Increasing propionate acid production in Bali cattle through ZnSO4 and Zn-Cu isoleusinate supplementation as a strategy to mitigate methane gas production

E Hartati1, A Saleh1, E D Sulistijo1, G Oematan1, I Benu1 and Y L Henuk2*
1Faculty of Animal Science, University of Nusa Cendana, Kupang, East Nusa Tenggara, Indonesia.
2Department of Animal Science, Faculty of Agriculture, Universitas Sumatera Utara, Medan, Sumatera Utara, Indonesia.

E-mail: *profesorhenuk@gmail.com

Abstract. The study aimed to evaluate zinc sulfate and Zn-Cu isoleusinate supplementation with concentrates on rumen fermentation of Bali cattle. The experiment design used randomized block design. The animal randomized, assigned into four groups of treatment diet. They were T0 = Ammoniated kume grass standing hay + concentrate (60:40); T1 = T0 + 150 mg ZnSO4/kg DM concentrate + 1% Zn-Cu isoleusinate; T2 = T0 + 150 mg ZnSO4/kg DM concentrate + 2% Zn-Cu isoleusinate; and T3 = T0 + 150 mg ZnSO4/kg DM concentrate + 3% Zn-Cu isoleusinate. Supplementation zinc sulfate and Zn-Cu isoleusinate in the concentrate did not increase significantly NH3 concentration, total VFA, acetate, and butyrate production. However, supplementation zinc sulfate and Zn-Cu isoleusinate increase significantly (P<0.01) propionate production, decrease propionate and acetate ratio. The best of rumen fermentation achieved at level of ZnSO4 and Zn-Cu isoleusinate supplementation 150 mg ZnSO4 kg⁻¹ diet. It can concluded supplementing Bali cattle with combination of 1.5% lemuru oil, 150 mg ZnSO4/kg DM concentrate and 2% Zn-Cu isoleucinate/kg DM ration had increased NH₃, VFA concentrations, C3 production, but decreased C2 and C2/C3 ratio which positively correlated with decrease in CH₄ gas production.

1. Introduction
Bali cattle are the predominant commodity in East Nusa Tenggara (ENT) Province of Indonesia due to their ability to adapt to low-quality feed [1]. The activity fattening of male Bali cattle in ENT Province had been conducted for generations. However, innovation to improve the quality of the feed on fattening had not much to do. Farmers still rely on forage as a single feed regardless of the balance of required nutrients for cattle. Be observed from nutrients obtained by the cattle, hence the need for protein as a body builder was relatively an abundant availability in farms. It was because the forage used in the fattening process was mostly good legume fodders, including Leucaena leucocephala, Gliricidia sepium, and Sesbania grandiflora [2]. However, long dry seasons (8-9 months) affect feed availability, both quantity,
and quality, which caused the low productivity of the animals. Also, the increasing variability of climate change due to global warming has impacted the farming system, particularly food production, which in turn caused low productivity of the animals.

Providing the animals with digestible concentrate and supplemented with oil comprising unsaturated fatty acids such as ZnSO4 and Zn-Cu Isoleusinat is one of the strategies to increase rumen fermentation through propionic acid (C3) formation in the rumen and therefore reduce methane production. The previous study reported that lemuru oil contained 21.9% arachidonic acid as a precursor to the synthesis of prostaglandin E2 (Pg E2) can increase Zn absorption [3]. Inclusion of 1.5% lemuru oil/kg DM in the diet can also increase Zn SO4 absorption [4]. The activity and growth of ruminal microbial are not only depending on the protein (source of nitrogen) and carbohydrate (source of energy and carbon skeleton) but also minerals such as Zn and Cu that provided from the feed. The previous study reported that Zn was potentially a limiting factor for rumen microbial growth [5]. It has been suggested that for the growth of ruminal microbial Zinc is needed in sufficiently high amounts around 130-220 ppm [6], while for ruminant animals in the growth period zinc needed around 40-50 ppm [7]. Feed provided to the animals is not sufficient to meet the requirements of Zn for both ruminal microbes and the animal itself due to low Zn content of forage in the tropics, which around 20 to 38 mg/kg dry matter [7, 8].

