American Journal of Animal and Veterinary Sciences

Original Research Paper

In vitro Ruminal Cumulative Gas and Methane Production, Enzyme Activity, Fermentation Profile and Nutrient Digestibility on Feed Supplemented with Organic Selenium

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Article history
Received: 22-08-2023
Revised: 19-09-2023
Accepted: 23-09-2023

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Abstract: Methane (CH_4) emissions are indeed a global environmental concern and ruminants are a significant source of these emissions; however, there is limited understanding regarding the contribution of organic Selenium (Se) in mitigating CH_4 emissions. Hence, this study investigated the impact of supplementing organic Se from Selenomethionine (Se-Met) on the *in vitro* ruminal cumulative gas and CH_4 production, fermentation profile, enzyme activities, and nutrient digestibility. The trial protocol has been approved by the ethical clearance committee and encompassed four distinct treatments implemented within a completely randomized design. The treatments were described in the following manner: (1) Basic feed without additional substances (CTL); (2) CTL with 0.15 mg of Se per kg of Dry Matter (DM)-denoted as low Se-Met (LS); (3) CTL with 0.30 mg of Se per kg DM-referred to as medium Se-Met (MS); and (4) CTL with 0.45 mg of se per kg DM-termed high Se-Met (HS). Each group consisted of five replications and was randomly allocated to the *in vitro* tubes. The research was conducted comprehensively for two months. The data underwent analysis using the General Linear Model (GLM) procedure and a probability value of $p<0.05$ was used to determine statistical significance. The findings indicated that both the MS and HS interventions led to a gradual rise in the activity of carboxyl methyl cellulose (CMC-ase) ($p<0.001$), amylase ($p<0.001$), and protease ($p = 0.002$). A linear and quadratic rise ($p = 0.001$) in total VFA was observed across all Se-Met doses. Notably, HS exhibited a linear increase in acetate ($p = 0.004$) and a linear decrease in butyrate ($p = 0.004$). Microbial crude protein exhibited a linear increase ($p = 0.003$) in both the MS and HS groups. Gas production notably increased ($p<0.05$) at the 6 h post-incubation, with the highest total GP ($p = 0.001$) occurring at 48 h in the MS and HS groups. MS and HS also showed a linear improvement in gas kinetics: B ($p = 0.003$) fraction and a + b fraction ($p = 0.002$). The LS, MS, and HS groups demonstrated improvement in the *in vitro* DM digestibility (linear, $p<0.001$; quadratic, $p<0.05$) and *in vitro* organic matter digestibility (linear, $p<0.001$; quadratic, $p = 0.006$) compared to the CTL group. Furthermore, the MS and HS groups exhibited a noteworthy linear increase ($p = 0.005$) in the *in vitro* crude protein digestibility and *in vitro* crude fiber digestibility compared to the CTL group. This research indicates that supplementation of Se-Met, as organic Se, at 0.30 and 0.45 mg of Se per kg DM could impact rumen enzyme activities, fermentation patterns, and nutrient digestibility *in vitro*. Nevertheless, the Se supplementation did not demonstrate the ability to reduce CH_4 gas emissions at these doses.

Keywords: Enzyme Activities, Methane Production, Nutrient Digestibility, Organic Selenium, Rumen Fermentation

Introduction

Enteric Methane (CH_4) does not just reflect significant energy lossaes in ruminants; it also exerts a potent greenhouse gas, boasting a global warming potential 28 times higher than Carbon Dioxide (CO_2) (Króliczewska *et al*., 2023). Ruminal methanogenic archaea play a role in CH_4 formation by using Hydrogen (H_2) and CO_2 to produce energy (Patra and Yu, 2012). Considering this, several efforts related to ruminant feed have been implemented to decrease enteric CH_4 production (Palangi and Lackner, 2022); nevertheless, knowledge about the impact of trace mineral supplementation in accomplishing this goal remains somewhat limited. Several studies have shown that supplementing Selenium (Se) as a trace mineral can potentially reduce CH_4 emissions by enhancing the utilization of H^+ ions in propionate production (Pan *et al*., 2021; Tian *et al*., 2022).

Se is an essential trace element required in minor amounts within animal feed to support various physiological processes. She holds significant importance for the metabolism of humans, animals, plants, and microorganisms (Zheng *et al*., 2022). Se functions within the antioxidant mechanism, serving as a crucial property of the glutathione peroxidase enzyme. This enzyme has the capability to counteract the effects of H_2 peroxide and lipid hydroperoxides (Kurutas, 2015). Se also contributes a significant role in the ruminal fermentation process. Feeding Se enhances the antioxidant capacity of rumen microorganisms, stimulates rumen fermentation, and facilitates microbial growth (Hendawy *et al*., 2021; Mehdi and Dufrasne, 2016; Zheng *et al*., 2022). Adding 0.5 mg se/kg Dry Matter (DM) to dairy bull diets significantly enhanced ruminal digestive enzyme activities (Liu *et al*., 2019). Including 0.4 mg Se/kg DM in sheep's diet improved nutrient digestibility and enhanced nitrogen absorption (Wang *et al*., 2019). Different Se administration forms increased ruminal Volatile Fatty Acids (VFA) production (Arshad *et al*., 2021; Hendawy *et al*., 2021). The rise of VFA levels confirms that Se inclusion in the feed can promote the growth of rumen microbes. Se contributes a crucial function in aiding ruminal microbes in synthesizing their proteins and constructing cellular walls (Liu *et al*., 2020).

