JEE Journal of Ecological Engineering

Journal of Ecological Engineering, 2026, 27(9), 512–524
https://doi.org/10.12911/22998993/220460
ISSN 2299–8993, License CC-BY 4.0

Received: 2026.02.17
Accepted: 2026.07.15
Published: 2026.08.01

Culturing rumen fluid-derived local microorganisms in media with varying C/N ratios: evaluation of growth, proteolytic activity, and ammonia reduction in chicken manure

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ABSTRACT

This research aims to optimize the growth of local microorganisms from rumen fluid waste cultured in medium with different C/N ratios and to evaluate ammonia removal from chicken manure using local microorganisms as starters. Rumen fluid waste, molasses, and tofu dregs were used in this study. Local microorganisms (LMOs) production was carried out by using three treatments of cultured growth medium with different carbon-to-nitrogen (C/N) sources formulated by molasses:tofu dregs liquid that is P1 (1:1), P2 (1:2), and P3 (1:3). Then, all treatment media were compared to a control P0 (natural rumen fluids). The results showed that P1, P2, and P3 had various initial C/N ratios of 25.30±1.00, 24.46±0.84, and 23.94±0.94, respectively. The local microorganisms product was represented by colonies through total plate count: P1 (4.17±0.60)×10^8 CFU/mL; P2 (3.58±0.06)×10^8 CFU/mL; P3 (0.41±0.24)×10^8 CFU/mL, and total lactic acid bacteria: P1 (1.37±0.40)×10^8 CFU/mL; P2 (2.90±0.40)×10^8 CFU/mL; P3 (0.41±0.26)×10^8 CFU/mL, whereas total coliforms were not detected. Next, the LMO exhibited proteolytic activity, as indicated by a clear zone in the growth of the plate media containing 1% skim milk. Moreover, the application of LMO to evaluate its ability was inoculated to the layer chicken manure, resulting in a significant ($P<0.05$) decrease in ammonia levels compared to the control (6463.52 ppm). Treatment P1 decreased the ammonia level at 4165.32 ppm, which indicated removing about 35.5% of the ammonia concentration. Finally, we concluded that preliminary laboratory results indicate potential local microorganisms from rumen fluid that have been cultured with a 1:1 carbon-to-nitrogen source provide optimal conditions for microorganisms growth and can be used to reduce the ammonia in chicken manure significantly; therefore they can be used as a potential bioactivators for livestock waste processing.

Keywords: ammonia, chicken layers excreta, C/N ratio, organic waste, rumen fluid, starters.

INTRODUCTION

The livestock industry generates large amounts of waste, especially from slaughterhouses, which can pollute the environment if not managed properly. Slaughterhouse waste contains various pollutants sourced from livestock's internal organs, which can cause pollution if discarded without being processed (Selormey et al., 2021; Trisnaningsih et al., 2025). One of the main types of waste from cows is cow's stomach contents, which consist of ruminant stomach fluids in the form of rumen, reticulum, omasum, and abomasum. The rumen is the most significant part of the ruminant stomach, producing a higher amount of ruminant stomach waste compared to other organs (Cherdthong, 2020). Rumen fluid waste contains microorganisms that can be utilised as a local microorganism (LMO) product to

treat waste generated from livestock activities (Sarteshnizi et al., 2020). The rumen contains various microorganisms, such as archaea, bacteria, yeast, and protozoa, which can be utilized as sources of microbes for processing manure, compost, and liquid organic fertilizers (Wei et al., 2022). One of the beneficial uses of rumen fluid is as a source of microbes that can be utilized to produce probiotic products (Reuben et al., 2022) as local microorganisms (LMOs). The use of probiotics is based on their ability to degrade complex organic compounds and suppress pathogenic microorganisms. The application of local microorganisms cultures has been widely developed for waste management, water, and sanitation. Belanche et al. (2019) stated that rumen fluid waste from ruminants, such as cattle and sheep, is being developed as a source of microorganisms for the production of local probiotic microorganisms. Lesik et al. (2019) stated that rumen fluid, which contained nutrients, microbes, and undigested food, was potential to be recycled into local microorganisms, enhancing nutrient content such as nitrogen, phosphorus, and potassium through microbial fermentation processes.

Microorganisms require carbon (C) and nitrogen (N) as essential nutrients for their growth. Carbon serves as an energy source for microorganisms, while nitrogen plays a significant role in increasing microbial populations (Pan et al., 2023). The C/N ratio indicates the nutritional level of a raw material, therefore microorganisms require an appropriate C/N ratio for metabolic processes (Anand et al., 2013). In this study, the carbon sources can be found in molasses used, and the nitrogen sources in tofu dregs fluids used. A suitable balance of carbon and nitrogen sources can optimize microbial growth (Silva-Sánchez et al., 2019). Pastawan et al. (2025) stated that a proper C/N ratio facilitates effective microbial decomposition of organic matter, leading to nutrient-rich and stable fertilizer products through aerobic and anaerobic conditions. Rumen fluid contained various microbes (Sarteshnizi et al., 2018), that could be applied to livestock waste to manage the pollution caused by ammonia. Ammonia (NH_3) emissions from livestock farms, particularly from animal manure waste, amount to 30 million metric tonnes, representing 64% of global anthropogenic NH_3 emissions (Ashworth et al., 2020). Chicken excreta produce significant ammonia emissions that can cause air pollution and reduce air quality (Seidavi et al., 2019). Microorganisms can utilize the organic matter in excreta as an energy source for growth, thereby degrading excreta through microbial activity. Moreover, the other use of local lactic acid bacteria to process the chicken manure could obtain the unconventional feed for catfish (Paradhipta et al., 2025). The results of a study by Ashayerizadeh et al. (2017) show that local culture bacteria comprising various microbes can break down chicken excreta through fermentation, increasing non-protein nitrogen (NPN), although the functional role of microorganisms needs to be further explored by the isolation and genome sequencing (Arista et al., 2025; Nakamura et al., 2022).