The Zn content of standing hay of Kume grass is only 4.42 mg/kg of dry matter, while nutrient-dense feed (NDF) based protein sources contains 18.51 mg/kg NDF dry matter [9], indicates a deficiency of Zn for ruminal microbes and animals. Because Zn is a metalloenzyme that widely involved in carboxy of peptidase A and B enzymes and alkaline phosphatase, which plays a vital role in protein synthesis, protein digestion, amino acid absorption and energy metabolism [10], therefore a deficiency of Zn will cause both ruminal microbial growth and animal productivity. Ruminants also need mineral Cu for several enzymes involved in various functions [8] though only 1-3% is absorbed [11]. The deficiency of Cu decreased the growth of animals [12]. Although Zn and Cu can be supplemented in inorganic form, the availability of organic form is much higher in terms of biological value than ZnSO4. Zn-Cu proteinate bonds formed through bioprocess with Neurospora sp were relatively degradable by rumen microbes but could be hydrolyzed by pepsin in the post-rumen where the optimum level of Zn and Cu was at the level of 3000 ppm Zn and 500 ppm Cu during that bioprocess [13]. The increasing of fibers digestibility in the rumen depends on cellulolytic bacteria; therefore, in this study, branched amino acid (BAA), especially isoleucine, was added to the media in producing the organic Zn-Cu.

Proteolytic and amylolytic enzymes are used to hydrolyze isoleucine and substrate carbohydrates into isoleucine and simple carbohydrates through fermentation with yeast. The results of in vitro study showed that addition of Zn and Cu in the substrate bond to the carboxyl isoleucine group or pure polysaccharide from the enzyme hydrolysis of the fungus produced isoleucine Zn-Cu complex compounds which are difficult to degrade in the rumen, but can be hydrolyzed in the post rumen and provide an optimal response against rumen fermentation at a concentration level of 3000 ppm Zn and 500 ppm Cu [4]. To increase the propionic acid production and decrease C2 and C2: C3 ratio, which is positively correlated with methane gas production from Bali cattle, and the present study, therefore, was aimed at determining the concentrations of Zn-Cu isoleucinate (3000 ppm Zn and 500 ppm Cu) supplementation.

2. Materials and methods

2.1. Animals

The experiment was conducted for two months (four weeks of preparation, two weeks for the adaptation period, and two weeks for the period of data collection). Bali cattle bulls (Year I) Bali cattle heifers (Year II) were used in the study. The basal ration was determining dry matter requirements, i.e., 3 % of body weight and used consisted of standing hay “Kume” grass and concentrates with a ratio of 60: 40%. The
prepared concentrates of locally available ingredients consisted of cornmeal, rice bran, coconut cake, lemuru oil flour, salt, and premix with a protein content of 17.07% and TDN 78.16% [3]. Ingredients and chemical composition of concentrate are shown in Table 1.

| Ingredients       | Composition (%) | % CP of Feedstuff | % TDN of Feedstuff | % CP of Concentrate | % TDN of Concentrate |
|-------------------|-----------------|-------------------|--------------------|--------------------|----------------------|
| Cornmeal          | 46.25           | 10.00             | 91.00              | 0.210416667        | 42.09                |
| Rice bran         | 20.50           | 0.478472222       | 66.00              | 2.23               | 13.53                |
| Coconut cake      | 23.00           | 23.10             | 74.00              | 5.31               | 17.02                |
| Fish meal         | 8.00            | 61.20             | 69.00              | 0.229166667        | 5.52                 |
| Coconut oil       | 1.50            | -                 | -                  | -                  | -                    |
| Salt              | 0.25            | -                 | -                  | -                  | -                    |
| Premix            | 0.50            | -                 | -                  | -                  | -                    |
| Total             | 100             |                   | 17.07              | 78.16              |                      |

