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## PREFACE

In this edition, Volume 30 No 2, we proudly present articles from animal and veterinary sciences including genetic, reproduction; animal physiology; and veterinary from scientist all over the world. The articles published in this edition are:
"An Overview of Performance and Influencing Factors of Frozen Semen Producers in Indonesia"; "Effect of Locally Extracted Phytase on Blood Hematological and Serum Biochemistry of Broiler Chickens"; "Screening Probiotics from Genetically Unselected Indonesian Local Chicken to Enhance Meat Quality in Broiler Chicken"; "Pathological Changes and Virus Detection in Chickens Infected with Fowl Adenovirus 8b"; "Assessing Greenhouse Gas Emissions from Urban Dairy Farming in Jakarta as an Indicator of Environmental"; and "Shelf Life of *Trichoderma* Mutant Inoculum with Dried Mud and Glutinous Rice as Carrier Materials".

We extent high appreciation to all peer reviewers who make this journal academically high value. Hopefully, these articles would offer any benefit to readers and the end-users of technological innovation, and attract interests from scientists to contribute their papers to the Indonesian Journal of Animal and Veterinary Sciences.

Chief Editor

Bogor, September 2025

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# Jurnal Ilmu Ternak dan Veteriner

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**Volume 30, Number 3, September 2025 ISSN 0853-7380 E-ISSN 2252-696X**

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# An Overview of Performance and Influencing Factors of Frozen Semen Producers in Indonesia

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**ABSTRAK**

Arifiantini RI, Gusman KT, Agil M, Susilawati T, Karja NWK, Said S, Mahendra HC.2025. Gambaran umum kinerja dan faktor-faktor yang memengaruhi produsen semen beku di Indonesia. JITV 30(3):132-139. DOI:http://dx.doi.org/10.14334/jitv.v30i3.3521.

Di Indonesia, terdapat 21 produsen semen beku, semua produsen tersebut mempunyai rumpun pejantan, jenis pengencer dan target produksi berbeda. Penelitian ini dilakukan dengan tujuan untuk menganalisis faktor-faktor yang memengaruhi kinerja produsen semen beku tersebut. Metode survei dilakukan dengan menggunakan kuesioner (melalui formulir Google) yang diisi oleh 21 petugas laboratorium produsen semen beku di Indonesia. Data yang terkumpul dianalisis secara deskriptif dan disajikan dalam bentuk angka dan persentase. Hasil penelitian menunjukkan bahwa kinerja para produsen bervariasi, dengan jumlah petugas laboratorium berkisar antara satu hingga lebih dari lima orang, dan didominasi oleh laki-laki dan perempuan (76,19%). Latar belakang pendidikan petugas laboratorium sebagian besar adalah sarjana peternakan (54,29%), jumlah sapi jantan yang dimiliki bervariasi mulai kurang dari 10 ekor hingga lebih dari 200 ekor (90,48% dan 9,52%). Sapi jantan yang dipelihara sebagian besar adalah sapi eksotik dan sapi lokal (76,19%). Pengencer yang paling banyak digunakan adalah pengencer buatan sendiri (71,43%) dan pengenceran semen sebagian besar dilakukan dalam satu tahap (57,14%). Konsentrasi sperma semen segar diukur oleh seluruh produsen, menggunakan fotometer (SDM atau accucell); kalibrasi internal dilakukan oleh 71,43% produsen. Evaluasi kualitas semen beku sebagian besar difokuskan pada motilitas sperma pasca-*thawing* (100%), dan hanya 61,90% produsen yang menghitung konsentrasi sperma. Penilaian tambahan, yang meliputi viabilitas dan morfologi sperma, hanya dilakukan oleh 52,38% produsen, dan hanya 4,76% produsen yang menilai integritas membran plasma sperma. Proporsi sampel semen beku yang melebihi, memenuhi, atau tidak memenuhi SNI masing-masing adalah 80,95%; 14,29%; dan 4,76%.

**Kata Kunci**: Balai Inseminasi Buatan, Produsen Semen Beku, Profil Produsen Semen Beku, Semen Beku

**ABSTRACT**

Arifiantini RI, Gusman KT, Agil M, Susilawati T, Karja NWK, Said S, Mahendra HC, 2025. An overview of performance and influencing factors of frozen semen producers in Indonesia. JITV 30(3):132-139. DOI:http://dx.doi.org/10.14334/jitv.v30i3.3521.

In Indonesia, there are 21 frozen semen producers, each with a different stud herd, diluent type, and production target. The present study was conducted to evaluate the performance of these producers. A survey method was employed, utilising a questionnaire (via Google Forms) completed by 21 laboratory personnel from frozen semen producers in Indonesia. The collected data were analysed descriptively and presented as numbers and percentages. The study revealed that producers' performance varied, with the number of laboratory workers ranging from 1 to more than 5, and that men and women (76.19%) were the dominant group. The laboratory personnel's educational backgrounds were predominantly in animal science (54.29%), while the number of bulls owned ranged from less than 10 to more than 200 (90.48% and 9.52%, respectively). The bulls kept were primarily exotic and local cattle (76.19% of the total). The most commonly used diluents were homemade (71.43%), and semen dilution was predominantly performed in a single step. The sperm concentration of fresh semen was measured by 100% of producers using a photometer (SDM or AccuCell); however, internal calibration was performed by 71.43% of producers. Moreover, the evaluation of frozen semen quality focused predominantly on post-thaw motility (100%), with only 61.90% of producers calculating sperm concentration. Furthermore, supplementary assessments, including sperm viability and morphology, were conducted in only 52.38% of cases, with only 4.76% of producers assessing sperm plasma membrane integrity. The proportions of frozen semen samples that exceeded, met, or did not meet the SNI were 80.95%, 14.29%, and 4.76%, respectively.

**Key Words:** Artificial Insemination Centers, Frozen Semen, Frozen Semen Producers, Frozen Semen Producer Performance

## INTRODUCTION

According to Livestock Statistics 2023, the cattle population in Indonesia in 2022 consisted of 17,602,538 beef cattle and 507,075 dairy cattle (Kementerian Pertanian 2023). The ratio of male to female cattle, as determined by the 2017 Livestock Business Household Cost Structure Survey (BPS 2017), is 36.86%: 63.14%. Total of 63.14% of cattle in the above category, 41.61% were mature females aged between two and over eight years, with a productive age of 38.05% or 6,881,652.94 heads.

Cattle breeding primarily uses artificial insemination, though some farmers still use natural mating. Assuming mating with artificial insemination at 60% and service per conception at two, the requirement for frozen semen in Indonesia is 8,257,983 straw units, which producers of frozen semen supply. According to the Regulation of the Minister of Agriculture (MOA) of the Republic of Indonesia Number 10/2016, the following entities are eligible to be frozen semen producers: Nasional Artificial Insemination Centers (NAICs), Regional Artificial Insemination Centers (RAICs), breeders, livestock companies, government, provincial or district/city governments, and universities.

In Indonesia, the production of frozen semen is currently overseen by various entities, including NAICs such as Singosari and Lembang AICs, 18 RAICs distributed across multiple provinces, and one Artificial Insemination Laboratory affiliated with universities, such as the Teaching Farm Airlangga. Regulation by MOA No. 10/2016 serves as a guideline for each frozen semen producer in producing and distributing its products and is outlined in each producer's standard operating procedure (SOP). Frozen semen producers generally follow similar SOPs; however, production targets, operational implementation, and application within each AIC distinguish producers.

The quality of frozen semen is influenced by various factors, including the male breed (Morrell et al. 2018), the freezing method employed (Zhang et al. 2024), the equipment used (Lieberman et al. 2016), the diluent (Sukirman et al. 2019; Zhang et al. 2024), and human resources (Pertiwi & Soesanto 2022). The variation is also influenced by the handling practices of frozen semen, its transportation and storage facilities, and the proximity of the region/district to the National Artificial Insemination Centre (Engidawork 2018).

The present study aimed to evaluate the laboratory human resources, identify the number and breeds of cattle, evaluate the equipment used, the type of semen diluents and dilution techniques, and the quality examination of frozen semen by frozen semen producers in Indonesia.

## MATERIALS AND METHODS

### Time and place of research

The research was conducted from September 2023 to November 2024 at two institutions: the Central Java RAIC in Ungaran, Central Java, and the School of Veterinary Medicine and Biomedical Sciences, IPB University.

### Research procedures

A survey was conducted to collect data on the performance of frozen semen producers. The survey method involved administering a questionnaire (Google Form) to laboratory personnel at frozen semen producers in Indonesia. The participating institutions included Singosari and Lembang NAIC, RAIC of Aceh, North Sumatra, Tuah Sakato, West Sumatra, Riau, Jambi, Bengkulu, South Sumatra, Lampung, Ciamis, Central Java, Blora, Yogyakarta, Banyumulek, West Nusa Tenggara, Baturiti Bali, South Kalimantan, South Sulawesi, Central Sulawesi, Southeast Sulawesi, and Teaching Farm Airlangga. The following questions were included in the survey: a) Number, composition, and education of laboratory personnel. b) Breeds and number of males used. c) Equipment used for quality testing of fresh and frozen semen. d) Diluent and dilution technique used. e) Quality testing of frozen semen performed by producers.