Research on optimizing the C/N ratio medium on the growth of rumen fluid waste as a local microorganism agent for livestock organic waste processing is still rare. Therefore, the use of rumen fluid waste to be cultured in medium with different C/N ratios should be evaluated to determine its potential use as a source of local microorganism production in organic waste processing, including livestock manure. Adding C and N sources to achieve an optimal C/N balance is necessary to support microbial growth and productivity (Zhu et al., 2021). In an effort to explore and evaluate the effectiveness of local microorganisms sourced from rumen fluid, it is necessary to apply the local microorganisms product to livestock waste, such as chicken manure. This study aims to optimize the growth of local microorganisms from rumen fluid waste cultured in medium with different C/N ratios and to evaluate ammonia removal from chicken manure using local microorganisms as starters.

METHODS

Rumen fluid preparation

Rumen fluids as a source of microorganisms were collected from the cow slaughterhouse in Yogyakarta, Indonesia. Rumen were taken from the Ongole breed under room temperature conditions and stored for 50 mL in a sterile conical tube. The rumen fluids were then immediately stored at refrigerator temperature (4 °C) to prevent contamination.

Local microorganisms medium producing preparation

Molasses, tofu dregs, and bottles are prepared. Molasses and tofu dregs are sterilized in an autoclave as formulated medium at 121 °C and 15 psi for 15 minutes to make the medium sterile and free of contamination by other bacteria. Furthermore, total organic C and total N were measured for each culture medium, serving as the initial C/N ratio for molasses and tofu dregs. The medium treatment with different C/N ratios was prepared using a mixture of molasses and tofu dregs in triplicate: P1 (1:1); P2 (1:2); P3 (1:3); and P0 (rumen fluids as the control). Then, the treatment medium was tested for C-organic content, total N, and the initial C/N ratio simultaneously to determine different C/N ratios for each treatment medium.

Preparation of solid and liquid culture media

Rumen fluids are filtered and prepared as a source of microorganisms for cultivation. Agar medium was made by adding 2.8 g of nutrient agar (NA) to a 100 mL beaker and then adding 100 mL of distilled water. The agar medium solution was transferred to an Erlenmeyer flask for further sterilization at 121 °C and 15 psi for 15 minutes. After that, the agar medium is poured into a petri dish and stored in the refrigerator as a solid medium stock. The solid medium stock will later be used to preculture rumen microorganisms using the spread/streak method. A liquid medium was prepared by dissolving 18.2 g of nutrient broth (NB) in 1400 mL of distilled water in a beaker. The liquid medium stock was poured into 36 test tubes, each containing 5 ml of NB 100/100 medium. 12 Erlenmeyer flasks were prepared, each containing 100 mL of NB 100/100 medium. The test tube and Erlenmeyer flask are then sterilized by using an autoclave.

Preculture of rumen microorganisms

Rumen fluids are inoculated into an agar medium prepared by the streak method to grow microorganisms. Next, 10 µl of rumen fluid was inoculated into each test tube containing a stock of 100/100 NB medium. The isolate was incubated on a shaker at 120 rpm for 24 hours at 28 °C. After 24 hours, the preculture propagation of rumen microbial isolates was continued by inoculating them in 100 ml Erlenmeyer containing 100/100 NB medium and incubating on a shaker at 120 rpm for 24 hours at 28 °C. Isolate culture was measured by determining the optical density (OD) of bacterial growth at 600 nm on a spectrophotometer to equalize the concentration of the microorganism used for inoculation in the molasses-and-tofu-dregs medium used to prepare the local microorganism product.

Culture of local microorganism production

The cultures of isolated microorganisms are inoculated into 1 L bottles for each treatment (P1, P2, P3) that were prepared previously. Fifty mL of cultures of isolated microorganism (determined by calculating the required cell culture necessary from the isolate concentration measured by the spectrophotometer) was inoculated into each bottle and incubated for 4 weeks in anaerobic conditions, constant temperature (28 °C), and without any sunlight intervention. After 28 days (4 weeks), the liquid obtained from the cultured microorganism is harvested, and samples are collected for local microorganism (LMO) product evaluation, including chemical properties, viable microbial population, proteolytic activity, and ammonia removal from chicken manure. LMO samples were collected from each fermentation bottle and observed for analysis (Figure 1).

Measurement of chemical and microbiological quality

The chemical properties of the local microorganism product were evaluated by measuring total, C-organic, and N content, pH, and the C/N ratio of the final product. Furthermore, the microbiological viable population of the LMO product was evaluated using the total plate count (TPC) test on plate count agar (PCA) media, the lactic acid bacteria (LAB) test on MRSA media, the total coliform test on BGLB media, and the total Yeast test on PDA media. The number of colonies that grow is calculated based on colony-forming units per ml (CFU/ml). Chemical and microbiological quality measurement was carried out at the Integrated Laboratory of the Animal Science Learning Center (ASLC) and the Laboratory of Leather Technology, By-products and Livestock Waste, Faculty of Animal Husbandry, Gadjah Mada University.

