Effect of Micronutrient Supplementation to Reduce Heavy Metal Toxicity in Rations from Local Feed Ingredients *in vitro* using Rumen Fluid of Ettawa Crossbreeds

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ABSTRACT
Poor environmental management including overexploitation of natural resources, use of pesticides, industrial waste and exhaust from motor vehicle combustion have caused high atomic weight and polyvalent (heavy metal) minerals to dissolve easily and their availability in and above the soil surface and the environment increases. Furthermore, these conditions decrease soil acid-base quality and can contaminate various plant products consumed by humans or animals. These result in a higher prevalence of heavy metal toxicity in plants, livestock, and even humans. Previous studies have been reported to increase the levels of Pb contained in livestock meat. If plants and livestock as human food sources have been contaminated by heavy metals, they will have a very fatal impact on human health. This study was conducted to evaluate the use of Zeolite minerals and organic minerals (Cr-organic) of the rations to reduce the malignancy of heavy metals in vitro using goat's rumen fluid. This study used a completely randomized design with 5 replications to test 4 types of treatment rations, namely: R1 (unprocessed cocoa pod-based ration), R2 (ammoniated cocoa pod-based ration), R3 (R2 + zeolite 1.5%), and R4 (R3 + organic chromium). The observed variables consist of fermentation characteristics (fermentation pH, production of total VFA and N-NH3) and digestibility of feed nutrients (digestibility of dry matter and organic matter). The results of the first study (in vitro) show that the addition of zeolite minerals and organic minerals in the diet containing heavy metals did not have a significant effect on the fermentation characteristics (pH, total production of VFA and N-NH3) and nutrient digestibility (digestibility of dry matter and organic matter).

Keywords: *heavy metal, local feed, micro nutrient, in vitro, ettawa crossbreed.*

1. INTRODUCTION

Poor environmental management including overexploitation of natural resources, use of pesticides, uncontrolled disposal of industrial waste, and exhaust of vehicle combustion have increased the accumulation and toxicity of heavy metals in various food products. Overexploitation of natural resources causes erosion, leading essential minerals (low valence and small atomic weight) to be washed away, while heavy metals (polyvalent and large atomic weight) appear on the surface and remain on the soil surface. This condition causes the potential Hydrogen (pH) of the soil to become acidic which finally increases the availability and solubility of heavy metals. The decrease in the pH of rainwater due to environmental pollution causes the soil pH to drop dramatically to 3. This certainly has implications and has a high potential for metal toxicity in plants (forage for livestock) and animals. [1] reported that the levels of heavy metal (Pb) in beef in Solo, Central Java, were based on age, namely at 1.5-years-old of 2.15 ppm, 2.5-years-old of 2.07 ppm, and 3.5-years-old of 2.24 ppm. It was also reported that the levels of Pb in the kidneys, liver and intestines were

2.28 ppm, 2.51 ppm, and 2.67 ppm, respectively. Whereas the lead content threshold set by the Indonesian National Standard is 0.3 ppm in food and 0.5 ppm in meat and milk [2].

Linder [3] reported that when Pb enters the body, it will be distributed through the blood, almost all of which enter the erythrocytes. About 90% of Pb will be stored in the bones and the rest in fatty tissue, especially the liver and kidneys. [4] Reported that it can accumulate in bones because Pb^{2+} ions can replace Ca^{2+} ions, causing calcium deficiency. The high doses will cause anemia, kidney failure, high blood pressure and damage to brain tissue and mental disorders. The lead on the brain can cause hyperactivity and problems with personality disorders in children, as well as mental retardation [5]. The risk of toxicity in children is greater given the higher absorption in children. Lead (Pb) contained in the meals for adults will be absorbed as much as 5-10%, while in infants and children it will be up to 40% and will stimulate P and Ca deficiency.

The essential mineral deficiency in livestock is relatively easier to be handled than heavy metal toxicity. In this case, mineral supplementation can be used to directly address the deficiency. However, the malignancy and toxicity of heavy metals require a special approach since heavy metals that enter the body will be difficult to remove. According to [3], the malignancy of heavy metals can be fought through the implementation of minerals against minerals at pharmacological doses and the provision of high protein and vitamins that function as redox coenzymes (oxidation-reduction). The excess minerals, in addition to their normal functions, will also act as drugs, which help the body fight heavy metals. The administration of excess protein is expected to increase the synthesis of metallothionein which has a high affinity for heavy metals so that heavy metals will be excreted through feces and urine.