### Data analysis

The data were analysed descriptively and presented as numbers and percentages of 21 frozen semen producers in Indonesia.

## RESULTS AND DISCUSSION

### Human resources

The findings indicated a variation in the number of laboratory staff among frozen semen producers. Some producers employed a single laboratory personnel member (4.76%), while 71.43% had 2-4 laboratory personnel, and 23.81% had 5 or more. Those with five or more staff were national producers with ambitious production goals. The gender composition of the laboratory staff was predominantly mixed, with 76.19% being both male and female, 14.29% female, and 9.52% male. Most personnel held a Bachelor of Animal Sciences degree, followed by qualifications as Veterinarians, High School Diplomas, Bachelor of Chemical Sciences, and Paramedic Diplomas (Table 1).

**Table** 1. Human resources at the Laboratory of frozen semen producers in Indonesia

| Variable | Criteria (Number) | Producers | Percentage (%) |
|---|---|---|---|
| Laboratory personal | 1 | 1 | 4.76 |
| | 2 | 4 | 19.05 |
| | 3 | 6 | 28.57 |
| | 4 | 5 | 23.81 |
| | 5 | 1 | 4.76 |
| | >5 | 4 | 19.05 |
| Gender of Laboratory personnel | All female | 3 | 14.29 |
| | All male | 2 | 9.52 |
| | Female dan male | 16 | 76.19 |
| Educational Background of Laboratory personnel | Highschool graduate | 4 | 11.43 |
| | Paramedic Diploma | 1 | 2.86 |
| | Bachelor of Animal Husbandry | 19 | 54.29 |
| | Bachelor of Chemical Sciences | 2 | 5.71 |
| | Veterinarian | 9 | 25.71 |
| Total | | 21 (100%) | |

The production of frozen semen involves a wide range of human resources, including administrators, animal nurses, veterinarians, feed quality supervisors (WASTUKAN), semen quality supervisors (WASBITNAK), bull masters, and laboratory personnel. The combined efforts of these professionals are essential in ensuring the quality of frozen semen (Pertiwi & Soesanto, 2022). This research focused on laboratory personnel who are responsible for preparing diluents, evaluating the quality of fresh semen, processing frozen semen, and assessing the quality of the final product. Furthermore, laboratory personnel meticulously document all data related to processes and operations in the frozen semen production laboratory. According to Regulation MOA No. 10/2016, human resources must meet competency standards and/or have expertise in their respective areas.

Human resources play a crucial role in meeting an organization's goals and objectives, significantly impacting the efficiency and overall performance of its systems. Having an adequate number of personnel with the right educational qualifications and skills is essential for determining the quality of frozen semen. Maintaining consistent quality and quantity of frozen semen is vital for producers. The production of frozen semen is a multifaceted process that begins with preparing diluents, assessing fresh semen, diluting, packaging in straws, equilibrating, freezing, storing the straws, and evaluating the frozen semen. This production process, which includes several stages, necessitates the involvement of multiple laboratory staff, ideally 2–3 for regional producers and a minimum of four for national producers.

**The number and breed of cattle owned by frozen semen producers**

The quantity and type of cattle owned by frozen semen producers showed variation, with ownership ranging from fewer than 10 to 50 bulls (90.48%) to more than 200 bulls (9.52%). The quantity and type of cattle owned by producers of frozen semen are linked to their production goals. These producers must evaluate the production capabilities of each breed and individual animal, as there are variations in frozen semen production potential among different breeds and individuals (Indriastuti et al. 2020; Shopian et al. 2025). Notably, some regional producers of frozen semen exclusively maintain domestic breeds (14.29%), a strategy implemented by the RAIC to preserve the genetic purity of domestic breeds in Indonesia and protect the genetic potential of cattle to develop superior breeding stock. In contrast, 9.52% of frozen semen producers solely keep exotic bulls in their herds. A large majority of frozen semen producers (76.19%) typically use a combination of domestic and exotic bulls. The production targets for frozen semen were broad, spanning from 20,000 to 3 million straws annually (Table 2). This table lists the number and breed of bulls, along with their respective production goals.

To ensure the production of high-quality frozen semen that aligns with the Indonesian National Standard

**Table 2.** The number and breed of cattle and the frozen semen production target of frozen semen producers in Indonesia

| Variable | Criteria (bull head) | Number of Producers | Percentage (%) |
|---|---|---|---|
| Number of Bulls | <10 | 6 | 28.57 |
| | 10 – 20 | 9 | 42.86 |
| | 20 – 50 | 4 | 19.05 |
| | 50 – 100 | 0 | 0.00 |
| | 100 – 200 | 0 | 0.00 |
| | >200 | 2 | 9.52 |
| Breed of Bulls | Only Exotic | 2 | 9.52 |
| | Only local cattle | 3 | 14.29 |
| | Exotic & local cattle | 16 | 76.19 |

**Table 3**. Potential frozen semen production from various breeds of cattle

| Breeds | Production Potential | | Source |
|---|---|---|---|
| | Straw/year | Straw/ejaculate | |
| Exotic cattle | | | |
| Limousin | 45.520* | 569 | Jakaria et al. (2018) |
| Simmental | 29.920* | 374 | Isnaini et al. (2019) |
| | 14.640 | 183 | Baharun et al. (2021) |
| Angus | 13.920* | 174 | Jakaria et al. (2018) |
| Domestic Cattle | | | |
| Aceh | 11.399 | 285 | Sophian et al. (2025) |
| Bali | 21.640 | 270 | Iskandar et al. (2022) |
| Brahman | 36.560* | 457 | Jakaria et al. (2018) |
| FH | 19.840* | 248 | Jakaria et al. (2018) |
| Madura | 7.980 | 99 | Komariah et al. (2020) |
| Ongole | 26.720* | 334 | Isnaini et al. (2019) |
| Pasundan | 13.400 | 167 | Santoso et al. (2021) |

*= The result of multiplying 80×number of straws/ejaculates

(SNI) for frozen bovine semen (4869-1:2021), frozen semen producers must own bulls of exceptional quality; this not only supports the success of artificial insemination (AI) in the field but also enhances the quality of local cattle in the areas where frozen semen is produced (Iskandar et al. 2022). The annual production target, measured in straws, reflects a producer's capacity to produce frozen semen, with a significant relationship between this target and the number and breed of bulls each producer owns. Prominent national frozen semen producers aim to exceed 3 million straws per year, made possible by a sufficient variety of bulls from different breeds. Conversely, regional producers have a more limited selection of bulls and breeds, resulting in lower production targets. As outlined in the Roadmap of Indonesian Superior Bulls 2018-2022, a bull is considered productive if it yields at least 7,500 straws of frozen semen annually for local cattle and 12,500 straws for exotic cattle.

The capacity of a bull to produce semen that can be effectively frozen is known as its potential for generating frozen semen. This potential can be evaluated for each ejaculate or over a year, typically based on 40 weeks of production annually or 80 semen collections, with two collections per week. The production of frozen semen from bulls is contingent upon the amount of motile sperm in the ejaculate (Santoso et al. 2021). There are significant variations in the ability to produce frozen semen among different cattle breeds (Sophian et al. 2025) and individual bulls (Indriastuti et al. 2020). The feasibility of producing frozen semen is influenced by both the volume of semen and the sperm concentration in each sample. While variations in sperm concentration do not impact the quality of frozen semen, these factors do affect the number of straws produced (Arifiantini 2017). Table 3 summarizes the potential of different cattle breeds to produce frozen semen for artificial insemination, based on previous studies.

**Semen diluents are used in the frozen semen producer.**

The study found that 71.43% of frozen semen producers utilized homemade diluents, with Tris-egg yolk and skimmed milk-egg yolk being the most common. In contrast, the other producers chose to use commercial diluents. The quality of frozen semen can differ significantly depending on the type of diluent and the dilution methods used. Nevertheless, using appropriate and effective diluents and techniques can make the production process more economical, leading to a lower cost per straw for breeders. The choice of commercial diluents by frozen semen producers is mainly influenced by the ease of preparation, established quality, and the need to produce in small quantities (Riwu et al. 2023). However, the use of commercial diluents is restricted by their non-local production, which can be affected by supply chain issues, potentially halting the production of frozen semen. Therefore, to ensure continuous production, producers need an alternative in-house supply of diluents. Table 4 lists the types of semen diluents used by frozen semen producers in Indonesia.

**Dilution techniques**

The results showed that 57.41% of frozen semen producers used a one-step dilution technique and 42.86% used a two-step dilution technique. The filling process was performed at 5 °C. Furthermore, the results indicated that 61.90% of producers conducted the packaging process at room temperature, whereas 38.10% performed it at 5°C. The semen dilution techniques employed by frozen semen producers in Indonesia are listed in Table 5.

The method of dilution used to produce frozen semen has been shown to affect semen quality significantly (Zhang et al. 2024). Techniques that are compatible with the process have been found to produce semen of notably higher quality (Dias et al. 2018). This study revealed that 57.41% of frozen semen producers used a one-step dilution method, while 42.86% used a two-step dilution method. When employing the one-step dilution method, the semen is packaged at room temperature (20–22°C). Then it undergoes an equilibration process, during which the temperature is adjusted to 5°C for 4 hours before freezing.