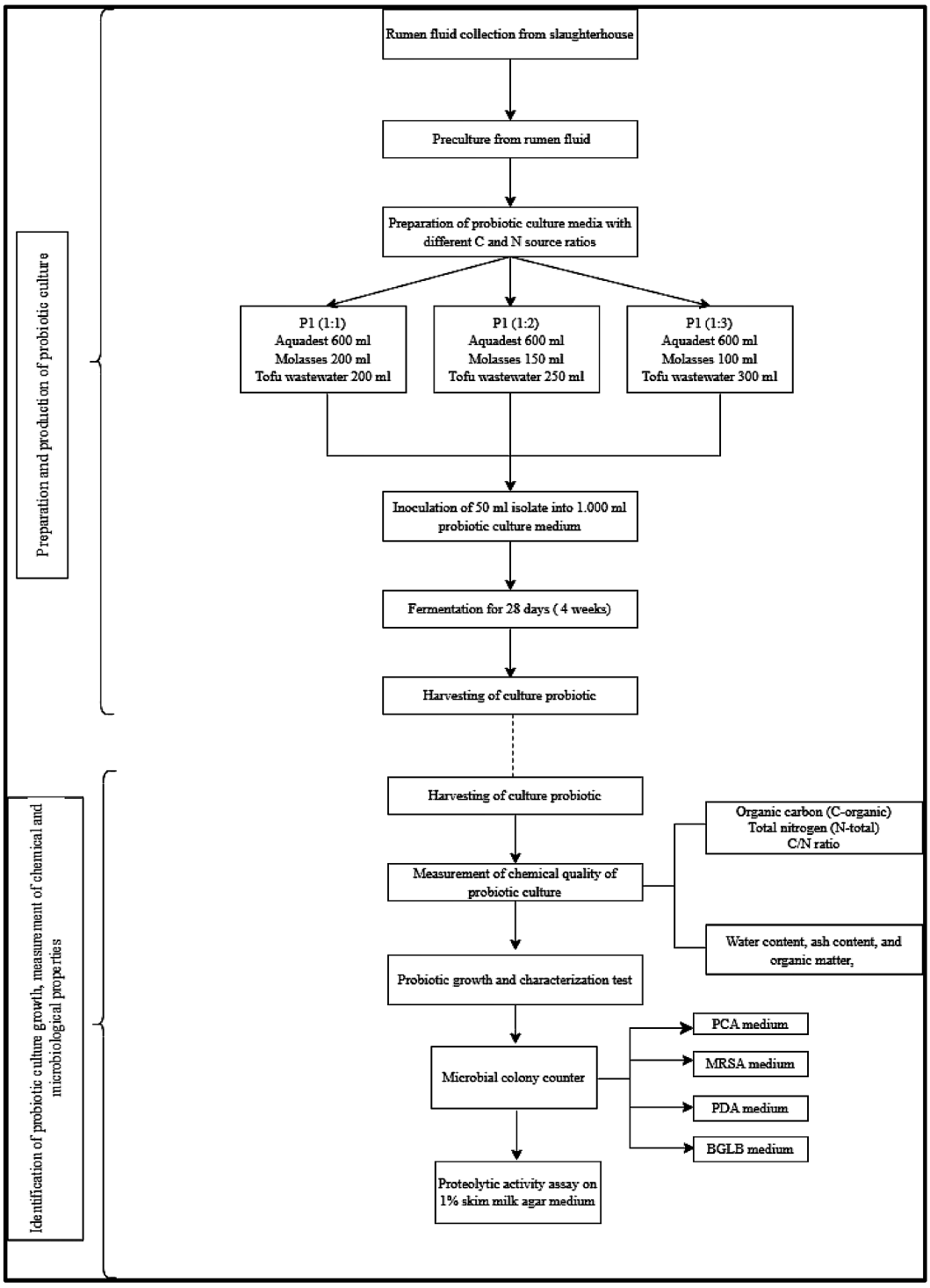

Figure 1. The diagram flow of fermentation processing local microorganism (LMO), proteolytic activity, measurement of chemical condition, and microbiological properties

Quantitative proteolytic activity of local microorganism production

The growth of local microorganisms that had been produced was evaluated by clear zone growth as viable proteolytic activity by growing the local microorganism on agar media with the addition of 1% skim, and compared with the growth of natural rumen fluid as a control (P0). The growth on the agar plate media was in three replications, and each replicate was provided with duplicate plates, then incubated for five days at 30 °C. Inoculation of LMO treatment into an agar plate following the well diffusion method.

The formation of a clear zone represents the putative proteolytic bacterial activity by measuring the diameter of the zone, which indicates the viable growth to utilize protein.

Evaluation of ammonia removal by local microorganisms in chicken manure

Chicken layer excreta were obtained from the Layer Chicken House of the Faculty of Animal Science, UGM. The excreta were diluted with aquabidest at a ratio of 1:3 (25 g excreta:75 mL aquabidest), then sterilised in an autoclave at 121 °C and 15 psi for 15 minutes. After sterilization, the excreta were aseptically inoculated by 1 mL of LMO product or rumen fluid as a control. The samples were then incubated at room temperature using a rotary shaker (120 rpm) for 5 days (120 hours) under sterile conditions (Figure 2). Ammonia concentrations were measured to the chicken manure processing after LMO application using the Nessler method by mixing the Nessler reagen A and B to react the ammonium and observed in spectrophotometre, as described by Trisnaningsih et al. (2025) with modifications. Ammonium standard solutions were prepared from $(NH_4)_2SO_4$, and the absorbances from the spectrophotometer were then calculated using the standard for ammonia final concentration. A total of 200 µL of Nessler reagents A and B were added to 9.8 mL of standard solution and samples chicken manure, then homogenised and incubated for 10 minutes in dark conditions. Absorbance was measured at 570 nm using a spectrophotometer. The ammonia concentration of the samples was calculated based on the standard curve regression equation. The ammonia measurement after LMO application was compared to the ammonia concentration from chicken manure without LMO inoculation.