As a tropical country, Indonesia has many areas with low Se in the soil (Stefani *et al*., 2020). The soil's Se content falls short, leading to inadequate levels of Se in plants, which in turn leads to a reduction of Se for livestock (Wang *et al*., 2019). Several nutritional problems may arise due to Se deficiency, contingent upon the animals' physiological state (Yatoo *et al*., 2013). Therefore, supplementing Se in the animal's diet is paramount to prevent Se deficiency-related problems and improve animal production (Anam *et al*., 2021; 2022; Khalil *et al*., 2019).

In general, there exist two types of Se: Inorganic and organic. Inorganic Se is commonly utilized due to its affordability. However, organic Se offers advantages such as higher absorption rates, increased biological activity, greater tissue accumulation, and lower toxicity than inorganic form (Samo *et al*., 2018; Zheng *et al*., 2022). Selenomethionine (Se-Met) is a frequently used organic source of Se and is particularly notable among them. This source is renowned for its exceptional bioavailability, surpassing inorganic sources. The high bioavailability can be attributed to its structural similarity to methionine, which enables efficient absorption in the body (Schrauzer, 2000).

To our knowledge, there is a lack of research on the impact of organic Se addition in the diets of Indonesian cattle. Furthermore, the influence of varying levels of organic Se on ruminal fermentation parameters, particularly on CH_4 emissions and enzyme activities, remains limited and unclear. Therefore, it necessitates additional research and investigation. Thus, the current research aimed to determine the impacts of using various doses of Se-Met, as organic Se source supplementation in the diet on *in vitro* ruminal cumulative gas and CH_4 production, enzyme activities, fermentation profile, and the digestibility of nutrients.

Materials and Methods

Design of Experimental Study

The methods employed in this research have received approval from the Research Ethics Committee at the Universitas Gadjah Mada, Indonesia (No: 025/EC-FKH/Eks./2023). The basal feed consisted of a blend of elephant grass (*Pennisetum purpureum*) and wheat bran obtained from Kalijeruk Farm, Sleman, Yogyakarta. The samples were measured using a weight ratio of 60:40, which was determined considering the Dry Matter (DM). Additionally, a mineral mix top-up of 0.5% was included. This feed composition reflects common agricultural practices in Indonesia. Prior to conducting analyses for DM, ash, Organic Matter (OM), Crude Protein (CP), Ether Extract (EE), and Crude Fiber (CF) using the (AOAC, 2005) method, all dried samples underwent processing by being ground using a Willey mill. The screen was adjusted to a size of 1 mm. The concentration of Se within the feed sample was quantified through Atomic Absorption Spectroscopy (AAS) (Anam *et al*., 2022). Briefly, 0.5 grams of DM were combined with a mixture of 10 mL HNO_3 and 2 mL HCl and then subjected to 15 min of homogenization. Subsequently, the sample underwent digestion at 200°C for 15 min. After the digestion process was completed, the sample was blended with demineralized water in a 25 mL flask. Finally, the solution was filtered and its absorbance was measured at 196 nm using AAS. The nutritional compositions of the basal feed are displayed in Table 1.

Table 1: Nutritional composition of basal feed

Item	Value
Ingredients (% of dry matter)	
Elephant grass	60.0000
Wheat bran	40.0000
Chemical composition	
Dry matter, %	88.0600
--- % of dry matter ---	
Organic matter	87.8900
Crude protein	12.6900
Crude fiber	23.8600
Extract ether	1.9100
Ash	12.1100
Nitrogen-free extract	49.4400
Total digestible nutrient	61.1100
Selenium, mg/kg	0.0028

The study consisted of four treatments, each with five replicates, following a completely randomized design. The interventions were outlined as (1) Basal feed without any supplementation (CTL); (2) CTL with 0.15 mg Se per kg DM-Low Se-Met (LS); (3) CTL with 0.30 mg Se per kg DM-Medium Se-Met (MS); and (4) CTL with 0.45 mg Se per kg DM-High Se-Met (HS). Certainly, to ensure transparency and rigor, the samples were randomly assigned to treatments using a computer-generated randomization process. The Se-Met was introduced into the foundational diet and meticulously blended with the concentrate. Subsequently, this mixture was uniformly incorporated with the forage. The Se-Met employed in the study was obtained from PT. Fenanza Putra in Indonesia, with a Se content level of 4,000 ppm.

Rumen Fluid Preparation

Rumen fluid utilized in the current research was collected from two fistulated Bali cattle weighing around 340 kg each, which were fed a diet comprising a mixture of elephant grass and commercial concentrate (60:40 w/w, DM basis) and kept at the livestock facility barn. The basal diet was provided at 3% of the animal's body weight. The diet, which included 13% CP per day and 75% TDN, met the requirements for beef cattle (NRC, 2000). Approximately 500 mL of rumen fluid (a total of 1,000 mL for both cattle) was collected before the animal's feeding. Recently obtained rumen fluid samples were gathered and placed within a thermos container at a temperature of 39°C. Afterward, the liquor underwent filtration using four layers of cheesecloth to separate feed substrate particles.