The diluents employed in the two-step dilution technique included those both with and without cryoprotectants. The initial diluent, constituting 50% of the required volume, was introduced at room temperature and subsequently stored at 5°C. In contrast, the second diluent, which contained glycerol, was maintained at this temperature and added 1 hour after the initial dilution. The filling process was conducted at 5°C, and the equilibration period was measured from the moment the semen was combined with the first diluent. The data indicated that 61.90% of producers conducted the packaging process at room temperature, while 38.10% preferred 5°C. The two-step dilution method is exclusively utilized by frozen semen producers who employ a cooling top (cooling cabinet) for equilibration, such as the NAIC. Producers engaged in smaller-scale frozen semen production primarily utilize refrigerators set between 4 and 5°C.

Arif et al. (2020) found that a one-step dilution method produced higher-quality frozen semen than two- or three-step dilution methods. The process involving two or three dilutions causes changes in temperature and osmotic pressure when the cooling. The countainer is

**Table** 4. Types of semen diluents used by frozen semen producers in Indonesia

| Type of Semen Diluents | Number of Producers | Percentage (%) |
|---|---|---|
| Commercial diluents | 6 | 28.57 |
| Egg yolk-skimmed milk | 6 | 28.57 |
| Tris-egg yolk | 9 | 42.86 |

**Table 5:** Dilution techniques of frozen semen producers in Indonesia

| Variables | Criteria | Producers | Percentage (%) |
|---|---|---|---|
| Dilution techniques | One-step dilution | 12 | 57.14 |
| | Two-step dilution | 9 | 42.86 |
| Dilution, filling, and sealing temperature | Room temperature | 13 | 61.90 |
| | 5°C | 8 | 38.10 |

opened and closed during the addition of diluent and the packing of the straw. These fluctuations in temperature and osmotic pressure place stress on sperm, leading to molecular alterations, DNA damage, changes in the plasma membrane, and increased reactive oxygen species (ROS). As a result, the motility and quality of the frozen semen are adversely affected.

**Semen freezing equipment used in frozen semen producers**

The equipment available to producers for creating frozen semen is generally adequate for the purpose. Both national and regional producers possess phase-contrast microscopes with monitors, automatic straw packaging and printing machines, and equilibration devices, such as a cooling cabinet or refrigerators. The freezing equipment at RAICs includes automatic machines and traditional tools, such as temperature-controlled Styrofoam boxes.

In addition to a microscope, a crucial tool for semen evaluation is a sperm concentration meter, as it is essential for calculating semen dilution. Sperm concentration refers to the number of sperm per milliliter of semen. An incorrect sperm evaluation can affect the sperm count in the straws. Importantly, all frozen semen producers in Indonesia utilize computer-based counters, such as the Photometer SDM 5 or SDM 6 (95.24%) and AccuCell (4.76%). These devices require calibration internally every two weeks. The results of this study indicated that only 71.43% of producers followed the internal calibration procedure, while the remaining producers did not comply with the protocol (Table 6).

**Semen quality testing by the producer**

The findings showed that all producers (100%) evaluated sperm motility, while only 61.90% checked sperm concentration. The quality of frozen semen produced was 4.76% not meeting SNI standards, 80.95% meeting SNI standards, and 14.29% surpassing the SNI standard (Table 7). Further analysis revealed that 52.38% of the studies included evaluations of sperm viability and morphology, and 4.76% concentrated on the integrity of the sperm plasma membrane. The National Standardization Agency (BSN) has established a standard for frozen bovine semen quality, as detailed in SNI 4869.1:2021, which requires post-thaw motility of over 40% and a minimum concentration of 25 million sperm/0.25 ml per straw.

The quality of frozen semen produced by producers must meet SNI standards. The study's findings indicated that although all producers conducted sperm motility tests, only 61.90% performed sperm concentration assessments. Recalculating the sperm concentration in a straw is vital to ensure the cell count aligns with the specified criteria. This step also evaluates the staff's competence in calculating the concentration of sperm in fresh semen and determining the correct amount of diluent

**Table 6:** Equipment for evaluation of sperm concentration

| Variables | Criteria | Producers | Percentage (%) |
|---|---|---|---|
| Sperm Concentration Tools | Photometer SDM/Accucell | 21 | 100 |
| Calibration | Internal | 15 | 71.43 |
| | External | 6 | 28.57 |

**Table 7:** The quality examination of frozen semen produced by frozen semen producers in Indonesia

| Variable | Producers | Percentage (%) |
|---|---|---|
| **Quality Examination** | | |
| Sperm motility | 21 | 100 |
| Sperm concentration | 13 | 61.90 |
| Sperm viability | 11 | 52.38 |
| Sperm morphology | 11 | 52.38 |
| Sperm plasma membrane integrity | 1 | 4.76 |
| **Frozen semen quality** | | |
| Below the Indonesian national standard | 1 | 4.76 |
| Compliant with Indonesian national standards | 17 | 80.95 |
| Exceeding the Indonesian national standard SNI | 3 | 14.29 |

to use. As shown in Table 7, while most centers adhere to the SNI standards for frozen semen quality, some even exceed these criteria, and some centers do not meet the required standards. It is essential to conduct a thorough evaluation of these producers to enhance their quality. The findings reveal that 52.38% of producers have implemented tests for sperm viability and morphology, whereas only 4.76% have examined the integrity of the sperm's plasma membrane.

To assess sperm viability and morphology, producers of frozen semen use eosin-nigrosine or 2% eosin staining methods, and the hypo-osmotic swelling technique to evaluate the integrity of the sperm plasma membrane. Those producers who did not perform these additional tests cited difficulties in acquiring raw materials and a lack of knowledge about the procedures and benefits of these examinations.

According to the Director General of Animal Husbandry and Animal Health Decree Number 9471/KPTS/HK.160/F/09/2023, additional tests for frozen semen quality are mandated to be gradually implemented over the next five years. Producers of frozen semen must understand these quality assessments, as they influence sperm fertility and the effectiveness of artificial insemination (AI). Evaluating sperm motility, along with viability and plasma membrane integrity, is particularly important because it ensures sperm remain viable and motile while also protecting the cytoplasm and DNA integrity within the cells (Arif et al. 2022; Ugur et al. 2019).

According to the analysis, there is significant diversity among frozen semen producers, which can be linked to several factors, including human resources, the number and breeds of bulls, production goals, types of semen diluents and dilution methods, equipment used, and the quality testing of frozen semen. Support and oversight are necessary from various entities, including the Livestock Service Office, universities, and research centers focused on semen quality in Indonesia. The involvement of professional certification bodies or animal product certification organizations is essential. Furthermore, producers should enhance their laboratory personnel's skills to ensure that the frozen semen produced meets the required standards for artificial insemination.

## CONCLUSION

The findings concluded that frozen semen producers differed in the number and gender of their laboratory personnel, as well as in the number and types of local and exotic bulls used. These producers typically use homemade diluents in a single-step process. Their equipment was generally comprehensive. All producers conducted quality tests on frozen semen for post-thaw motility, although only a few recalculated the sperm concentration in the straws. Additional assessments, such as sperm viability and morphology, were conducted by 52.38% of producers, while 4.76% tested sperm plasma membrane integrity. Most producers met or exceeded the SNI standards, but 4.76% failed to meet the SNI criteria.

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# Effect of Locally Extracted Phytase on Blood Hematological and Serum Biochemistry of Broiler Chickens

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**ABSTRAK**

Lemma M, Girma M, Ameha N, Beker A, Zeryehun T, Yirgalem M. 2025. Pengaruh fitase yang diekstrak secara lokal dalam hematologi darah dan biokimia serum ayam broiler. JITV 30(3):140-149. DOI:http://dx.doi.org/jitv.v30i3.3409

Penelitian dilakukan untuk menguji pengaruh fitase yang diekstraksi secara lokal pada performa ayam pedaging dengan atau tanpa suplementasi fitase pada hematologi darah dan biokimia serum ayam pedaging. Sejumlah ayam pedaging Cobb 500 umur 180 hari secara acak dikelompokkan ke dalam empat perlakuan pakan menggunakan rancangan acak lengkap (RAL) yang direplikasi tiga kali dengan masing-masing sebanyak 15 ekor anak ayam. Rancangan percobaan adalah rancangan acak lengkap (RAL) dan ayam diberi pakan yang mengandung fitase pada laju dosis 0, 300, 600, dan 1200 FTU/kg selama 42 hari. Terdapat perbedaan yang signifikan (P<0,05) di antara perlakuan terhadap persentase volume darah yang terdiri dari sel darah merah (PCV), Hb, dan WBC. Namun, tidak ada perbedaan yang signifikan (P>0,05) dalam jumlah RBC, MCV, MCH, atau MCHC pada ayam dengan pakan yang disuplementasi dengan enzim. Nilai total protein, albumin, dan globulin tidak meningkat secara signifikan (P>0,05) pada kelompok yang diberi suplemen dibandingkan dengan kelompok kontrol T1 selama seluruh periode pertumbuhan. Konsentrasi kalsium berkisar antara 1-1,49% dan 0,75-0,89%, sedangkan fosfor berkisar antara 0,37-0,52% dan 0,28-0,44% pada pakan starter dan finisher, masing-masing, dan berada dalam kisaran yang direkomendasikan dalam ransum broiler. Berdasarkan hasil analisis anggaran parsial ini, ayam broiler T4 (1200 FTU/kg) mencapai keuntungan yang lebih tinggi dibandingkan ayam yang diklasifikasikan sebagai T2, T3, dan T1. Penggunaan fitase mungkin lebih menguntungkan daripada pemberian pakan kontrol. Tidak terdapat perbedaan mortalitas yang signifikan antar perlakuan (P>0,05).