Referring to the aforementioned background, an effort was made to reduce the severity of heavy metal toxicity through the manipulation of nutrients in the ration. The approach used zeolite-amino acid complex (zeolite-methionine) and organic minerals (Cr-organic). The use of zeolite-amino acids as an agent is to reduce the toxicity of heavy metals based on the physical properties of zeolites that can be used to bind heavy metals. This property is its ability to act as a cation exchanger. Heavy metal ions are expected to replace ions in zeolite so that heavy metals are not deposited in the body but will be excreted through feces. The use of zeolite-amino acid complex in addition to supplying bypass amino acids because the amino acids that enter the rumen will be remodeled into N-NH3 [6]. The presence of zeolite-amino acid bonds makes more amino acids are available to synthesize metallothionein to reduce heavy metal accumulation as well as to increase mineral availability in reducing heavy metals in livestock products (milk and meat). The study was conducted to evaluate the use of Zeolite minerals and organic minerals (Cr-organic) in the rations to reduce the malignancy of heavy metals in the rumen fluid of the ettawa crossbreed.

Table 1. Composition of raw materials of ration in vitro experiments

Composition	Treatment			
	R1	R2	R3	R4
a. Raw Materials				
Grass (%)	60.00	60.00	60.00	60.00
Corn Yellow (%)	25.00	25.00	25.00	25.00
Sago pulp (%)	8.00	8.00	6.00	5.80
Rice bran (%)	7.00	7.00	7.50	7.50
Pb (ppm)	0.00	3.00	3.00	3.00
Zeolite (%)	0.00	0.00	1.50	1.50
Cr-Organic (%)	0.00	0.00	0.00	0.20
b. Ration nutrients				
Crude protein (%)	10.55	10.55	10.55	10.55
Crude fat (%)	3.47	3.47	3.47	3.47
Crude fiber (%)	18.35	18.36	18.20	18.17

Where: R1 (basal ration); R2 (basal ration containing 0% Zeolite and 3 ppm heavy metal Pb; R3 (basal ration containing 1.5% Zeolite and 3 ppm heavy metal Pb), R4 (basal ration containing 1.5% zeolite and 0.2% Cr-organic and 3 ppm heavy metal Pb

2. MATERIALS AND METHODS

This study was carried out in the Laboratory of Nutrition Science and Feed Technology and the Field Laboratory of Ruminants Livestock, Faculty of Animal Science, Halu Oleo University, Kendari. The samples were analyzed in the Laboratory of Nutrition Science and Feed Technology. The in vitro study used a completely randomized design with 4 treatments and 5 replications, i.c.: (i) R1 (basal ration); (ii) R2 (basal ration containing 0% Zeolite and 3 ppm Pb; (iii) R3 (basal ration containing 1.5% Zeolite and 3 ppm Pb, (iv) R4 (basal ration containing 1.5% zeolite and 0.2% Cr-organic and 3 ppm Pb). The composition and nutrient content of the in vitro experimental treatment rations are presented in Table 1.

The source of microbial inoculum used in the in vitro study was goat PE rumen fluid. Each treatment was carried out 5 times based on the period of rumen fluid collection (group). The fermentation variables consisting of levels of N-NH_3 and total VFA production, nutrient digestibility (digestibility of dry matter and organic matter) rations were measured.

2.1. Production of organic mineral

Six hundred grams of cassava was thinly sliced then added with 400 ml of mineral water of chromium and selenium. The cassava was autoclaved for 45 minutes or cooked and then placed in a plastic container. When it cold, 0.5 grams of tryptophan and *Saccharomyces cerevisiae* were added. Then it was incubated for 3 days and dried in an oven at 40°C until dry and then ground. The sample was taken for analysis of mineral content incorporated into yeast protein using an Atomic Absorption Spectrophotometer (AAS). One gram of the sample was put into a tube and 10 ml of 20% Titrocholoro Acetic Acid (TCA) solution was added. The tube was centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded, the precipitate obtained was weighed as much as 0.8 grams and put into a digestion flask and then 10 ml of concentrated HNO_3 was added. The flask was heated first over low heat and increased. The solution was allowed to boil for 5 minutes. After the cold solution was added, 2 ml of concentrated H_2SO_4, 2 ml of 70% $HClO_4$ and 0.2 ml of 10% $AgNO_3$ were added, and reheated until the solution was clear. The clear solution was read for its Cr mineral content using AAS as the Cr mineral incorporated in yeast. Based on these levels, it was supplemented into the ration according to the needs of the livestock.