2.2. Experimental design
The experimental design employed was a Randomized Block Design with 4 treatments and 3 replications. The animals were randomly assigned to one of four treatments as follows: T0 = Standing day grass kume ammoniation + concentrate (60:40); T1 = T0 + 150 mg ZnSO4/kg DM concentrate + 1% Zn-Cu isoleucinate (3000 ppm Zn and 500 ppm Cu); T2 = T0 + 150 mg ZnSO4/kg DM concentrate + 2% Zn-Cu isoleucinate (3000 ppm Zn and 500 ppm Cu); T3 = T0 + 150 mg ZnSO4/kg DM concentrate + 3% Zn-Cu isoleucinate (3000 ppm Zn and 500 ppm Cu). The parameters observed are pH (pH meter), NH3 concentration (Conway Microdiffusion), total VFA (steam distillation), and partial VFA (gas chromatography). Data obtained in this study were analysed by ANOVA using the IBM SPSS Statistic for windows, version 17 software package. Duncan's test was also used to analyse the difference.

2.3. The procedure of parameters collection
2.3.1. Determination of pH and partial VFA. Rumen fluid sample was taken three hours after feeding. Rumen fluid sample was used to assess pH, NH3 concentration, and partial VFA concentration. pH rumen fluid determination used to pH meter. Partial VFA concentration was analysed using gas chromatography. Rumen fluid was taken through a stomach syringe tube with a speed of 10.000 rpm for 15 minutes at 4°C to produce "supernatant". Then, 2 ml supernatant inserted into a closed plastic tube and added 30mg 5 sulphosalicylic acid (C6H3(OH)SO3H.2H2O, rubbed it and centrifuges again on 3000 rpm speed for 10 minutes on 4°C, filter with millipore to gain clear supernatant which ready to be injected into gas chromatography.

Calculation formula of VFA concentration sample (c):

\[
\text{VFA concentration (mM) } = (\text{Sample high/standard high}) \times \text{ standard Concentrate}
\]
2.3.2. Determination of N-Ammonia concentration. N-Ammonia concentration in rumen fluid was determined by Conway micro diffusion method. Put 1 ml supernatant rumen fluid in one side of the Conway dish, and 1 ml natrium carbonate (Na₂CO₃) saturated on the other side of the dish, then the dish was placed inclined. Put 1 ml borax acid red methyl indicator and brown-green "kresol" on the center of the dish, then close it tide with vaseline cap, rubbed it so that supernatant and saturated Na₂CO₃ mixed well. Borax acid shown by color changes will cache free ammonia. After 24 hours, borax ammonia was titrated with H₂SO₄ 0.005N solution until blue color changes into reddish concentration. N-Ammonia concentration in rumen fluid can be calculated using the formula:

\[
\text{SSN-Ammonia} = (\text{ml H}_2\text{SO}_4 \times \text{NH}_2\text{SO}_4 \times 1000\text{mM})
\]

3. Results and discussion

Ammonia (NH₃) and volatile fatty acids (VFAs) production through feed fermentation occurred in the rumen and are often used to describe feed quality. High NH₃ production demonstrates that rumen microbes quickly degrade protein feed. Moreover, high VFA production describes the amount of carbohydrate polysaccharide in the feed that is easily degraded by rumen microbes. The average production of pH, NH₃, total VFA, and partial VFA (acetate; C2), propionate; C3 and butyrate; C4) in young bulls and heifers are presented in Tables 2 and 3.