In vitro Gas Production Test

The *in vitro* gas production method assessed the potential degradability and degradation rate of feeds supplemented with organic Se. The feed sample was exposed to incubation using a glass syringe (Fortuna, Poulten, and Graft GmbH, Germany) with a capacity of 100 mL, containing a blend of rumen fluid and saliva buffer mixture. The saliva buffer was prepared to contain a reducing solution micro mineral blend, buffering solution, macro mineral combination, resazurin, and reducing solution (Menke and Steingass, 1988). The feed samples, which had been dried and weighed 300 mg of DM, were meticulously inserted into a glass syringe lubricated with Vaseline on its piston. Furthermore, the feed samples in the glass syringe are incubated at 39°C for one night.

During the morning, a combined volume of 10 mL of rumen fluid was completely blended with 20 mL of salivary buffer during the running time and pre-warmed to a temperature of 39°C. Simultaneously, a stream of CO_2 gas was passed through to maintain anaerobic conditions. Subsequently, all samples are incubated at 39°C within a controlled-temperature cabinet (incubator) for a duration of 48 h. Five replicates were conducted for each sample treatment. Furthermore, a control fermentor (lacking substrate; composed of rumen fluid and buffer-saliva) was employed to quantify overall gas production.

During incubation, the cumulative Gas Production (GP) values were measured at specific intervals (0, 2, 4, 6, 8, 10, 12, 24, 38 and 48 h). These values were then inserted into the equation following Ørskov and McDonald (1979):

$$GP = a + b \times (1 - exp(^{-ct}))$$

where, the fraction "*a*" signifies the rapidly degrading portion (ml), fraction "*b*" represents the fraction undergoing slower degradation (ml), and the sum of the fraction "*a* + *b*" denotes the potential gas (ml). The fraction "*c*" denotes the constant rate (%/h).

Determination of CH_4 and Rumen Fermentation Profile

The assessment of CH_4 was conducted to investigate the influence of organic Se supplementation on CH_4 production, which is also a component of the rumen fermentation process. After recording the complete gas volume at the 48 h mark, the gas contained within the syringe was moved into an ordinary 10 mL vacutainer. Gas Chromatography (GC) assessed CH_4 content (Gunun *et al*., 2018). The detected CH_4 gas was expressed in terms of ml as yield, CH_4 per digested DM (DDM), and CH_4 per digested OM (DOM).

The syringe plunger was released after gas measurement to evaluate the ruminal fermentation profiles and a pH measurement was immediately performed. The liquid content was subsequently subjected to filtration and centrifugation at 3,000 g for 15 min to isolate feed particles from the rumen fluid. Afterward, rumen liquor was combined with 25% of met phosphoric acid (5:1 v/v) for VFA analysis. For assessing ammonia-nitrogen (NH_3-N) content, a volume of five milliliters of

rumen fluid was readied. Furthermore, another 1.5 mL of freshly collected rumen fluid underwent subsequent centrifugation at 10,000 g. The precipitate-pellet was utilized to measure Microbial Crude Protein (MCP) content, while the supernatant was employed to evaluate the activity of rumen microbial enzymes.

The VFA levels were assessed utilizing Gas Chromatography (GC) (GC-8A series, Shimadzu), following the approach outlined by Filípek and Dvořák (2009). The Flame Ionization Detector (FID) was employed as the detector at a temperature of 260°C, while the injector temperature was maintained at 250°C. The chromatographic column utilized was CPFFAP CB/CP7485, with dimensions of 60°C in temperature, 25 meters in length, and an inner diameter of 0.32 mm. Helium gas was employed as the conveying medium with a 3 mL/min flow rate and a division ratio of 27.5. Concentrations of VFA, including acetate, propionate, butyrate, and the acetate to propionate ratio were calculated. The estimations for MCP were conducted following the Blümmel *et al*. (1997) protocol. The concentration of NH_3-N was evaluated utilizing colorimetry with a spectrometer, as described in the work by Chaney and Marbach (1962). Carboxyl methyl cellulase (CMC-ase) enzyme activity was measured using carboxyl methyl cellulose substrate (Halliwell and Lovelady, 1981). The activity of the protease enzyme was assessed by gauging its capacity to degrade casein into peptides and amino acids (Malathi and Chakraborty, 1991). Amylase enzyme activity was measured using 2% amylum substrate in Na-phosphate buffer at pH 6 (Halliwell, 1961).