2.2. In Vitro Fermentation

After organic mineral products were produced, ruminant ration formulations were made and in vitro ration fermentability testing was carried out. In vitro fermentation was carried out using the [7]. Samples of treatment ration (50 mg) and 40 ml of McDougall's solution were put into a 100 ml fermentation tube. In each tube, 10 ml of ettawa crossbreed rumen fluid was added, stirred slowly, CO2 gas flowed, and incubated in a shaker bath at 39^0C. The rumen fluid of ettawa crossbreed as a source of bacterial inoculum was taken. After 4 hours, the goat was fed in the morning by mouth using a stomach tube connected to a vacuum pump. Variable NH3, Total VFA in fermented liquid samples in fermenter tubes was taken 4 hours after incubation. The measurement of dry matter and organic matter digestibility was carried out by incubating the fermenter tube containing the treatment sample in a shaker bath at 39^0C for 48 hours.

Analysis of the nutritional content of the ration consisting of dry matter, organic matter, crude protein, crude fiber and crude fat using the [8]. Total Digestible Nutrient content is calculated referred to [9]. Total VFA production [9] and nutrient digestibility were measured using the method proposed [7].

The collected data were analyzed using Analysis of Variance (ANOVA) of SPSS 16 with 5% and 1% confidence levels. If there was a significant effect, then continued with the Duncan's Multiple Range Test (DMRT).

3. RESULTS AND DISCUSSION

3.1. Fermentation Characteristics

The result showed that the concentration of NH_3 and VFA total of rumen fluid was not significantly different (P>0.05) among the treatments (Table 2).

The production of N-NH_3 was a compound that resulted from the protein digestion process by fermenting microorganisms in the rumen. The conversion of protein into amino acids and peptides was further degraded by rumen microbes, mostly producing intermediate products in the form of ammonia (NH_3). The addition of zeolite and organic chromium in the ration containing heavy metal Pb did not significantly different (P>0.05) on the concentration of NH_3. The average concentration of NH_3 in all treatments ranged from 5.16-5.82 mM, which was lower than [6] the normal level of NH_3 for the fermentation process in the rumen was 6-21 mM. Insufficient levels of N-Ammonia in the fermentation process would have implications for growth and microbial activity to degrade feed during the fermentation process [10]. Ammonia levels were very important as a nitrogen source [11] [12] and the main nitrogen source for microbial protein synthesis in the rumen [13]. The low concentration of N-NH_3 with the addition of 3% heavy metal Pb in the ration might be caused the effect of Pb mineral on several bacterial species such as *Bacteroides succinogenes*, *Ruminococcus albus*, *Bacteroides amylophilus*, and *Eubacterium ruminantium* [14] which play a very important role in the process of feed protein degradation. Based on its toxicity rating based on its inhibition against gas formation, Pb is the most toxic element, followed by Arsenate (As), Cadmium (Cd) and mercury (Hg) [14].

The low ammonia levels in this study were probably due to the inhibition of the population and activity of the bacteria *Ruminococcus albus* and *Bacteroides amylophilus* that play a major role in protein degradation to be ammonia [15] during fermentation process. The exposure of Pb reduced the level of degradation in large particles because of toxicity to the microorganisms [16]. [17] reported that heavy metals can also inhibit several enzymes in the rumen. The disruption of these enzymes in the fermentation process certainly has implications for the production of fermentation products such as pH, ammonia, total VFA levels and the microbial population. The results show that Pb exposure had an impact on rumen metabolism through the formation of organometallic lipid components that inhibit the growth and respiration of microorganisms [18] and reduce the production of total gas and energy and fatty acid production [19].

The combination of the addition of zeolite (1.5%) and organic chromium (2.0%) based on the dry matter of the ration did not affect microbes and in vitro incubation ecosystems. The combination of zeolite (1.5%) and organic chromium (2.0%) in the ration also did not have a significant effect on the total production of VFA during the in vitro incubation process. This is indicated by the average total VFA production achieved 140.56-161.20 mM. The volatile fatty acids (VFA) were the result of the degradation of feed carbohydrates [20]. Most of the carbohydrates are broken down into VFA, CO_2, and methane (CH4).

The total VFA concentration produced is in the optimum range (140.56-161.20 mM) to provide energy and a carbon skeleton source for microbial growth during the in vitro fermentation process. However, the concentration of $N\text{-}NH_3$ has not been able to optimally provide ammonia compounds as raw materials for the synthesis of certain amino acids which are used as precursors of protein-forming microorganisms. Although ruminants can use NPN as a source of N for microbial amino acid synthesis, they still need amino acids because basically livestock cannot synthesize amino acids. The non-optimal concentration of $N\text{-}NH_3$ obtained during the incubation process resulted in the available N source due to the lack of -keto acid for transaminases to synthesize amino acids. This condition implies the lack of essential amino acids for the growth of microorganisms such as methionine (Met), leucine (Leu), isoleucine (Ile), valine (Val), lysine (Lys), and threonine (Thr). The essentiality of these amino acids is based on the transfer of Met and branched amino acids (Leu, Ile, Val) into rumen microbial protein which is large enough to account for one third of the portion. The carbon skeletons of the amino acids lysine and threonine are remodeled in the rumen, making them impossible to use in the de novo synthesis of lysine and threonine. Meanwhile, ketothreonine acid was not found in the rumen or digesta samples [21]. In addition to these six types of amino acids, ruminants also need aromatic amino acids, namely phenylalanine (Phe) and tryptophan (Trp), and a number of semi-essential or co-essential amino acids [22].