Statistical analysis

The differences in the data means were analyzed using a one-way ANOVA, SPSS 28. The difference between means was considered homogeneous and was followed by Duncan's Multiple Range Test (DMRT). The obtained data were expressed as means ± standard errors.

RESULTS AND DISCUSSION

Medium-producing condition of a local microorganism

We observed the initial conditions of the raw substrate and culturing medium treatments for the

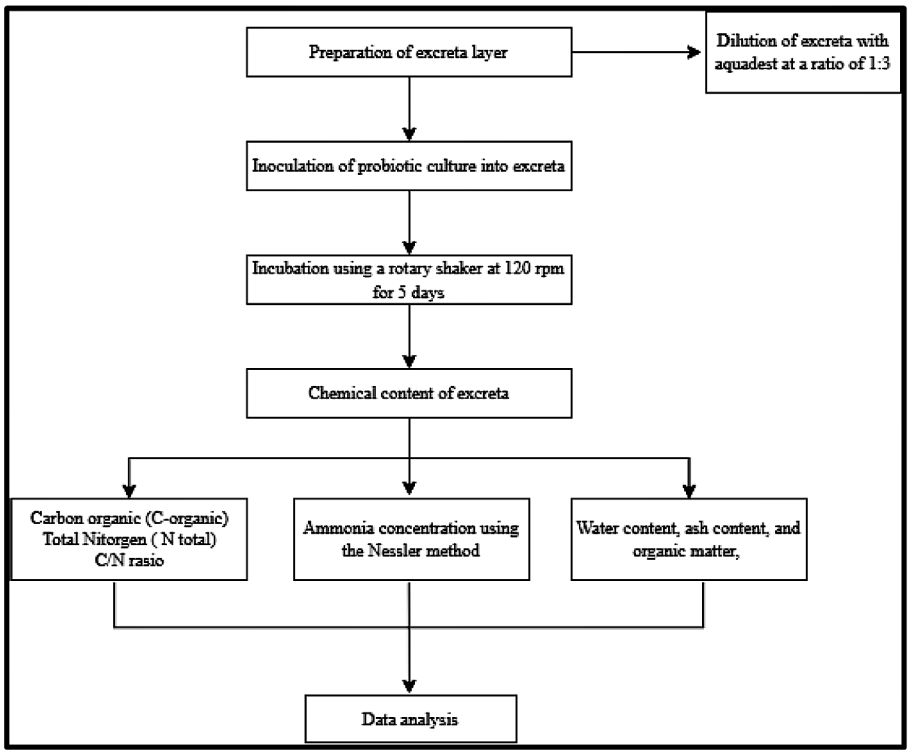

Figure 2. Application of the local microorganism product to the layer chicken manure

growth of rumen microbes. The original substrate and the medium treatment content at the initial condition are shown in Table 1. We showed that the C/N ratio of the mixed materials for tofu dregs is 6.98±0.01 and for Molasses is 18.77±**0**.58. Meanwhile, the initial C/N ratio measurement of P1, P2, and P3 for the treatment medium used to optimize local microorganisms was 25.30±1.00, 24.46±**0**.84, and 23.94±0.94, respectively.

The treatment P1 has the highest C/N ratio compared to P2 and P3 because it contains more C sources, resulting in a higher initial C/N ratio (Table 1). The P3 treatment has a lower initial C/N ratio because its N content is higher, thereby reducing the initial C/N ratio. A high C content causes the C/N ratio value in a material to be high (Gioacchini et al., 2024). The initial C/N ratio is a very important parameter because it determines whether rumen microorganism growth and distribution during the LMO production is running optimally. The C/N ratio should be between 20 and 30, with 25 optimal for bacterial growth (Meng et al., 2019; Macias-Corral et al., 2019). Although all treatments showed an initial C/N ratio within the appropriate range for microbial growth (20-30), where as P1 had the optimal C/N ratio according to Anand et al. (2013): 25. The initial C/N ratio value in each treatment is affected by the composition ratio between the used molasses and tofu dregs, therefore the C/N ratio value of each treatment can also influence bacterial growth.

Chemical properties of LMO production

Based on the results of the measurement final C/N ratio in LMO product (Table 1), it can be seen that the average final C/N ratio levels from three replications of P1, P2, and P3 showed significant differences (P<0.05) with successive values; 28.79±1.66, 32.64±8.30, and 36.67±7.92, respectively, with a control C/N ratio of rumen fluids of 13.70±5.48. Treatment P3 obtained the highest C/N ratio (36.67 ± 7.92) among the other treatments, while P1 had the lowest C/N ratio (28.79 ± 1.66), and the control P0 had a C/N ratio of 13.70 ± 5.48. In the growth activity, microorganisms used carbon and nitrogen to grow and metabolize, which resulted in a C/N ratio medium between initial and final conditions (Gonzalez and Aranda, 2023). Gioacchini et al. (2024) stated that a balanced amount of carbon content in the material compound is required for the C/N ratio to be optimally used by microorganisms. Total organic carbon tended to increase compared to the initial value before the fermentation process in P2 and P3. This indicates that microorganisms actively utilized nitrogen compounds from tofu dregs in their cell metabolism (Hu et al., 2024), which gained in a high final C/N ratio value. According to Philben et al. (2019), the fermentation process can increase carbon content by accumulating organic acids, such as acetic acid. Conversely, microbial degradation of carbon can reduce a material's carbon content (Meng et al., 2019).