**Table 2.** Average pH, production of NH₃, total VFA, acetate, propionate, and butyrate for each diet of treatment Bali cattle bulls

| Parameters          | T0          | T1          | T2          | T3          |
|---------------------|-------------|-------------|-------------|-------------|
| pH                  | 6.78 ± 0.53a | 6.88 ± 0.11a | 6.68 ± 0.18a | 6.68 ± 0.20a |
| NH₃ production (mM) | 6.06 ± 0.66a | 6.04 ± 0.75a | 6.19 ± 0.33a | 5.34 ± 0.29a |
| VFA production (mM) | 133.3 ± 6.58a | 148.68 ± 7.11a | 152.49 ± 12.4a | 171.35 ± 3.34a |
| C₂ production (mM)  | 29.13 ± 6.0a | 27.42 ± 4.38a | 30.51 ± 4.36a | 35.05 ± 1.90a |
| C₃ production (mM)  | 14.96 ± 2.21a | 17.45 ± 0.98a | 24.57 ± 4.02b | 25.23 ± 3.65b |
| C₄ production (mM)  | 1.56 ± 0.86a | 1.17 ± 0.61a | 1.04 ± 0.59a | 1.37 ± 0.35a |
| C₂:C₃ ratio         | 1.93 ± 0.15a | 1.57 ± 0.26b | 1.25 ± 0.11b | 1.40 ± 0.14b |

In the present study, supplementing Bali cattle (bulls and heifers) with ZnSO₄ and different levels of Zn-Cu isoleucinate fed ammoniated kume grass, and concentrat did not cause a significant decreased (P>0.05) of NH₃ concentration in their rumen. This indicates that supplementation of ZnSO₄ and different levels of Zn-Cu isoleucinate to Bali cattle (bulls and heifers) are resistant to degradation in the rumen, which is in line with the expectations that Zn-Cu isoleucinat is expected pass through rumen degradation and being available in the post rumen. The result of the present study is in agreement with the previous *in vitro* study [9]. However, NH₃ production in bulls and heifers with 150 mg ZnSO₄ / kg concentrate and Zn-Cu isoleucinate at 3000 ppm Zn and 500 ppm Cu at 2% tended to be higher (P <0.06) than other treatments. Supplementing bulls and heifers with 150 mg ZnSO₄/kg concentrates and Zn-Cu isoleucinate at the level of 2%, and 3% is thought to be insufficiently available C period results from polysaccharide
reshuffle so that NH3 released from protein reorganization flows to the post rumen which causes rumen microbial growth is not optimal. The N-NH3 production in the present study ranged from 5.34-6.19 mM, indicating that the amount is still sufficient to support the optimum growth of microbial and so ensuring the fermentation process.

Table 3. Average Production of NH3, VFA Total, and Profile of VFA (Acetate, Propionate, and Butyrate) for each Ration Treatment in Bali cattle heifers

| Parameters               | Treatment     |
|--------------------------|---------------|
|                          | T0            | T1            | T2            | T3            |
| NH3 (mM)                 | 6.57 ± 0.29a  | 6.16 ± 0.13a  | 5.90 ± 0.37a  | 5.83 ± 0.39a  |
| Total VFA production (mM)| 189.20 ± 39.29a | 199.51 ± 27.4a | 194.98 ± 17.3a | 195.57 ± 12.3a |
| C2 production (mM)       | 132.17 ± 29.76a | 134.92 ± 19.78a | 119.17 ± 11.68a | 119.17 ± 11.68a |
| C3 production (mM)       | 32.88 ± 5.70a  | 41.63 ± 6.18ab | 47.38 ± 6.09b  | 45.14 ± 8.2b  |
| C4 production (mM)       | 20.34 ± 0.60a  | 21.01 ± 2.05a  | 23.550 ± 5.7a  | 24.15 ± 5.47a |
| C2:C3 ratio              | 4.03 ± 0.72a  | 3.27 ± 0.46b  | 2.55 ± 0.51b  | 2.74 ± 0.37b  |

Rumen microbial growth required about 5-8 mM N-NH3/L for their maximum growth. The range of NH3 levels that can support microbial growth in the rumen is around 3.57 to 12 mM; however, the fermentation process is disrupted if the concentration is less than 3.57 mm [14, 15]. Supplementing Bali cattle (bulls and heifers) with ZnSO4 and different levels of Zn-Cu isoleucinate fed ammoniated kume grass and concentrates had a significant effect on total VFA production. This means that a combination of 150 mg of ZnSO4 / kg BK and Zn-Cu isoleucinate at 3000 ppm Zn and 500 ppm Cu had the ability to in degrading the organic matter of feed which increased total VFA production. The highest increase in total VFA production was obtained at the level of 2%, and this result was following the results of in vitro studies reported by [3,4].