**Kata Kunci:** Broiler, Hematologi, Mortalitas, Serum, Fitase, Fitat

**ABSTRACT**

Lemma M, Girma M, Ameha N, Beker A, Zeryehun T, Yirgalem M. 2025. Effect of locally extracted phytase on blood hematological and serum biochemistry of broiler chickens. JITV 30(3): 140-149. DOI:http://dx.doi.org/jitv.v30i3.3409

Experiments were conducted to examine the effect of locally extracted phytase on the performance of broilers with or without phytase supplementation on the blood hematological and serum biochemistry of broiler chickens. A total of 180-day-old Cobb 500 broilers were randomly assigned to four treatment diets in a completely randomized design (CRD), with 15 chicks per replicate and three replications. The experiment used a completely randomized design (CRD), and the birds were fed experimental feed containing phytase at 0, 300, 600, and 1200 FTU/kg for 42 days. There were significant differences (P<0.05) among treatments for Packed cell volume (PCV), Hb, and WBC. However, there were no significant (P>0.05) differences in RBC count, MCV, MCH, or MCHC of broilers fed the diet supplemented with enzymes. Total protein, albumin, and globulin values did not increase significantly (P>0.05) in the supplemented groups compared with the T1 control group throughout the growth period. The calcium concentration ranged from 1-1.49% and 0.75-0.89% in starter and finisher diets, respectively, whereas phosphorus ranged from 0.37-0.52% and 0.28-0.44% in starter and finisher diets, respectively, and it is within the range recommended in the broiler ration. According to the results of this partial budget analysis, T4 (1200 FTU/kg) broiler chickensachieved higher profits than chickens classified as T2, T3, and T1, respectively. Using phytase may be more profitable than feeding control. There was no significant difference in mortality between treatments (P>0.05).

**Key Words:** Broiler, Hematology, Mortality, Serum, Phytase, Phytate

## INTRODUCTION

The poultry industry in developing countries faces challenges due to the high costs of conventional feed ingredients, such as yellow corn and soybean meal, which are primarily used in poultry rations (Alagawany & Attia 2015). Thus, there is an urgent need for nutritious and affordable feeds. Nowadays, feed accounts for about 75% of total animal production costs. Most poultry feed ingredients have historically been of plant origin, with the anti-nutritional factor phytate present as mixed phytate salts (AOAC 2006).

Feedstuffs such as corn and soybean meal have low phosphorus bioavailability due to phytate (Adeola 2004). Phytate has low solubility in the small intestine, poultry poorly absorb it, and its negative charge makes it a potent mineral chelate that forms insoluble salts with minerals. Birds do not produce the enzymes needed to digest NSP. Supplementing with NSP-degrading enzymes not only reduces the anti-nutrient effect of NSP but can also release nutrients available to birds from NSP (Balamurugan & Chandrasekaran, 2009).

Phytase is the only known enzyme that can initiate the phosphate hydrolysis at carbon 1, 3, or 6 in the inositol ring of phytate. The removal of the phosphate group by phytase results in the release of calcium, iron, zinc, and other metals (Konietzny & Greiner 2006). Phytase increased feed intake and body weight in poultry (Murugesan et al. 2005). The inclusion of phytase in broilers' diets with a low concentration of non-phytate phosphorus increased phosphorus retention with concomitant reduced excretion (Juanpere et al. 2004). As more phosphorus is released from phytate, leading to more breakdown of intact IP-6, the less able it is to bind or chelate minerals, starch, or proteins directly or via ionic bridges (Selle 2007).

However, the effects of extrinsic enzymes vary and depend on a variety of factors, including the age of the bird and the quality and type of the diet (Bedford 2000; Acamovic 2001). To solve this problem, phytase is commonly used as a feed additive to release P bound to phytic acid. Extrinsic phytase dietary supplements are effective in improving P digestibility (Walk et al. 2013; Selle & Ravindran 2007; Adeola & Cowieson 2011). Many commercially available multienzyme complexes can improve the nutritional value of protein-rich plant-based feed ingredients. However, imported commercial phytase is expensive and unavailable in Ethiopia. Therefore, to replace the commercial phytase enzyme, this research is locally extracting a phytase enzyme from available material (rye). The immune system in poultry can be identified from hematological profiles of the animal's blood. Hematological testing is used not only for diagnosis and therapeutic purposes but also for monitoring treatment responses (Oloche 2015). Therefore, this study aimed to determine the effects of phytase supplementation on hematology profiles in broilers.

## MATERIALS AND METHODS

### Description of the study area

The study was carried out at the Poultry farm of Haramaya University of Agriculture, which is located at a distance of 510 km east of Addis Ababa, at 42°3' east of longitude, 9°26'north latitude, and an altitude of 1980 meters above sea level. The mean annual rainfall of the area is 780 mm, and the average minimum and maximum temperatures are 8.5 and 24.4°C, respectively (Fedis Agricultural Research Centre)). Experimental work was conducted at Haramaya University's poultry farm for 42 days, from September to October 2021.

### Ethical approval

The protocols for this experiment, use, and care of broilers were carried out in accordance with the guidelines of the Animal Care and Use Committee of Haramaya University, Ethiopia. Name of the approving committee: 1. Dr. Mulatu Wagari (chair) 2. Mr. Bacha Daba (Secretary) 3. Dr. Sisay Girma 4. Mr. Nega Baraki 5. Dr. Anteneh Belayneh 6. Dr. Dereje Tadese 7. Dr. Hirut Yirga 8. Dr. Teshome Seyum

### Extraction procedure of phytase from rye

Rye grains were bought from a nearby market in Harar town, Ethiopia. The average phytase activity in rye kernels will be around 3.7 U/g. Based on this, 81 grams of rye were used to extract 300 U/g, 162 grams of rye were used to extract 600 U/g, and 324 grams of rye were used to extract 1200 U/g. In total, 568 g of rye yielded 2100 U/g of phytase. Small amounts of rye germ were ground in an Ultra-Turrax (5-10 min) at 4 °C in ice-cold 100 mM sodium acetate buffer, pH 5.0, whereas larger amounts were ground in a kitchen blender in the same buffer. Afterward, the soluble compounds were extracted by shaking for 2 h at 40°C. The cell debris was removed by centrifugation at 20,000 g for 30 min. Samples were weighed and placed in 250 ml Erlenmeyer flasks, then extracted using the procedures described by Harland and Oberleas (1977). The enzyme extraction was conducted at the Biotechnology Laboratory of Haramaya University.

The effect of pH on enzyme stability was tested over the pH range 1.0-9.0 at 4°C. In the pH range from 4.0 to 7.5, the phytase was relatively stable, while below pH 3.0 and above pH 7.5, a rapid decline in activity was observed. Within 10 days, more than 90% residual activity was measured at pH 4.0. At pH 2.5 and 8.0, 70% and 46% of the original activity were lost over 24 h of incubation, respectively.

### Purification of the phytase

The cell-free culture supernatant was dialyzed and concentrated using the Lab scale TFF filtration system (Millipore, Bedford). The dialyzed and concentrated culture was then applied to an anion-exchange chromatographic column (diethylaminoethyl, HiPrep 16/10 DEAE-Sepharose FF, Pharmacia, Sweden) equilibrated with 20 mM Tris-HCl buffer, pH 8. After washing the column, the bound enzyme was eluted at a flow rate of 1 ml/min using a linear gradient from 0 to 100% of 1 M NaCl in 20 mM Tris-HCl buffer, pH 8. Fractions were collected in volumes of 5 ml. Fractions containing phytase activity were pooled, dialyzed, and concentrated using the Labscale TFF filtration system (Millipore, Bedford). The concentrated enzyme was applied to a gel filtration column (HiPrep 16/60 Sephacryl S-100 HR, Pharmacia, Sweden) pre-equilibrated with 50 mM phosphate buffer and 200 mM NaCl at pH 8, and eluted using the same buffer at a flow rate of 1 ml/min. Fractions were collected every 5 min, and those with high phytase activity were pooled. The pooled fractions were dialyzed using ultrafiltration tubes at 5000 rpm for 20 min at 4°C in Centricon 10 (Amicon, USA) ultrafiltration concentrators (membrane cutoff of 10 kDa). During all the purification procedures, all collected and pooled fractions were tested for absorption (wavelength 280 nm), total protein (wavelength 595 nm), and phytase activity (wavelength 355 nm).