The final condition of medium characteristics showed that the lowest moisture content (83.27±0.16) was found in the treatment with a 1:1 ratio of carbon:nitrogen sources (P1). In contrast, the highest moisture content (93.12±0.40) was found in the treatment with a 1:3 ratio of carbon:nitrogen sources (P3). The addition of carbon and nitrogen sources in a 1:1 ratio (P1)

Table 1. Initial and final medium characteristic conditions

Substrate/ medium treatment	Initial condition (day 0)				Final condition (day 28)						
	C-organic content (ppm)	N total content (ppm)	C/N ratio	pH	C-organic content (ppm)	N total content (ppm)	C/N ratio	Water content (%)	Organic matter (%)	Ash/ inorganic content (%)	pH
Tofu dregs fluid	6534.48 ±45.66	935.90 ±4.72	6.98 ±0.01	-	-	-	-	-	-	-	-
Molasses	260584.98 ±1758.10	13900.60 ±482.59	18.77 ±0.58	-	-	-	-	-	-	-	-
P0	-	-	-	-	97.71 ±26.50^{a}	7.35 ±0.82^{a}	13.70 ±5.48^{a}	95.84 ±0.30^{d}	3.04 ±0.30^{a}	1.13 ±0.30^{ab}	6.10 ±0.00^{ns}
P1	58934.29 ±57.54^{c}	2334.84 ±95.62^{ns}	25.30 ±1.00^{ns}	4.80 ±0.00^{ns}	59762.80 ±4389.72^{c}	2077.18 ±124.41^{c}	28.79 ±166^{b}	83.27 ±0.16^{a}	15.02 ±0.14^{c}	1.70 ±0.40^{c}	3.43 ±0.25^{ns}
P2	56923.50 ±62.73^{b}	2330.26 ±79.76^{ns}	24.46 ±0.84^{ns}	4.80 ±0.00^{ns}	58116.74 ±3034.93^{c}	1850.85 ±435.71^{b}	32.64 ±8.30^{b}	88.75 ±1.32^{b}	9.91 ±1.33^{b}	1.34 ±0.12^{b}	3.43 ±0.15^{ns}
P3	54901.75 ±109.93^{a}	2.296.41 ±90.73^{ns}	23.94 ±0.94^{ns}	4.73 ±0.58^{ns}	50492 ±1683.26^{b}	1376.92 ±323.58^{b}	36.67 ±7.92^{b}	93.12 ±0.40^{c}	5.96 ±0.44^{b}	0.91 ±0.04^{a}	3.56 ±0.30^{ns}

Note: ^{ab} Means in the same row without common letter are different at P<0.05. ^{ns} non-significant.

resulted in a lower water content due to the greater amount of molasses added, producing a more concentrated solution. Meanwhile, the addition of carbon and nitrogen sources in a 1:3 ratio (P3) with more tofu dregs content resulted in higher water content. We recycled the liquid waste into an added-value product represented by chemical properties, which is obtained through C-organic, N total, C/N ratio, water content, organic matter, ash content, and pH, that showed the improvement value as the good quality of the cultured medium product compared to the P0 (Table 1). The microorganism in each of the medium treatments was successfully grown to be a potential starter local microorganism, indicated by changes in the C/N ratio (Table 1) in the medium. The optimal water content in the product, as for treatment P1 (83.27%), was the most appropriate condition to keep the microorganism surviving in the medium (Gonzalez and Aranda, 2023) because too high water content of the medium could decrease the viability of the microorganism (Šipailienė and Petraitytė, 2018). The highest organic matter content (15.02±0.14) was observed in the treatment with a 1:1 carbon-to-nitrogen ratio (P1), indicating sufficient nutrients for microorganisms.

In contrast, the lowest organic matter content (5.96±0.44) was found in the treatment with a 1:3 carbon-to-nitrogen ratio (P3). The organic matter content in the rumen fluid (P0) was lower (3.04±0.30) than in other treatments. Organic matter is a critical component because it provides essential nutrients such as carbon and nitrogen, which are necessary for the growth and function of microorganisms (Kaur and Vyas, 2024). Furthermore, Table 1 showed that the highest ash content (1.70±0.40) was found in the treatment with a 1:1 balance of carbon:nitrogen sources (P1), while the lowest ash content (0.91±0.04) was found in the treatment with a 1:3 addition of carbon:nitrogen (P3). Organic matter will be oxidized and burned during combustion, while inorganic substances remain as a residue or ash (Gollmer et al., 2019).