This is presumably due to the optimal availability of substrate (N-NH3) in the rumen, thus increase the synthesis and growth of rumen microbes. Consequently, the digestibility of carbohydrates increases, which causes the optimum production of total VFA. Low availability of C skeleton is thought to be the limiting factor of rumen microbial fermentation, particularly for the treatment of the combination of 150 mg ZnSO4 and 3% Zn-Cu isoleucinate at 3000 ppm Zn with 500 ppm Cu and thereby resulting in low production of total VFA.

The proportion of C skeleton and C-NH3 is fundamentally crucial in determining the protein synthesis and VFA availability [16]. The highest VFA production obtained in the present study was in R2 (150 mg ZnSO4 / kg DM and 2% Zn-Cu isoleucinate at 3000 ppm Zn and 500 ppm Cu), where the VFA concentration increased 12.6% compared to the animals consuming ammoniated standing hay and concentrated without supplementation. Zn-Cu isoleucinate supplementation at 3000 ppm and 500 ppm Cu, in the present study is expected to pass through rumen degradation and is available in the post rumen and also as a branched amino acid, which may increase the growth of cellulolytic bacteria. As a result, the total VFA production produced in this treatment was higher than that without supplementation.

ZnSO4 and Zn-Cu isoleucinate supplemented to Bali cattle provide inorganic and organic minerals, which may change the VFA profile of animals in the present study where the acetic acid production (C2) did not differ between treatments. This is probably due to the isoleucine contained in Zn-Cu isoleucine
that increases the role of both cellulolytic bacteria and non-cellulolytic bacteria [18]. As a result, Zn-Cu isoleucinate supplementation at 3000 ppm Zn and 500 ppm Cu did not differ between treatments (C2) since the microbes also digested other non-fiber feed. In contrast, feeding Bali cattle (bulls and heifers) with ZnSO4 and different levels of Zn-Cu isoleucinate fed ammoniated kume grass and concentrates had a significant effect (P<0.01) on propionic acid (C3) but decreased acetate: propionate concentration (C2/C3) which positively correlated with CH4 production. This indicates that feed fermentation occurred in the rumen and was associated with propionic acid production, which is essential as the primary source of glucose formation in ruminants.

Approximately 50% of glucose in ruminants derives from propionic acid [17], which is the primary energy source for essential organs such as the brain, nerves, mammary glands, and fetus. Therefore, supplementation that can increase propionic acid synthesis is essential not only to reduce CH4 gas production but also beneficial for ruminants since it can increase the availability of energy for the production process. The increase in energy availability may due to lemuru oil contained in concentrates, which increases the production of propionic acid and thus reducing energy loss in the form of methane as reported by [3,4].

The results of the present study are in agreement with [3,4] where concentrates (16% CP, 70% TDN, lemuru oil 1.5%/kg DM and 75 mg ZnSO4 / kg DM rations) fed to cattle had decreased acetic acid production and conversely an increased in acid production propionate, decreased C2/C3 ratio and NGR number due to addition of 1.5% lemuru oil. The NGR number is positively correlated with methane (CH4) gas production. Therefore, the decrease in the C2/C3 ratio and NGR reflects a decrease in methane gas production. Furthermore, it was reported that the energy wasted through methane gas production was reduced by 41% compared to the animals without lemuru oil supplementation.

4. Conclusions

It can be concluded that supplementing Bali cattle (bulls and heifers) with a combination of 1.5% lemuru oil, 150 mg ZnSO4/kg DM concentrate and 2% Zn-Cu isoleucinate/ kg DM ration had increased NH3, VFA concentrations, and C3 production, but decreased C2 and C2/C3 ratio which was positively correlated with the decrease in CH4 gas production.

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