**Seed germination**

Rye grains were soaked in the following solutions: (1) 0.1% Tween-80 for 5 minutes, (2) 0.5% NaOCl for 2 minutes, (3) 0.75% H2O2 for 1 minute. After soaking, the rye grains were thoroughly rinsed in sterile water. The seeds were then allowed to germinate in a sterile box in a dark place at 20°C. Once a day, the seeds are washed with sterile water; after rinsing, the water is completely removed (Gibson et al., 1988).

The seeds were culled for broken seeds, then weighed out for sprouting. For each time segment, samples of each seed were placed in quart glass jars. The mouths of the jars were covered with flexible plastic screening held in place with a string. The grains had been soaked in deionized water for 12 hours before sprouting was considered underway. After 12 hours, the deionized soaking water is drained, and half a liter of deionized water is dispensed into the jar. Seeds were rinsed with sterile water once a day; the water was removed entirely after each rinse.

Excess water is shaken out of the bulb before placing the bulb, mouth facing down, to empty the seeds. The seeds were then allowed to germinate in sterile trays indoors in the dark at 20°C. Every afternoon, the grains are rinsed with sterile water, which is then removed. One hundred milliliters of 1.2% HCl is delivered to every flask. The flask becomes sealed with plastic wrap. The flasks were shaken at 2 hundred rpm for 2 hours at 26°C. The samples had been vacuum filtered with #1 Whatman Filter paper. The filtrate was stored for no more than 1 week at 1 °C in the refrigerator. During the first 7-10 days, phytase activity started.

**Experimental animals and management**

The experiment was conducted at Haramaya University poultry farm for 42 days. Before starting the actual experiment, the experimental house was cleaned and washed, and the floor was covered with a 7cm layer of wood shavings, which was thoroughly disinfected before placement of the experimental birds. Two infrared lamps, each providing 250 watts, were fitted for each pen as a source of heat and light. A circular plastic feeder and waterer were placed in each pen a day before the birds were placed.

A total of one hundred eighty (180) day-old unsexed broiler chicks were purchased from available farms around Debre Zeit's private farm. Water was available at all times, and the experimental ratio was measured on an ad libitum basis twice a day at 8:00 and 16:00 hrs. The refusals were recorded every morning to determine feed and nutrient intakes. Broilers were weighed by pen at 0, 7, 14, 21, 28, 35, and 42 days of age using a sensitive balance. There are three phases: 0 to 10, 11 to 24, and 25 to 42 days of age. Standard biosecurity protocols were employed throughout the experimental period, and the chicks were vaccinated against viral diseases.

**Design of the Experiment and Ingredients**

A total of 180-day-old Cobb 500 broilers were randomly assigned to four treatment diets in a completely randomized design (CRD), with 15 chicks per replicate and three replications. The treatment rations were formulated using FeedWin Interactive software to be isocaloric and isonitrogenous, meeting the nutrient requirement standards for broilers (NRC, 1994), as shown in Table 1 below. Accordingly, each of the starter treatment rations contained about 3100 kcal ME/kg of energy and 22% crude protein, while each of the finisher's treatment rations contained 3200 kcal ME/kg of energy and 20% crude protein. The starter phase lasted until 3 weeks of age (days 1st to 21st), and the finisher phase lasted from the beginning of the third week until slaughter (days $22^{nd}$ to $42^{nd}$). The starter and finisher diets were formulated separately. The treatments consisted of a control diet for each phase, and three other diets were formulated, each with increasing levels of the phytase enzyme added to the feed at 0, 300, 600, and 1200 FTU/kg for T1, T2, T3, and T4, respectively.

**Hematological parameter**

Blood samples were collected from the wing vein of 2 birds per replication into a 5 ml sterile syringe using a 23-gauge needle for hematological and bioche-

**Table 1.** Composition of ingredients fed to broilers from 0-3 weeks and 4-6 weeks of age

| Ingredients (%) | 0-3 weeks (Starter Phase) | 4-6 weeks (Finisher Phase) |
|---|---|---|
| Maize | 56.76 | 61.39 |
| Soyabean | 18.45 | 13.65 |
| Wheat | 2.92 | 8.2 |
| Methionin | 0.5 | 0.1 |
| Dicalcium | 0.5 | 0.5 |
| Nougseed cake | 17.46 | 13.66 |
| Salt | 0.97 | 0.5 |
| Premix | 0.5 | 0.2 |
| Limestone | 0.97 | 1.3 |
| Lysine | 0.97 | 0.5 |
| Total | 100 | 100 |

mical parameters at the finisher phase (42 days of age). From each chicken, 1-2 mL of blood was collected aseptically through the brachial (wing) vein as described by Kelly (2013).

Each blood sample was immediately transferred into a tube containing ethylenediaminetetraacetic acid as an anticoagulant. The red blood cell (RBC) and white blood cell (WBC) counts were determined using a hemocytometer (Irizaary-Rovira 2004). The hemoglobin (Hb) concentration was evaluated by matching the acid hematin solution to a standard colored solution in the Sahlis hemoglobinometer. Packed cell volume (PCV) was measured by the microhaematocrit method. Mean corpuscular volume (MCV), Mean Corpuscular Haemoglobin Concentration (MCHC), and mean corpuscular hemoglobin (MCH) were computed as = (Ross et al. 1976; Irizaary-Rovira, 2004). These parameters were determined using blood harvested into the tube containing EDTA.

### Serum biochemical parameters

Serum was separated after centrifugation at 3,000 (rpm x g) for 15 min and stored at -20° C until used. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) activities, cholesterol, glucose, and triglycerides concentrations were measured by using enzyme/ buffer and substrate kits, such as AP (Alkaline Phosphatase), at Haramaya University Health Center Laboratory. Total serum protein was determined by refractometer (George 2001). Total serum immunoglobulin concentration was determined by the serum zinc sulfate turbidity test, measuring the optical density of the test and the control separately at 545nm using a spectrophotometer (Kassaye et al. 2016).

### Economic efficiency analyses

The net profits from broilers were calculated based on the cost of broiler birds and the feed each bird consumed in the respective treatments, along with other costs. To estimate the net benefit of phytase feeding, a partial budget was prepared, accounting for full feed costs according to the principles developed by Upton (1979). The partial budget analysis involves calculating variable costs and benefits. Partial budget measures the cost of the chicken, feed, and other inputs (if any), and the profit after the experiment—the difference between gains and losses from the proposed change.

The costs of experimental feed ingredients and basal feeds were used to calculate the feed cost for each treatment. Total variable cost includes feed and other costs. The total rate of return (TRR) was calculated as the difference between the sale and purchase prices in the partial budget analysis. The net income (NI) was expressed by subtracting the total variable cost (TVC) from the total return between the changes in the total rate of return (ΔTRR) and total variable cost (ΔTVC).

The marginal rate of return (MRR) measures the increase in net income (ΔNI) per additional unit of expenditure (ΔTVC), as shown below.

$$MRR = \frac{\Delta \text{NI}}{\Delta \text{TVC}}$$

The chick sale price-to-feed cost ratio was also calculated as an additional parameter to evaluate the efficiency of the change in feed ingredients.

### Statistical analysis

All data collected in this study were analyzed using SAS (2016). Differences between treatment means were separated using Duncan's Multiple Range Test. The following model was used for data analysis. $Y_{ij} = \mu + T_i + e_{ij}$, where $Y_{ij}$= represents the jth observation in the ith treatment level, $\mu$= overall mean, $T_i$= treatment effect, and $e_{ij}$= random error.

## RESULTS AND DISCUSSION

The chemical composition of the experimental feed ingredients was presented in Table 2 and determined by proximate analysis, which analyzed the dry matter (DM), ash, crude protein (CP), ether extract (EE), and crude fiber (CF). Two experiential diets used in the experiment are chick starter and finisher diets. They were made from local feed ingredients commonly used in poultry feeding in Ethiopia. The diets were formulated to meet the NRC (1994). The metabolizable energy of the experimental diets was determined by the indirect method using the formula given by Wiseman (1987): ME (Kcal/kg DM) = 3951 + 54.4 EE - 88.7 CF - 40.8 Ash. The formulation, calculated, and determined analyses of the diet are shown in Tables 2 and 3 below, respectively.

The experimental birds were fed on the starter diet for the first three weeks (1-21 days) of the experiment. Thereafter, they were fed on the finisher diet (22-42 days) supplemented with four levels of phytase enzyme having 0.0 FTU/Kg, 300 FTU/Kg, 600FTU/Kg, and 1200FTU/Kg, for the last four weeks of the experiment (22-42 days). The phytase has been added to the feed in powder form, and it should be added from the starter to the finisher phase. The feed was formulated twice, once at the starter phase and once at the finisher phase. So the chickens were fed with phyase for 42 days. The calcium concentration ranged from 1-1.49% and 0.75-0.89% in starter and finisher diets, respectively, whereas phosphorus ranged from 0.37-0.52% and 0.28-0.44% in starter and finisher diets, respectively, and are within the range recommended in the broiler ration (NRC 1994).