Viable microbial population in the local microorganism product

Next, we showed the total microbial count as the viable microbial population in the LMO growth product, which is presented in Table 2. The total plate count of the local microorganism (LMO) product, which was produced the highest number of colonies after the fermentation process, was on the treatment P1 with a total number of colonies of 4.17±0.60 × 10^8 CFU/ml, while the treatment produced the smallest number of colonies was on treatment P0 with a colony number of 0.38±0.06 × 10^8 CFU/ml. It is indicated that the microorganism contained in rumen fluid without treatment of the fermentation process with different C/N ratios didn't show growth activity by keeping total colony significantly below the other treatments, and seemed to be decreased over time. Cui et al. (2024) reported that an unbalanced C/N ratio can lead to nutritional limitations, thereby affecting microbial growth. This indicates that the C/N ratio plays an essential role in microorganism growth (Meng et al., 2019). The results of measuring the number colonies of lactic acid bacteria in local microorganism products showed that the treatment produced the highest number of lactic acid bacteria colonies was on treatment P2 with a ratio carbon:nitrogen of 1:2 with a total number of colonies of 2.90±0.40 × 10^8 CFU/ml, while the natural rumen fluids (P0) showed the lowest number colonies of 0.12±0.01 × 10^8 CFU/ml. The growth of lactic acid bacteria is affected by several key factors, including temperature, pH of the medium, and nutrient availability (Nasrollahzadeh et al., 2023). Lactic acid bacteria, such as *Lactobacillus* sp., utilize glucose as the primary substrate (Zou et al., 2024); in this study, glucose from molasses is converted into end products, including lactic acid, ethanol, and CO_2 (Sharma et al., 2020). Lactic acid produced in the LMO product is due to the activity of lactic acid bacteria in order to metabolize the glucose as a substrate from molasses, resulting in a decrease in pH (Cao et al., 2024). Nitrogen sources such as tofu residue liquid are used by lactic acid bacteria to hydrolyse proteins into peptides and amino acids for growth (Hu et al., 2024).

The LMO product colony counted during 48 hours of growth showed that the addition of carbon and nitrogen sources with a ratio of 1:1 (P1) had the highest number of yeast of 0.24±0.02 × 10^8 CFU/mL compared to other treatments (Table 2). The ratio of 1:1 obtained the condition that microbial can optimal growth by using sufficient carbon (C) and nitrogen (N) (Gioacchini et al. (2024). Yeast could optimally grow in the condition of sufficient nutrient including carbon and nitrogen sources, and can break down various organic materials, including carbohydrates, fatty acids, and hydrocarbon compounds, making

Table 2. Viable microbial population of cultured rumen fluid waste-local microorganism

Parameters	Total colony (x10^8 CFU/ml)			
	P0	P1	P2	P3
Total plate count	0.38 ± 0.06^{a}	4.17 ± 0.60^{b}	3.58 ± 0.06^{b}	0.41 ± 0.24^{a}
Total lactic acid bacteria	0.12 ± 0.01^{a}	1.37 ± 0.40^{b}	2.90 ± 0.40^{c}	0.41 ± 0.26^{a}
Total yeast	0.08 ± 0.00^{a}	0.24 ± 0.02^{b}	0.19 ± 0.05^{b}	0.22 ± 0.13^{b}
Total coliform	0.00 ± 0.00nd	0.00 ± 0.00nd	0.00 ± 0.00nd	0.00 ± 0.00nd

Note: ^{ab} Means in the same row without a common letter are different at P<0.05. nd non-detected.

it effective in reducing chemical oxygen demand (COD) (Mohiuddin et al., 2024). Based on the coliform colony counted, ther is no coliforms detected in P1, P2, or P3. This result indicated that the local microorganism cultured was suppressed the pathogen activity by increasing the lactic acid bacteria population (Che et al., 2024; Gwiazdowski et al., 2023), and potentially used as biological starter. These data showed that during the LMO fermentation process, pathogenic bacteria and coliforms from rumen fluid waste were successfully decreased, therefore the local microorganism product in this study will be safe for the environment when used as a starter.

Proteolytic growth ability of LMO

Next, we observed the proteolytic activity of the LMO product by measuring the diameter of colonies on plate media (Figure 3, Figure 4). The local microorganism cultured in P1, P2, and P3 exhibited proteolytic activity, forming clear zones in media containing skim milk. Although the control treatment (P0) also exhibited a clear zone, it also showed proteolytic activity. It showed that rumen fluid waste naturally contains proteolytic bacteria (Belanche et al., 2019; Cherdthong, 2020; Wei et al., 2022), and in this study, we optimize the growth of proteolytic bacteria as an LMO

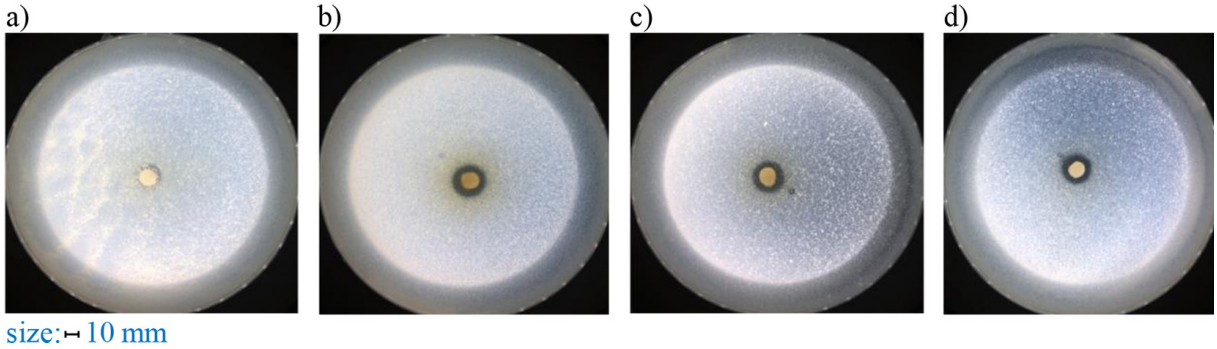

Figure 3. Observation of clear zone (day-1) of all treatments LMO: a) clear zone of P0; b) clear zone of P1; c) clear zone of P2; and d) clear zone of P3