3.2 Digestibility of dry matter and organic matter

Data on dry matter and dry matter digestibility and organic matter digestibility of ration containing heavy metal Pb supplemented with a mixture of zeolite-organic chromium minerals at different levels in vitro are presented in Table 3.

The use of zeolite and organic chromium in feed containing heavy metal Pb had no significant effect (P>0.05) on dry matter digestibility and organic matter digestibility. The data in Table 3 shows that the average

Table 2. Characteristics of in vitro fermentation of rations using inoculum sources for Ettawa crossbreeds

Treatment	$N\text{-}NH_3$ (mM)	VFA total (mM)
R1	5.82	161.20
R2	6.16	145.92
R3	5.36	141.98
R4	5.44	140.56
Total	22.78	589.66
Average	5.70	147.42

Treatments where: R1 (basal ration); R2 (basal ration containing 0% Zeolite and 3 ppm heavy metal Pb; R3 (basal ration containing 1.5% Zeolite and 3 ppm heavy metal Pb), R4 (basal ration containing 1.5% zeolite and 0.2% Cr-organic and 3 ppm heavy metal Pb)

Table 3. Digestibility of ration nutrients in vitro using rumen fluid inoculum of Ettawa crossbreed

Treatment	Dry matter digestibility (%)	Organic matter digestibility (%)
R1	52.92	50,17
R2	52.11	49,17
R3	48.99	45,97
R4	51.67	48,95

Treatments were : R1 (basal ration); R2 (basal ration containing 0% Zeolite and 3 ppm heavy metal Pb; R3 (basal ration containing 1.5% Zeolite and 3 ppm heavy metal Pb), R4 (basal ration containing 1.5% zeolite and 0.2% Cr-organic and 3 ppm heavy metal Pb.

dry matter digestibility and organic matter digestibility feed ranged between 48.99%-52.92% and 45.97-50.17%, respectively. The results of nutrient digestibility (both dry and organic matter) obtained in this study may be one of the implications of the non-optimal conditions of fermentation characteristics obtained during the in vitro incubation process. The high value of both dry and organic matter digestibility in the rumen fermentation process is closely related to the population and activity of the rumen micro flora [23].

The average dry matter and organic matter digestibility values obtained in this study were not optimal. This is possibly because there was no balance between energy availability (VFA production) and the availability of N sources (N-NH_3 concentration) to support population development and microbial activity during the fermentation process. Some of the conditions for these microorganisms to live in the rumen include a) rumen pH between 5.5 – 7.0, b) anaerobic conditions (strictly anaerobic), (c) temperature, 38 – 40^oC, d) presence of fermentation products and residues from ingredients food, and e) presence of food ingredients available to the landlady [24].

The digestibility values of dry matter and organic matter nutrients in all treatments were relatively the same, indicating that the addition of organic zeolite-chromium minerals in the ration with the levels used in the study had not been able to suppress the effect of Pb toxicity on rumen microbes [16]. The depressed microbial population due to Pb toxicity further reduces the contribution of rumen microbes in degrading feed particles which has implications for the non-optimal digestibility of dry matter and organic matter. The effect of Pb toxicity on the population and activity on feed fermentation will certainly reduce the production of VFA organic acid compounds in the rumen. It is known that the availability of VFA products, especially acetic acid, is very important to provide a carbon skeleton that supports the synthesis of microbial cells. As reported [25] that the low concentration of total VFA due to the presence of heavy metal Pb can be understood because the increase in heavy metal Pb^{2+} in rumen culture media will reduce the proportion of acetic acid. In addition, [16] reported that exposure to Pb in the ration can reduce the digestibility of large feed particles due to toxicity to rumen microorganisms. Furthermore, the total production of VFA and the total gas production were lower in the rumen fluid contaminated with Pb compared to the feed that did not contain Pb.-Based on the phenomenon, it seems necessary to improve the concentration of organic zeolite-chromium minerals in the ration with a higher level so that its effectiveness in optimizing its role in suppressing Pb toxicity to microorganisms and the rumen ecosystem is necessary.

4. CONCLUSION

Nutrient manipulation by adding zeolite and organic minerals to rations containing heavy metals has not been able to increase the concentration of VFA, NH_3, organic matter digestibility, and organic matter digestibility.

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