In vitro Digestibility of Nutrient

Digestibility is an important factor in measuring the nutritional value of animal diets. The influence of organic Se addition on nutrient digestibility was tested using the *in vitro* method following Tilley and Terry (1963). To conduct this test, 200 mL tubes were prepared, each containing 250 mg of DM from the feed substrate. These tubes were then supplemented with McDougall buffer solution and rumen liquor in a 4:1 ratio. A total of 50 mL of the buffer solution was introduced to the feed substrate. Some tubes were left empty as blanks without any feed substrate. To maintain anaerobic conditions, each tube for every treatment was flushed with CO_2. Subsequently, the tubes were subjected to incubation at a temperature of 39°C. After incubating for 48 h, the fermented liquid and feed substrate were separated using a crucible containing glass wool. The *in vitro* DM Digestibility (IVDMD), OM Digestibility (IVOMD), CP Digestibility (IVCPD), and CF Digestibility (IVCFD) were evaluated by calculating the discrepancies in nutritional content between the initial state before fermentation and the residual material after a 48-h fermentation period, as outlined in the study by Yao *et al*. (2020).

Statistical Analysis

We ensured our statistical analysis's robustness by systematically checking our tests' assumptions. Normality and homoscedasticity assumptions were assessed using an appropriate statistical test. Regarding missing data, we adopted a rigorous approach to handling any missing values in our dataset. This information ensures the transparency and reliability of our statistical analysis. The data underwent analysis using the General Linear Model (GLM) procedure within the open-access SAS on demand for academics® software (www.sas.com). In the context of the statistical examination, the model incorporated the varying levels of organic Se supplementation as fixed factors. The study examined the linear and quadratic impacts of additional organic Se by employing orthogonal polynomial contrasts. A probability value of $p<0.05$ was used to define statistical significance. Moreover, linear relationships between the examined variables and their visualization were established utilizing Pearson's correlation within the OriginPro® 2022 version software.

Results

In vitro Rumen Enzyme Activities and Fermentation Parameters

Table 2 shows data on *in vitro* ruminal enzyme activities and rumen fermentation parameters. The MS and HS group demonstrated improved CMC-ase ($p<0.001$), amylase ($p<0.001$), and protease ($p = 0.002$) enzyme activities linearly compared to the supplemented group. The inclusion of various Se-Met doses into the feed led to significant linear ($p<0.001$) and quadratic ($p = 0.004$) increases in total VFA. The HS group displayed the highest acetate value ($p = 0.004$) compared to all other treatments, while a linear decrease ($p = 0.004$) in the butyrate proportion was noted. The MCP increased linearly ($p = 0.003$) in the MS and HS groups. However, no noteworthy distinctions were detected ($p>0.05$) in rumen pH values, propionate levels, the ratio of acetate to propionate, and NH_3-N concentrations across all dietary interventions.

In vitro Cumulative Gas and CH_4 Production

Table 3 displayed that adding Se-Met at various doses did not have a notable impact on GP following 2 and 4 h of incubation ($p>0.05$). The MS group exhibited a linear rise ($p<0.05$) in GP at 6 and 8 h, in contrast to the other treatments. Additionally, both the MS and HS groups exhibited a linear increase at 12 h ($p = 0.002$), 24 h ($p = 0.001$), 36 h ($p = 0.002$), and 48 h ($p = 0.001$) after incubation, in comparison to the CTL groups. Furthermore, introducing 0.30 and 0.45 mg of Se per kg DM from Se-Met led to a linear rise in the b ($p = 0.003$)

and a + b (p = 0.002) fractions. Nonetheless, no significant distinctions were witnessed in fractions a and c across all treatments (p>0.05). Regarding CH_4 emissions, which were presented as ml yield, CH_4/DDM, and CH_4/DOM, the administration of Se-Met at various doses showed no significant impact (p>0.05).

Table 2: Effect of Se-Met supplementation on the *in vitro* ruminal enzyme activity and fermentation profile

	Treatment					p-value	
Item	CTL	LS	MS	HS	SEM	Linear	Quadratic
Enzyme activities							
CMC-ase (U/g)	3.36[a]	3.83[a]	4.53[b]	4.96[b]	0.17	<0.001	0.933
Amylase (U/g)	16.98[a]	18.07[ab]	19.14[bc]	19.90[c]	0.31	<0.001	0.678
Protease (U/g)	143.26[a]	149.45[ab]	155.95[b]	157.11[b]	1.86	0.002	0.408
Fermentation profile							
pH	6.54	6.62	6.59	6.61	0.01	0.156	0.295
TVFA (mM)	40.60[a]	51.57[b]	56.91[b]	51.22[b]	1.68	0.001	0.001
Acetate (%)	68.45[a]	69.52[ab]	69.71[ab]	70.68[b]	0.28	0.004	0.910
Propionate (%)	20.11	19.89	19.41	19.64	0.19	0.305	0.576
Butyrate (%)	11.44[b]	10.59[ab]	10.88[b]	9.68[a]	0.21	0.004	0.605
Ace: Pro ratio	3.42	3.50	3.61	3.60	0.05	0.139	0.663
NH_3-N (mg/ml)	23.48	25.06	22.25	23.42	0.42	0.393	0.789
MCP (mg/ml)	0.18[a]	0.20[ab]	0.23[b]	0.23[b]	0.01	0.003	0.667

