta ARSIĆ, Milica STOJKOVIĆ, Slavica DRAŽIĆ, Sreten NEDIĆ, Ljubomir JOVANOVIĆ

Uzgoj preživara doprinosi globalnoj emisiji metana (CH_4) i pored negativnog uticaja na životnu sredinu, enterični CH_4 kod krava izaziva gubitak energije unete hranom. Cilj ove studije je bio da se izvrši procena emisije CH_4 i metaboličkog statusa krava određivanjem profila koncentracije metana kao alata kojim se analizira način produkcije CH_4. Istraživanjem je obuhvaćeno osamnaest krava čija je enterička emisija CH_4 merena tokom tri uzastopna dana u tri perioda: 2 sata pre (P1), 2–4 sata (P2) i 6–8 sati (P3) posle jutarnjeg hranjenja. Na osnovu emisije CH_4, krave su podeljene u dve grupe (n=6, pojedinačno): HM (prosečna CH_4 koncentracija: 5430,08 ± 365,92 ppm) i LM (prosečna CH_4 koncentracija: 1351,85 ± 205,20 ppm). Nakon merenja CH_4, trećeg dana, uzorkovana je venska krv radi utvrđivanja indikatora metaboličkog statusa. HM krave su imale statistički značajno veće prosečne koncentracije CH_4, maksimalnu i prosečnu amplitudu CH_4 pika u odnosu na LM krave u svim periodima merenja (P1-P3), dok je broj CH_4 pikova imao tendenciju da bude veći kod HM nego kod LM krava u P2. Nije bilo razlika u maksimalnoj i prosečnoj širini pika CH_4 i prosečnoj udaljenosti između dva pika CH_4 između ispitivanih grupa krava. HM krave su imale značajno veće koncentracije ukupnih proteina i značajno niže koncentracije ukupnog bilirubina i NEFA od LM krava. Zaključuje se da su HM krave imale veći broj ruktusa i time oslobađale više CH_4 nego LM krave, a razlike u metaboličkom statusu između HM i LM krava su najverovatnije bile povezane sa razlikama u funkciji jetre.