### Haematological profiles

The effects of phytase supplementation on the blood hematological parameters of the broiler chickens are listed in Table 4. Packed cell volume (PCV), Hb, and WBC values were significantly ($P<0.05$) influenced by the dietary treatments, while all other parameters were not significantly ($P>0.05$) different. The values for PCV ranged from 27.783 – 33.053%, Hb from 9.11 – 11.05g/dl, and WBC from 4.8 – 6.09×109/L, respectively. It was observed that PCV, Hb, and WBC values were reduced across dietary treatments. PCV, Hb, and WBC were significantly ($P<0.05$) influenced by the dietary treatment; the values obtained were within the normal range for broiler chicken reported by (Oloche et al. 2015).

Table 4 shows that white blood cell counts in the control and treated groups were 6.31, 6.17, 5.92, and 5.62 × 10^9/l at T0, T1, T2, T3, and T4, respectively. The experimental birds were fed on different groups that were significantly different from one another. This indicates a potential effect of diet on white blood cell levels, as the treated groups showed lower average counts than the control group, and there were statistically significant differences between the diet groups.

The present results were in disagreement with the findings of Shareef and Al-Dabbagh (2009), and Alkhalf et al. (2010) observed a non-significant ($P>0.05$) difference in PCV of broilers fed the diet supplemented with enzymes. In contrast, Cowieson et al. (2006), Rahman et al. (2013), and Kaushal et al.

**Table 2.** Chemical composition of ingredients and treatment diets (percentage on DM basis)

| Ingredient | (%) | Chemical Composition | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | DM | ash | EE | CF | CP | Ca | P | ME |
| Maize | 46 | 87.74 | 5.35 | 3.4 | 3.85 | 9.1 | 0.055 | 0.375 | 3576.2 |
| Soyabean | 13.39 | 92.22 | 6.2 | 9.6 | 10.26 | 31.08 | 0.37 | 0.885 | 3310.218 |
| Wheat Short | 15.5 | 88.6 | 8.25 | 2.6 | 9.9 | 14.01 | 0.24 | 0.705 | 2877.71 |
| Nougseed cake | 15 | 93.56 | 11.9 | 7.25 | 15.7 | 28.24 | 0.6 | 0.8 | 2467.29 |
| Phytase | - | 94.52 | 1.6 | 0.95 | 1.5 | 13 | 1.2 | 0.6 | 3804.35 |

ME= Metabolizable energy, DM= total dry matter, CP= Crude protein, EE= Ether extract, Kcal= Kilocalorie, Kg= kilogram

**Table 3**. Chemical composition of ingredients and treatment diets (% on DM basis)

| Ingredients | Item | | | | | | | Chemical composition |
|---|---|---|---|---|---|---|---|---|
| | %DM | CP | CF | EE | ash | Ca | P | ME(Kcal/Kg) |
| Maize | 89.88 | 9.06 | 3.54 | 3.86 | 4.42 | 0.075 | 0.50 | 3666.65 |
| Wheat short | 90.46 | 14.90 | 8.85 | 3.58 | 5.95 | 0.18 | 0.59 | 3117.997 |
| Soybean meal | 92.64 | 35.85 | 9.6 | 6.51 | 6.22 | 0.34 | 0.74 | 3199.848 |
| Nougseed cake | 92.95 | 29.61 | 16.46 | 7.43 | 11.26 | 0.41 | 0.57 | 2435.782 |
| Phytase | 94.52 | 35.32 | 0.5 | 4.00 | 4.53 | 1.2 | 0.6 | 3939.426 |
| Starter phase | %DM | CP | CF | EE | ash | Ca | P | ME(Kcal/kg ) |
| T1 | 92.27 | 22.82 | 9.3 | 7.4 | 10.7 | 1.0 | 0.51 | 3092.09 |
| T2 | 91.63 | 22.92 | 9.14 | 8.35 | 11.65 | 1.0 | 0.52 | 3119.202 |
| T3 | 91.07 | 22.45 | 9.21 | 7.2 | 10.2 | 1.49 | 0.51 | 3109.593 |
| T4 | 92.45 | 22.42 | 8.7 | 7.03 | 10.3 | 1.37 | 0.37 | 3141.502 |
| Finisher phase | %DM | CP | CF | EE | Ash | Ca | P | ME(Kcal/kg ) |
| T1 | 92.88 | 21.1 | 8.37 | 8.17 | 9.83 | 0.89 | 0.32 | 3251.965 |
| T2 | 92.62 | 20.76 | 7.8 | 7.45 | 9.6 | 0.89 | 0.32 | 3272.74 |
| T3 | 91.52 | 20.31 | 8.87 | 8.1 | 10.3 | 0.87 | 0.44 | 3184.631 |
| T4 | 93.62 | 20.12 | 9.62 | 7.01 | 11 | 0.75 | 0.28 | 3030.25 |

T1, T2, T3, and T4 0, 300, 600, and 1200 FTU/Kg, respectively, DM= dry matter, CP= crude protein, EE= ether extract, CF= crude fiber, Ca= calcium, P= phosphorus, ME= Metabolisable Energy, Kcal= kilocalorie, Kg= kilogram

(2015) found a significant (P<0.05) increase in PCV and Hb content of broilers fed the diet supplemented with enzymes. Ferdous et al. (2018) noted a significant (P<0.01) increase in PCV and Hb in broilers fed a diet supplemented with enzymes and multivitamins. Hosseini (2011) noted a significant (P<0.05) increase in Hb of broilers fed the diet supplemented with yeast (*Saccharomyces cerevisiae*). Ahmed et al. (2007) found a significant (P<0.05) increase in PCV and Hb values in broilers fed a diet supplemented with enzymes (Alquerzim). Milanovic et al. (2008) noted an increase in PCV and Hb of broilers fed the diet supplemented with organic iron. The analyzed data show that there were no significant differences (P>0.05) in MCV, MCH, and MCHC among broilers fed the enzyme-supplemented diet. The present results were consistent with those of Shehab et al. (2012), who observed non-significant (P>0.05) differences in MCV in broilers fed a diet supplemented with enzymes. Chuka (2014) found no significant differences (P>0.05) in MCV, MCH, and MCHC in broilers fed a diet supplemented with enzymes and probiotics. The results of hematological variables in this study suggest that the test diets did not pose any severe effects on the health status of the experimental birds.

**Serum biochemistry**

The effects of phytase supplementation on the blood serum biochemistry parameters of the broiler chickens are listed in Table 5 below. Total protein, albumin, and globulin values did not increase significantly (P>0.05) in the supplemented groups compared with the T1 control group throughout the growth period. The results of the present experiment were in accordance with the findings of Chuka (2014) noted non-significant (P>0.05) differences in serum globulin, and serum albumin of broilers fed the diet supplemented with enzymes and in contrast with the findings of Hassen & Chauhan (2003) who found a significant (P<0.05) increase in serum globulin levels in broilers fed a diet supplemented with enzymes. Paryad and Mahmoudi (2008) and Yazhini et al. (2018) noted a significant (P<0.05) increase in total protein, albumin, and globulin concentrations in broilers fed the diet supplemented with probiotic (S. cerevisiae). Shareef and Al-Dabbagh (2009) and Chaudhary et al. (2017) observed a significant (P<0.05) increase in serum total protein of broilers fed the diet supplemented with probiotics. Chuka (2014) and Kaushal et al. (2019) found a significant (P<0.05) increase in total protein in broilers fed a diet supplemented with enzymes. The dietary treatment did not influence all measured serum biochemical parameters, indicating that the animals well tolerated the diets.

### Partial budget analysis

The economic returns associated with subbudgets are shown in Table 6. A producer's goal in poultry production is to achieve the highest growth rate at the lowest feed cost per unit of live weight gain. According to this partial budget analysis, T4 broiler chickens achieved higher profits than those classified as T2, T3, or T1. T4 birds had the highest cost of selling chicks for feed compared to other treatments. Therefore, the results of this study suggest that using phytase may be more beneficial than feeding control. T4 was the most cost-effective diet per chick reared in this study.

### Mortality

The death is recorded in Table 7 below. According to the present study, there was no significant difference in mortality rate between the treatments (P>0.05). The cause of chicken deaths during this period was transportation stress and the inability of individuals to adapt to the environment. Examination in all phases of dead chicken showed the presence of watery fluid in the abdominal cavity and pericardial sac and sudden death; this may be due to the high growth rate of broiler chicken and rapid growth, which causes the heart and lungs not to develop well enough to support the remainder of the body, resulting in congestive heart failure and tremendous death losses (Martin 1997).