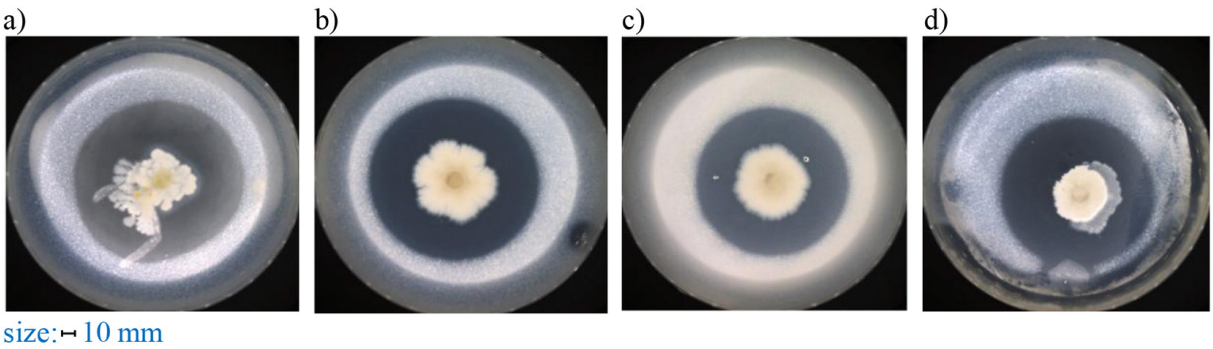

Figure 4. Observation of clear zone (day-5) of all treatments LMO: a) clear zone of P0; b) clear zone of P1; c) clear zone of P2; and d) clear zone of P3

Note: ^{abc} Means in the same row without a common letter are different at P<0.05

product. All local microorganisms, P1, P2, and P3, utilized protein from skim milk during their growth until day-5 to support metabolic activity. Kieliszek et al. (2021) stated that proteolytic bacteria can utilize proteins, especially casein, by producing proteolytic enzymes that break them down into peptides and amino acids. Casein, contained in skim milk, is used as the primary substrate for the production and testing of protease activity because it is easily hydrolysed and the results can be observed visually on agar media (Natsir et al., 2024; Senthilkumar et al., 2021).

Table 3 shows that the addition of carbon and nitrogen sources at a 1:1 ratio (P1) produced a clear zone (49.60 ± 1.31) larger than P2 and P3 on the fifth day of observation. Although the rumen fluid only (P0) showed the largest clear zone (Figure 4) indicated that the natural rumen fluid already contained proteolytic bacteria, which supports the beneficial further purposes. Carbon content plays a significant role as the main energy source for microorganisms (Talapko et al., 2022; Wang et al., 2023), while nitrogen sources are needed for enzyme synthesis, including protease enzymes (Sun et al., 2021). Optimal nutrient availability enables microorganisms to produce protease enzymes, allowing them to degrade milk proteins, especially casein in skim milk. According to Hossain et al. (2021), the ability of microbes to break down proteins varies across species, partly due to the secretion of different enzymes, leading to varying levels of activity. Differences in the diameter of the clear zone (Figure 4) can be attributed to variations in the addition of carbon and nitrogen sources in the cultured medium of the local microorganism product.

Ammonia removal in chicken manure by LMO

Finally, we investigated the application of LMO as a starter in animal waste, represented by chicken manure, which commonly occurs in the environment. The addition of LMO cultured in P1, P2, and P3 to layer chicken excreta had a significant effect ($P<0.05$) compared to the control (only layer chicken manure) and P0 treatments. Meanwhile, the addition of local microorganism product between the P1, P2, and P3 treatments did not show any significant differences ($P>0.05$) (Figure 5). Treatment P1 with a ratio of 1:1 had the highest reduction of ammonia concentration because the number of total count bacteria was also high, therefore, the organic matter degradation proceeds optimal (Silva-Sánchez., et al 2019). The common bio-starter used by agricultural businesses was only for certain purposes; for example, lactic acid bacteria were used for fermented organic matter (Paradhipta et al., 2025), and nitrifying bacteria were used for protein-derived organic compounds in the nitrification process as a liquid organic fertilizer product (Pastawan et al., 2025).

The addition of LMO with a 1:1 balance of carbon and nitrogen sources (P1) showed in a higher removal of ammonia levels (4165.32 ppm) than other cultured LMO products (P2 and P3) (Figure 5). The optimal of reducing ammonia concentration in chicken manure was occurred in treatment P1 compared to the control P0 and control as chicken excreta without addition of LMO (Table 4). All treatments showed a significant ($P<0.05$) removal of ammonia concentration compared to P0. The use of LMO as a starter in ammonia removal from chicken manure showed the current ability of LMO activity, because we limited the conditions: the chicken manure was sterilized to determine the LMO activity in degrading organic matter, particularly ammonia removal, without any interruption from other bacterial growth and activity. The ability of local microorganisms to remove ammonia is due to the activity of group microorganisms that suppress ammonia (Zhang et al., 2024), such as nitrifying bacteria. Cao et al. (2021) stated that the treatment for ammonia removal is based on the alteration of volatile N into non-volatile N,

Table 3. The diameter of the clear zone of proteolytic activity with skim milk media by local microorganisms

Days of incubation	Diameter of clear zone (mm)			
	P0	P1	P2	P3
Day-1	0.00 ± 0.00^{a}	10.47 ± 0.36^{b}	8.54 ± 0.79^{b}	8.77 ± 0.90^{c}
Day-2	17.53 ± 0.56^{b}	12.52 ± 1.72^{a}	11.78 ± 1.73^{a}	11.17 ± 0.90^{a}
Day-3	30.87 ± 0.40^{b}	25.73 ± 1.11^{a}	21.70 ± 3.79^{a}	21.08 ± 3.56^{a}
Day-4	38.87 ± 0.45^{b}	34.68 ± 0.52^{ab}	32.60 ± 3.30^{a}	30.94 ± 5.08^{a}
Day-5	50.77 ± 0.30^{ns}	49.60 ± 1.31^{ns}	46.67 ± 3.74^{ns}	40.90 ± 10.41^{ns}

Note: ^{ab} Means in the same row without a common letter are different at $P<0.05$.