CTL = basal feed without any supplementation, LS = CTL with 0.15 mg Se per kg DM-low Se-Met, MS = CTL with 0.30 mg Se per kg DM-medium Se-Met, HS = CTL with 0.45 mg Se per kg DM-high Se-Met. CMC-ase = Carboxyl Methyl Cellulase, TVFA = Total Volatile Fatty Acids, Ace: Pro = Acetate to Propionate ratio, NH_3-N = Ammonia-Nitrogen, MCP = Microbial Crude Protein. SEM = Standard Error of the Mean. Superscript letters within the same row signify statistically significant distinctions at a significance level of p<0.05

Table 3: Effect of Se-Met supplementation on the *in vitro* gas production, kinetics and methane production

	Treatment					p-value	
Item	CTL	LS	MS	HS	SEM	Linear	Quadratic
Gas production (mL/300 mg DM)							
Incubation time (h)							
2	13.97	14.26	16.33	15.86	0.42	0.059	0.624
4	21.35	21.64	24.06	23.19	0.46	0.053	0.505
6	29.32[a]	29.41[a]	32.67[b]	31.71[ab]	0.51	0.014	0.546
8	32.08[a]	32.26[a]	35.34[b]	34.48[ab]	0.51	0.016	0.554
12	40.93[a]	41.89[a]	46.51[b]	44.99[b]	0.69	0.002	0.230
24	59.13[a]	61.06[a]	66.11[b]	65.30[b]	0.91	0.001	0.324
36	67.69[a]	67.94[a]	74.82[b]	73.92[b]	1.05	0.002	0.721
48	72.61[a]	74.23[a]	80.96[b]	80.76[b]	1.16	0.001	0.595
Gas kinetics							
a (ml)	7.27	7.05	8.56	8.38	0.43	0.240	0.978
b (ml)	69.86[a]	71.37[a]	77.19[b]	77.81[b]	1.19	0.003	0.822
c (%/h)	0.06	0.06	0.06	0.05	0.00	0.380	0.351
a + b (ml)	77.13[a]	78.41[a]	85.74[b]	86.19[b]	1.33	0.002	0.839
CH_4 production							
CH_4 (ml)	9.53	10.01	10.87	9.93	0.23	0.259	0.139
CH_4/digested DM (ml/g)	76.21	71.77	74.92	68.81	1.97	0.347	0.861
CH_4/digested OM (ml/g)	84.10	81.83	85.58	78.48	1.93	0.535	0.576

CTL = basal feed without any supplementation, LS = CTL with 0.15 mg Se per kg DM-low Se-Met, MS = CTL with 0.30 mg Se per kg DM medium Se-Met, HS = CTL with 0.45 mg Se per kg DM-high Se-Met. DM = Dry Matter, h = Hour, GP = Gas Production, a = rapidly degrading portion, b = fraction undergoing slower degradation, c = the constant rate, a + b = the potential gas. CH_4 = Methane. SEM = Standard Error of the Mean. Superscript letters within the same row signify statistically significant distinctions at a significance level of p<0.05

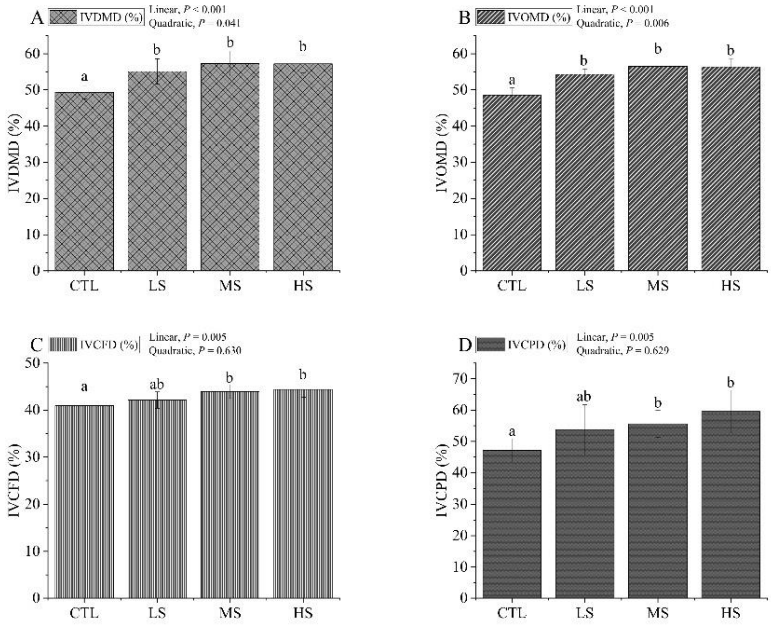

Fig. 1: The *in vitro* nutrient digestibility; (A) *in vitro* Dry Matter Digestibility (IVDMD); (B) *in vitro* Organic Matter Digestibility (IVOMD); (C) *in vitro* Crude Fiber Digestibility (IVCFD); (D) *in vitro* Crude Protein Digestibility (IVCPD). CTL = basal feed without any supplementation, LS = CTL with 0.15 mg Se per kg DM low Se-Met, MS = CTL with 0.30 mg Se per kg DM medium Se-Met, HS = CTL with 0.45 mg Se per kg DM-high Se-Met. Superscript letters within the same row signify statistically significant distinctions at a significance level of $p<0.05$