**Table 4.** Effects of phytase supplementation on the blood hematological parameters of broiler finishers from (0-42 days)

| Parameters | Treatments | | | | | |
|---|---|---|---|---|---|---|
| | T1 | T2 | T3 | T4 | SEM | P.value |
| PCV (%) | 33.053$^{a}$ | 32.82$^{a}$ | 30.503$^{ab}$ | 27.783$^{b}$ | 0.95 | 0.014 |
| Hb(g/dl) | 11.05$^{a}$ | 10.12$^{ab}$ | 9.94$^{ab}$ | 9.11$^{b}$ | 0.3536 | 0.03 |
| RBC($\times 10^{12}$/L) | 2.76 | 2.78 | 2.76 | 2.95 | 0.01 | 0.21 |
| WBC($\times 10^{9}$/L) | 6.09$^{a}$ | 5.95$^{a}$ | 5.73$^{ab}$ | 4.8$^{b}$ | 0.29 | 0.03 |
| MCV(fl) | 138.60 | 138.33 | 139.07 | 139.09 | 0.86 | 0.90 |
| MCH(pg) | 31.81 | 31.23 | 30.85 | 29.63 | 0.01 | 0.66 |
| MCHC (%) | 23.45 | 23.19 | 22.23 | 21.25 | 35.61 | 0.31 |

PCV= Packed cell volume; Hb= Hemoglobin; RBC= Red blood cell; WBC= White blood cell; MCV= Mean Corpuscular Volume; MCHC= Mean Corpuscular Hemoglobin Concentration; fL= femtoliters, pg= picograms, dl= deciliter; T1, T2, T3, and T4= 0, 300, 600, and 1200 FTU/Kg, respectively

**Table 5.** Effects of phytase supplementation on the blood serum biochemistry parameters

| Parameters | Treatments | | | | | |
|---|---|---|---|---|---|---|
| | T1 | T2 | T3 | T4 | SEM | P.value |
| Total protein(g/dl) | 3.65 | 3.65 | 3.79 | 3.65 | 0.48 | 0.99 |
| Albumin (g/dl) | 1.90 | 2.31 | 2.04 | 1.95 | 0.51 | 0.89 |
| Globulin (g/dl) | 1.75 | 1.67 | 1.75 | 1.69 | 0.52 | 0.99 |
| Total immunoglobulin(g/dl) | 0.61 | 0.91 | 0.83 | 1.06 | 0.03 | 0.08 |

T1, T2, T3, and T4 = 0, 300, 600, and 1200 FTU/Kg, respectively, SEM = Standard Error of Mean; dl = deciliter

**Table 6.** Economics of phytase supplementation in broiler chicken

| Particulars | Treatments | | | |
|---|---|---|---|---|
| | T1 | T2 | T3 | T4 |
| Purchase price/bird (birr) | 40.00 | 40.00 | 40.00 | 40.00 |
| Total feed consumed(kg)/chick | 3.89 | 3.93 | 3.89 | 3.93 |

| Particulars | Treatments | | | |
|---|---|---|---|---|
| | T1 | T2 | T3 | T4 |
| Selling price/bird(chick) | 207.9 | 238.2 | 221.25 | 256.35 |
| Feed cost/bird (birr) | 56.40 | 56.98 | 56.40 | 56.98 |
| Other cost /bird (birr) | 22.22 | 22.22 | 22.22 | 22.22 |
| TVC/bird (birr) | 78.62 | 79.2 | 78.62 | 79.2 |
| TR (birr) | 167.9 | 198.2 | 181.25 | 216.35 |
| NR (birr) | 89.28 | 119 | 102.63 | 137.15 |
| ΔTR(birr) | 0.00 | 30.3 | 13.35 | 48.85 |
| ΔNR(birr) | 0.00 | 29.72 | -16.37 | 34.52 |
| ΔTVC | 0.00 | 0.87 | -0.72 | 0.58 |
| MRR | - | 34.13 | 22.73 | 59.51 |
| Chicks sale/feed cost | 3.68 | 4.18 | 3.92 | 4.49 |
| Feed cost/Chick sale ratio | 0.27 | 0.24 | 0.25 | 0.22 |

Meat price @ Rs 150 per kg of live weight, Feed price @ Rs 14.5 per kg; Net Return, ΔTVC = Change in total Variable Cost, ΔNR = Change in Net Return; MRR = Marginal Rate of Return; T1, T2, T3 and T4 = 0, 300, 600 and 1200 FTU/Kg, respectively, TR= Total Return, NR = net return

**Table 7.** Effect of adding phytase on the mortality percentage of broilers

| Parameters (%) | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | T1 | T2 | T3 | T4 | SEM | P value |
| Mortality of the starter | 2.95 | 2.93 | 0.73 | 0.3333 | 0.73333 | 0.09 |
| Mortality of finisher | 0.733 | 2.22 | 0.733 | 0.000 | 0.2357 | 0.08 |
| Mortality of the entire | 3.63 | 5.12 | 1.42 | 0.31 | 0.01 | 0.6775 |

Meat price @ Rs 150 per kg of live weight, Feed price @ Rs 14.5 per kg.T1, T2, T3, and T4 = 0, 300, 600, and 1200 FTU/Kg, respectively, SEM= Standard error of the mean

## CONCLUSION

There were significant differences (P<0.05) among treatments for Packed cell volume (PCV), Hb, and WBC. However, there were no significant (P>0.05) differences in RBC count, MCV, MCH, or MCHC of broilers fed the diet supplemented with enzymes. Total protein, albumin, and globulin values did not increase significantly (P>0.05) in the supplemented groups compared with the T1 control group throughout the growth period. The calcium concentration ranged from 1-1.49% and 0.75-0.89% in starter and finisher diets, respectively, whereas phosphorus ranged from 0.37-0.52% and 0.28-0.44% in starter and finisher diets, respectively, and it is within the range recommended in the broiler ration (NRC, 1994). According to the present partial budget analysis, broiler chickens in T4 yielded higher profits than those in T2, T3, and T1, respectively. Using phytase may be more profitable than feeding control. No significant difference (P>0.05) in mortality percentage between the treatments in all phases.

## AUTHOR CONTRIBUTION STATEMENT

Mengistu Lemma came up with the idea and wrote the paper. Negasi Ameha, Meseret Girma, and Ali Beker, who also authorized the final version for publication, critically edited the manuscript for key intellectual content.

## CONFLICT OF INTERESTS

The authors state that they have no conflicts of interest.

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# Screening Probiotics from Genetically Unselected Indonesian Local Chicken to Enhance Meat Quality in Broiler Chicken

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**ABSTRAK**

Wardhani R, Afidah U, Syamsurya D, Lee J, Fufa AJ, Gani F, Husain DR. 2025. Skrining probiotik pada ayam lokal Indonesia yang belum terseleksi secara genetik untuk meningkatkan kualitas daging ayam pedaging. JITV 30(3):150-158. DOI:http:dx.doi.org/jitv.v30i3.3456.

Penelitian ini bertujuan untuk mengisolasi dan mengkarakterisasi bakteri probiotik dari usus ayam lokal Indonesia (*Gallus gallus domesticus)* yang secara genetik belum terseleksi, untuk meningkatkan kualitas daging ayam pedaging. Ayam kampung berperan sebagai sumber bakteri probiotik yang menjanjikan dikarenakan ketahanan dan kemampuan dalam beradaptasi. Probiotik dipilih menggunakan media MRSA (de Man Rogosa Sharpe Agar) dengan suplementasi 1% $CaCO_3$. Potensi probiotik isolat bakteri dievaluasi berdasarkan ketahanannya terhadap keasaman lambung (pH) dan garam empedu. Karakterisasi meliputi morfologi koloni, uji antibiotic menggunakan *Escherichia coli* dan *Salmonella typhi*, dan uji biokimia seperti MR-VP, TSIA, motilitas, dan katalase. Empat isolat bakteri probiotik diperoleh, dengan tiga diantaranya bersifat Gram-positif (J2, J3, and J4) dan satu bersifat Gram-negatif (J1). Semua isolat berbentuk batang (basil), katalase-negatif, tidak motil, dan menunjukkan efek penghambatan terhadap *E. coli* dan *S. typhi*. Isolat J2 menunjukkan aktivitas penghambatan terkuat, dengan zona penghambatan berukuran 15,5 mm terhadap *S. typhi* dan 11,5 mm terhadap *E. coli*. Ketika digunakan sebagai aditif pakan, isolat J2 meningkatkan kualitas daging ayam pedaging dengan mempertahankan pH optimal, meningkatkan kapasitas menahan air, dan mengurangi kadar kolesterol dan lemak, meskipun meningkatkan kehilangan pemasakan. Hasil ini menunjukkan bahwa isolat J2 memiliki potensi signifikan sebagai agen probiotik dalam nutrisi unggas, dengan implikasi yang menjanjikan untuk meningkatkan kualitas daging dan keamanan pangan. Penelitian di masa depan harus fokus pada pengoptimalan formulasi probiotik dan mengeksplorasi efek jangka panjang pada kesehatan dan produktivitas unggas.

**Kata Kunci:** Ayam Lokal, Kualitas Daging, Sumber Probiotics

**ABSTRACT**

Wardhani R, Afidah U, Syamsurya D, Lee J, Fufa AJ, Gani F, Husain DR. 2025. Screening probiotics from genetically unselected Indonesian local chicken to enhance meat quality in broiler chicken. JITV 30(3):150-158. DOI:http:dx.doi.org/jitv.v30i3.3456.