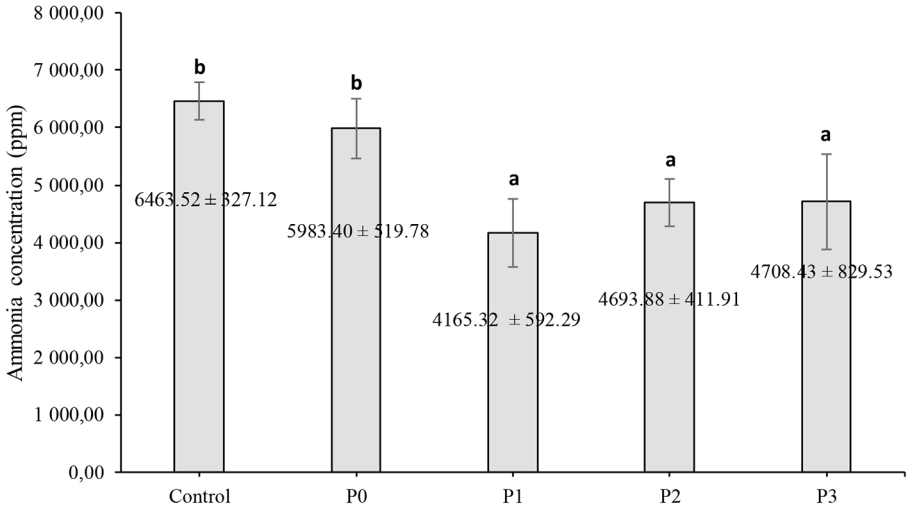

Figure 5. Ammonia content as ammonium in chicken manure oxidized by local microorganism for five days, [ab] Means in the figure indicate significant differences P<0.05. Error bars indicate the standard deviation of three replicates

Table 4. Ammonia reduction (%) in chicken manure with/without local microorganism addition

Treatment(s)	Ammonia reduction (%)
Without treatment	0
P0	7.4 ± 3.2[a]
P1	35.5 ± 5.0[b]
P2	27.4 ± 2.5[c]
P3	27.2 ± 7.5[bc]

comprising nitrification and denitrification processes by heterotrophic nitrifying and denitrifying bacteria. Nitrifying bacteria reduce ammonia through a two-stage nitrification process (Nishi et al., 2023). The first stage is carried out by ammonia-oxidizing bacteria (AOB), which convert ammonia into nitrite using the enzymes ammonia monooxygenase and hydroxylamine oxidoreductase (Li et al., 2024). The ammonia monooxygenase enzyme converts ammonia into hydroxylamine (NH_2OH), while hydroxylamine oxidoreductase catalyses hydroxylamine into nitrite (Musiani et al., 2020). The second stage is carried out by nitrite-oxidizing bacteria (NOB) (Zhao et al., 2023), such as Nitrobacter and Nitrospira, which convert nitrite to nitrate via the enzyme nitrite oxidoreductase. Several factors, such as pH, influence the effectiveness of ammonia reduction by nitrifying bacteria (Wang et al., 2023; Zhao et al., 2023). AOB bacteria can survive in acidic environments and remain active in reducing ammonia (Wang et al., 2021). The results of this study indicated that culturing LMO product with varying carbon and nitrogen sources can reduce ammonia in layer chicken manure compared to without LMO product as a starter (control), due to the presence of putative nitrifying bacteria that convert ammonia to nitrate. The initial C/N ratio in the fermentation process of LMO obtained the optimal nitrifying process by putative nitrifying bacteria contained in the LMO.

CONCLUSIONS

Based on the results, we concluded that the C/N ratio in medium for culturing microorganisms is an important compound needed by the microorganism to optimally grow, which improved the microbial growth ability. The growth of LMO product cultured in different C/N ratio media presented proteolytic ability and nitrification ability, which referred to a beneficial environment. The mixture of molasses and tofu dregs obtained a good initial C/N ratio that can be used for optimizing the growth of microorganisms. Local microorganism (LMO) obtained by fermenting rumen fluid waste with molasses as a carbon source and tofu dregs as a nitrogen source improved activity of microorganisms. Providing carbon and nitrogen sources at a 1:1 ratio (P1) resulted in a balanced nutrient medium that supported LMO growth optimally. The growth of rumen microorganisms through the fermentation process, as a local microorganism product, could be used as an alternative biological agent for organic waste management. These LMOs can significantly reduce ammonia levels in chicken

manure, suggesting their potential as bioactivators for livestock waste processing. Moreover, we managed to recycle the liquid waste from the rumen into a new product as a biological agent. The LMO production carried out for 28 days has omitted the pathogenic bacteria, which indicated safe to be used in the environment.

Acknowledgement

The author would like to thank the Final Assignment Recognition Program (RTA) Universitas Gadjah Mada Number 5286/UN1.T1/PT.01.03/2024 for funding the research.

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