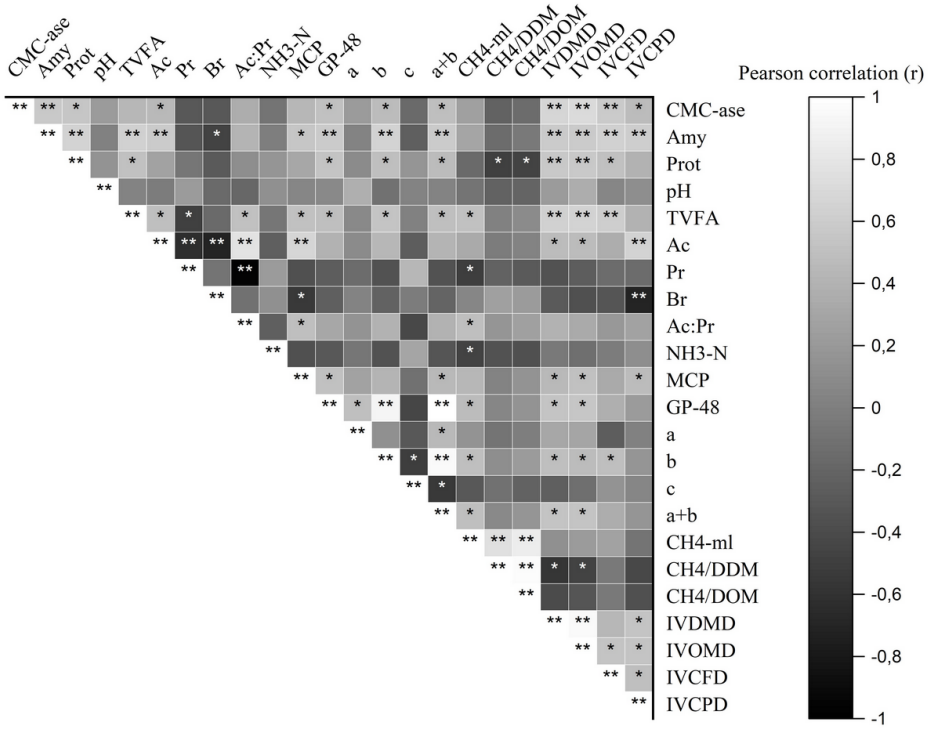

Fig. 2: Correlation between assessed *in vitro* nutrient digestibility and fermentation profile. Abbreviations = see Tables 2-3 and Fig. 1. * = correlation is significant at the 0.05 level, ** = correlation is significant at the 0.01 level

In vitro Nutrient Digestibility

The impact of organic Se supplementation on nutrient digestibility is illustrated in Fig. 1. The LS, MS, and HS groups exhibited improvement in the IVDMD, with both linear and quadratic impacts ($p<0.001$ and ($p<0.05$, respectively) in comparison to the CTL group. Comparable results were noticed, showing a linear increase ($p<0.001$) and a quadratic increase ($p = 0.006$) in IVOMD. The MS and HS groups also demonstrated a linear improvement ($p = 0.005$) in IVCPD and IVCFD compared to the CTL group.

Correlation Between Assessed in vitro Nutrient Digestibility and Fermentation Profile

The IVDMD demonstrated noteworthy positive associations with CMC-ase ($r = 0.670$), amylase ($r = 0.587$), protease ($r = 0 .567$), TVFA ($r = 0.631$), IVOMD ($r = 0.955$) ($p<0.01$), acetate ($r = 0.472$), MCP ($r = 0.453$), GP-48 ($r = 0.536$), b fraction ($r = 0.480$), a + b fraction ($r = 0.537$), IVCPD ($r = 0.521$) ($p<0.05$) and exhibited an inverse relationship with CH_4/DDM ($r = -0.572$) ($p<0.05$). The IVOMD displayed significant positive correlation with CMC-ase ($r = 0.718$), amylase ($r = 0.599$), protease ($r = 0.570$), TVFA ($r = 0.631$), IVDMD ($r = 0.955$) ($p<0.01$), acetate ($r = 0.473$), MCP ($r = 0.515$), GP-48 ($r = 0.543$), b fraction ($r = 0.472$), a + b fraction ($r = 521$), IVCFD ($r = 0.533$), IVCPD ($r = 0.532$) ($p<0.05$) and exhibited an inverse relationship with CH_4/DDM ($r = -0.463$) ($p<0.05$). The IVCFD demonstrated noteworthy positive associations with CMC-ase ($r = 0.584$), amylase ($r = 0.632$), TVFA ($r = 0.603$) ($p<0.01$), protease ($r = 0.512$), b fraction ($r = 0.482$), IVOMD ($r = 0.533$) and IVCPD ($r = 0.497$) ($p<0.05$). The IVCPD exhibited significant positive correlations with amylase ($r = 0.608$), acetate ($r = 0.654$) ($p< 0.01$), CMC-ase ($r = 0.479$), MCP ($r = 0.461$), IVDMD ($r = 0.521$), IVOMD ($r = 0.532$) and IVCFD ($r = 0.497$) ($p<0.05$) and exhibited an inverse relationship with butyrate ($r = -0.710$) ($p< 0.01$) (Fig. 2).

Discussion

In vitro Rumen Enzyme Activities and Fermentation Profile

Bacteria represent the most abundant microorganisms and hold a pivotal function in feed digestion in the rumen. Enzymes, produced and released by rumen microorganisms, are essential for breaking down the feed (Hao *et al*., 2021; Stewart *et al*., 2019). The current research observed an elevation in rumen microbial enzyme activity upon adding Se-Met to the feed, which can be attributed to Se's antioxidant properties, influencing both rumen microorganisms and enzyme secretion. Rumen microorganisms utilize the Se present in the feed to synthesize proteins and construct cellular wall constituents in selenomethionine's form. Additionally, Se is a protective agent for cell membranes, protecting them from oxidative harm by effectively neutralizing free radicals (Hendawy *et al*., 2021). Including Se supplements in sheep, feed increased Se levels, and glutathione peroxidase enzyme activities on rumen microorganisms (Čobanová *et al*., 2017). In separate research, Liu *et al*. (2019) illustrated that supplementing dairy bull diets with Se up to 0.5 mg per kg of DM substantially improved the activities of amylase and protease enzymes. Furthermore, Zhang *et al*. (2020a) showed that incorporating coated sodium selenite at 0.30 ppm to mid-lactation dairy cow rations increased ruminal CMC-ase enzyme activity by 19.53%.

One of the end products of feed fermentation in the rumen is VFA, which contributes to microbial growth, body metabolism, and animal productivity (Hao *et al*., 2021). Within this investigation, the elevation in the levels of total VFA and acetate suggested that the inclusion of dietary Se could potentially promote the microorganism's growth in the rumen and enhance enzyme activities (Amin *et al*., 2022; Wang *et al*., 2009), as supported by the increase in MCP resulting from Se inclusion to the feed (Liu *et al*., 2020). Additionally, certain enzymes, like the CMC-ase enzyme, act on the less structured portions of cellulose fibers. This enzyme will specifically break down the β-1,4-glucan bond and produce a smaller monomer (Hao *et al*., 2021). The acetate concentration response indicates improved fiber digestibility and is linked to the beneficial impact of Se addition on the activities of cellulolytic microbial enzymes. Cellulolytic bacteria release cellulolytic enzymes, breaking the feed fiber into acetate (Liu *et al*., 2019). In this study, additional Se reduced the ruminal propionate proportion. The findings were consistent with Zhang *et al*. (2020b), who conducted a study on administering coated sodium selenite with Se content doses varying from 0.10-0.30 mg per kg of DM to lactating Holstein dairy cows. They reported an increase in VFA levels, while the butyrate concentration decreased by 10.66-22.73% compared to the unsupplemented group. However, in contrast, Du *et al*. (2019) explained that the rumen fermentation pattern would lead to greater propionate production due to amylase and Ruminobacter amylophilus activities. Furthermore, the proportion of butyrate is also linked to protease activity as it is one of the degradation products of feed protein. We suspected several factors could contribute to these differences, such as feed composition, mineral content, source and form of minerals, and interactions among other minerals (Spears *et al*., 2022).

The increase in VFA levels did not coincide with changes in rumen fluid pH across the treatments. Nonetheless, the pH of the rumen media after incubation for each treatment stayed within the standard range of 5.5-7.5 (McDonald *et al*., 2011).

This research revealed that the addition of organic Se did not result in any changes in NH_3-N concentration, which is consistent with the findings of Sun *et al*. (2023), who introduced Se-yeast into the diet of deer, Almaraz-Buendia *et al*. (2018) when applied to oat hay and Faixová *et al*. (2016) when supplementing organic and inorganic Se in sheep. A segment of NH_3-N synthesis leads to the production of MCP through microorganisms, while the remainder is absorbed into the bloodstream and participates in the cycle of ruminal nitrogen. The host animals receive their protein supply from MCP (Hailemariam *et al*., 2021). According to Arshad *et al*. (2021), in the formation of MCP, rumen microorganisms utilize various nutrients, including NH_3-N, carbon chains, and Adenosine Triphosphate (ATP). The observed improvement in microbial protein synthesis due to Se inclusion might be associated with the elevated production of total VFA and the enhanced rumen bacteria population. Nonetheless, in this current research, the organic Se addition did not change the ruminal NH_3 levels, whereas the Se-supplemented group exhibited higher MCP levels. This might be due to the Se-supplemented group's microbiota having a greater capacity for synthesizing MCP. It is also consistent with the observed trend of increased MCP in response to See addition in the feed. Du *et al.* (2019) noted that the Se inclusion in dairy cows' ration led to a notable augmentation in the combined ruminal populations of bacteria and fungi.

In vitro Cumulative Gas and CH_4 Production

In this study, the GP in the Se-Met treatment showed an increasing trend from 6 h until the end of the 48-h fermentation period. The GP at 48 post-incubations positively correlated with CMC-ase, amylase, and protease activity. Furthermore, adding Se-Met in the feed increased the gas fractions b and a + b. This could be a result of incorporating Se-Met into the fermentation substrate. This inclusion enhances the antioxidant levels of microorganisms in the rumen, consequently fostering microbial proliferation and the fermentation processes. This encompasses the breakdown of soluble carbohydrates through fermentation (Zheng *et al*., 2022). Chen *et al*. (2019) also noted that the bacterial activity of rumen gas and heightened microbial functioning could impact the generation of resultant gas. Moreover, the GP is frequently linked with a more extensive breakdown of feed through fermentation processes. Similar findings were noted in the study by Dehghani *et al*. (2019) that the nano-Se inclusion improved cumulative GP at 48 and 96 h after incubation under *in vitro* conditions. The pace of GP is likewise linked to the digestibility of the feed. The cumulative gas produced, including carbon dioxide, CH_4, and other gases, represents the outcome of the fermentation process involving structural or readily digestible carbohydrates (Kara, 2019).

In this current research, the Se-Met inclusion in the feed did not significantly impact CH_4 emission. Similarly, Del Razo-Rodriguez *et al*. (2013) found that supplementing different levels of Se (0.30-0.90 ppm) on corn-based diets did not significantly alter CH_4 production among Suffolk × Dorset crossbred goats. However, Pan *et al*. (2021) demonstrated reduced CH_4 production and increased energy utilization through Se supplementation. This effect may be attributed to the elevation in propionate levels during rumen fermentation. Hendawy *et al*. (2021) explained that elevated propionate concentrations can alter a reduction in CH_4 emission, indicating improved energy utilization. Incorporating Se into the diet can affect the proportion of propionate by reducing the methanogen population. However, in this current study, no alterations in the propionate proportion were observed by adding Se until 0.45 mg/kg DM to the feed. We suspected that the level of Se supplementation provided was still relatively low to have a significant impact on CH_4 emissions. Tian *et al*. (2022) observed that a higher level of organic Se (2.4 mg/kg) from Se-yeast decreased CH_4 emissions and impacted microbial diversity and the microbial metagenome in goats.

In vitro Nutrient Digestibility

A higher *in vitro* nutrient digestibility corresponds to a higher concentration of total VFA and is associated with an increase in MCP and enzyme activity with organic Se supplementation. This is also supported by correlations between nutrient digestibility and various fermentation parameters, including enzyme activities. (Fig. 2). The IVDMD, IVOMD, and IVCFD were positively correlated with CMC-ase, amylase, and protease enzymes. Similarly, a finding reported by Shi *et al*. (2011) indicated that Se inclusion in the feed elevated ruminal fermentation processes and feed conversion due to the enhanced microorganism's abundance and activity of several enzymes. Another form of organic Se, such as Se-yeast, improved lactating dairy cows' DM digestibility. Indeed, the supplementation of Se has been linked to a heightened presence of cellulose-degrading bacteria (Arshad *et al*., 2021; Hendawy *et al*., 2021). Therefore, introducing Se could potentially impact the proportional prevalence of *Prevotella_1* and *Ruminococcaceae_UCG-005*, leading to an augmentation in rumen fermentation processes (Zheng *et al*., 2022). Alimohamady *et al*. (2013) concluded that organic Se from Se-yeast exhibited greater effectiveness in enhancing nutrient digestibility than inorganic form in female goats. Using Se-yeast at 0.30 mg of Se per kg DM in ration improved DM digestibility, CP digestibility, and CF digestibility compared to the unsupplemented group in goats (Samo *et al*., 2018). The

ability of microorganisms to counteract oxidative damage will increase, accompanied by an increase in the antioxidant capacity of microorganism cells due to the addition of Se (Matthews *et al*., 2019).

Conclusion

In conclusion, this research indicates that supplementation of Se-Met, as organic Se, at 0.30 and 0.45 mg of Se per kg DM could impact rumen enzyme activities, fermentation patterns, and nutrient digestibility *in vitro*. Nevertheless, the Se supplementation did not demonstrate the ability to reduce CH_4 gas emissions at these doses.

Acknowledgment

The authors extend their acknowledgment to the PT. Agromix Lestari (Kalijeruk Farm) for their role in providing materials for this study.

Funding Information

This study was supported by the National Research and Innovation Agency (BRIN)-Research, Indonesia Endowment Fund for Education (LPDP) Ministry of Finance Republic of Indonesia. Innovation for Advanced Indonesia (Riset dan Inovasi untuk Indonesia Maju, RIIM Batch III) (No: B-733/II.7.5/FR.06/4/2023).

Author's Contributions

Moh. Sofi'ul Anam: Responsible for the design, conducted laboratory analyses, data analysis and initial manuscript preparation.

Andriyani Astuti: Contributed to the methodology and data analysis.

Budi Prasetyo Widyobroto: Contributed to data analysis and reviewed of the manuscript.

Gunawan: Contributed to the manuscript reviewed and revision.

Ali Agus: Responsible for the design, data analysis, and manuscript reviewed.

Ethics

This article presents unique content that has yet to be previously published. The corresponding author verifies that all co-authors have reviewed and endorsed the manuscript, with no ethical concerns arising. The author ensures that the methods employed in this research have received approval from the Research Ethics Committee at Universitas Gadjah Mada, Indonesia (No: 025/EC-FKH/Eks. /2023).

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