This study investigated the isolation and characterization of probiotic bacteria from the intestines of genetically unselected Indonesian local chickens (*Gallus gallus domesticus)* to improve the quality of broiler chicken meat. Due to their resilience and adaptability, *Indonesian local* chickens serve as a valuable source of probiotic bacteria. Probiotics were selected using MRSA (de Man, Rogosa, and Sharpe Agar) medium supplemented with 1% $CaCO_3$. The probiotic potential of bacterial isolates was assessed based on their resistance to gastric acidity (pH) and bile salts. Characterization included colony morphology, pathogenic inhibition assays using *Escherichia coli* and *Salmonella typhi*, and biochemical tests such as MR-VP, TSIA, motility, and catalase. Among the four probiotic bacterial isolates obtained, three were Gram-positive (J2, J3, and J4) and one was Gram-negative (J1). All isolates were rod-shaped (bacilli), catalase-negative, non-motile, and inhibited *E. coli* and *S. typhi*. Isolate J2 exhibited the most potent inhibitory activity, with inhibition zones measuring 15.5 mm against *S. typhi* and 11.5 mm against *E. coli*. When used as a feed additive, the J2 isolate improved broiler meat quality by maintaining optimal pH, enhancing water-holding capacity, and reducing cholesterol and fat content, although it increased cooking losses. These results indicate that the J2 isolate has significant potential as a probiotic agent in poultry nutrition, with promising implications for improving meat quality and food safety. Future studies should focus on optimizing probiotic formulations and exploring the long-term effects on poultry health and productivity.

**Key Words:** Local Chicken, Meat Quality, Probiotics Source

## INTRODUCTION

Genetically unselected Indonesian local chicken (*Gallus gallus domesticus)*, commonly known as free-range chicken, possesses several advantages, including its ability to adapt to diverse environments and greater disease resistance compared to modern broiler chickens. Free-range chickens are allowed to roam freely and consume natural food sources available in their surroundings. This natural diet, combined with their robust adaptability, influences the intestinal microflora, leading to the development of a bacterial community that is well-adapted and resistant to the chickens' environment (Husain et al. 2023).

Probiotics derived from free-range chickens have been the subject of extensive research due to their significant potential in enhancing poultry health and productivity. These probiotics, primarily comprising lactic acid bacteria (LAB), have shown remarkable efficacy in improving gut health and growth performance in poultry (Shi et al. 2022; Johnson et al. 2024). Research has demonstrated that LAB isolated from the gastrointestinal tracts of chickens can inhibit the colonization of harmful pathogens, thereby promoting a healthy gut environment (Husain et al., 2023).

Kobierecka et al. (2017) highlighted that certain Lactobacillus strains from chicken digestive tracts effectively inhibited the growth of *Campylobacter jejuni*, a common poultry pathogen, thereby enhancing gut health and reducing infection risks (Kobierecka et al., 2017). Additionally, Wang et al. (2023) explored the survival and antibacterial properties of LAB from Tibetan chickens, demonstrating their potential as a natural alternative to antibiotics in broiler chicken diets (Wang et al. 2023). Probiotics play a crucial role in optimizing digestive tract function by fostering the growth of beneficial bacteria and enhancing the activity of digestive enzymes, thereby improving feed digestibility and nutrient absorption (Zhang et al. 2012; Ghodrati et al. 2021). These findings underscore the importance of chicken-derived probiotics in promoting sustainable, health-oriented poultry farming practices.

Probiotics have been shown to positively influence various physiological parameters in broiler chickens, including blood parameters, immune responses, and meat quality (Kim et al. 2017; Bohatko 2023; Purba et al. 2023; Yang et al. 2023). These beneficial microorganisms promote growth and enhance antioxidant enzyme activities, contributing to overall health improvements (Aluwong et al. 2013). Additionally, probiotics are known to boost immune responses, which are critical for broiler immune development, as well as to improve feed conversion efficiency, water-holding capacity, and the oxidative stability of meat, thereby enhancing carcass quality (Khan et al. 2023). Furthermore, probiotics have been reported to alleviate the effects of heat stress in broilers by increasing antioxidant enzyme activity and reducing oxidative stress (Ogbuagu et al. 2018). They also improve intestinal morphology, which facilitates better nutrient absorption and overall health (Aluwong et al. 2017). In this study, we focus on isolating and characterizing probiotic bacteria from the intestines of genetically unselected Indonesian local chicken (*Gallus gallus domesticus)* to enhance the quality of modern broiler chicken meat.

## MATERIALS AND METHODS

### Isolation of probiotic bacteria

Probiotic bacteria from the intestines of genetically unselected Indonesian local chickens were collected in Luwu Timur Regency, South Sulawesi, Indonesia. The samples were collected from the inner walls of the chicken intestines and placed in sterile physiological saline. This suspension was subjected to a series of serial dilutions. One milliliter of the diluted sample was inoculated onto De Man, Rogosa, and Sharpe (MRS) agar supplemented with 1% calcium carbonate ($CaCO_3$). The inoculated plates were incubated at 37°C for 24–48 hours.

### Purification, morphological characterization, and stock culture preparation of probiotic bacteria

Purification was performed by selecting single colonies that exhibited clear zones on MRS agar, indicative of potential probiotic activity. These colonies were incubated at 37°C for 48 hours. The purification process was repeated 2–3 times to ensure the purity of the colonies. The morphology of each purified colony was examined for characteristics such as shape, margin, color, surface elevation, and odor. Purified colonies were then cultured on slant MRS agar to prepare stock cultures for further analysis.

### Acid resistance testing

The resistance of isolates to gastric acidity was assessed using MRS broth (MRSB) adjusted to pH 2.5–3.0 with 0.1 N HCl, simulating stomach conditions. The ability of the bacteria to grow in this acidic MRSB environment indicated positive acid resistance, while the absence of growth indicated a lack of resistance.

### Biochemical characterization and bile salt resistance

Biochemical characteristics were assessed using the Methyl Red Voges-Proskauer (MR-VP) test, catalase test, and Triple Sugar Iron Agar (TSIA) motility test. To evaluate bile salt resistance, MRSB was supplemented with synthetic bile salts (ox bile) at concentrations of 1% and 5%. Each bacterial isolate from the stock culture was inoculated into the MRSB-bile salts medium and incubated at 37°C for 2–3 hours. Colony counts were conducted before and after incubation to assess bile salt tolerance.

### Inhibitory activity against pathogenic bacteria

The inhibitory potential of the probiotic isolates against pathogenic bacteria, specifically *Salmonella typhi* and *Escherichia coli*, was evaluated using the agar diffusion method on Mueller-Hinton agar (MHA) (Husain et al., 2023). Paper disks were soaked in bacterial supernatant for 15 minutes. One mL suspension of each test pathogen was added to sterile petri dishes, followed by the addition of MHA medium at 45°C, which was then allowed to solidify. The soaked disks were placed on the solidified MHA, and the plates were incubated at 37°C for 24 and 48 hours. The diameters of the inhibition zones were measured to assess the antibacterial activity. Ciprofloxacin was used as a control because of its broad-spectrum antibiotic activity against gram-positive and gram-negative bacteria.

### Broiler chicken feed and the addition of probiotics

The probiotic isolate that performed best in the initial screening was then evaluated for its effect on meat quality in broiler chickens. The starter probiotics were prepared by dilution. Initially, 9 mL of 0.9% NaCl physiological saline was transferred into a test tube and homogenized. Subsequently, a 0.5 mL aliquot of the bacterial stock was drawn using a syringe and added to the dilution tube. The concentration of probiotic bacteria was determined using the Standard Plate Count (SPC) method, achieving a final concentration of $10^7$ cells/mL. The artificial feed was composed of 30 grams of rice bran, 40 grams of ground corn, and 30 grams of fish meal, a composition sourced from a local farmer. Every 100 grams of the artificial feed received a spray of the probiotic starter. A commercial feed, BP-11, was used as the control.

The groups were divided into two: a control group that received feed without probiotic supplementation and a treatment group that received feed supplemented with probiotics. Six broiler chickens per treatment group were fed ad libitum for 6 weeks. The chickens were raised following standard broiler husbandry practices. Meat quality parameters, including color (L* for lightness, a* for redness, and b* for yellowness), pH, water-holding capacity (WHC), cooking losses (%), cholesterol (mmol/L), and fat content (%), were subsequently analyzed.

## RESULTS AND DISCUSSION

### Isolation and purification of probiotic bacteria

Six isolates of lactic acid bacteria (LAB) were isolated. The presence of clear zones around the colonies, as shown in Figure 1, indicates the growth of LAB. These six LAB isolates were further purified to obtain pure bacterial colonies. After three rounds of purification, four distinct bacterial isolates were successfully obtained. The morphology of these four LAB isolates was then observed and documented.

Observations of bacterial colony morphology revealed that all four isolates exhibited distinct colony morphologies. The isolates, labeled as J1, J2, J3, and J4, each displayed a circular shape. J1 and J2 isolates showed smooth colony edges and a convex surface, but J1 was milky white while J2 was clear white. Additionally, both J3 and J4 exhibited smooth colony edges. However, J3 had a clear white colony with flat elevation, whereas J4 had a milky white colony with raised elevation. Furthermore, the results of the bacterial cell morphology, as determined by Gram staining, are presented in Table 1.

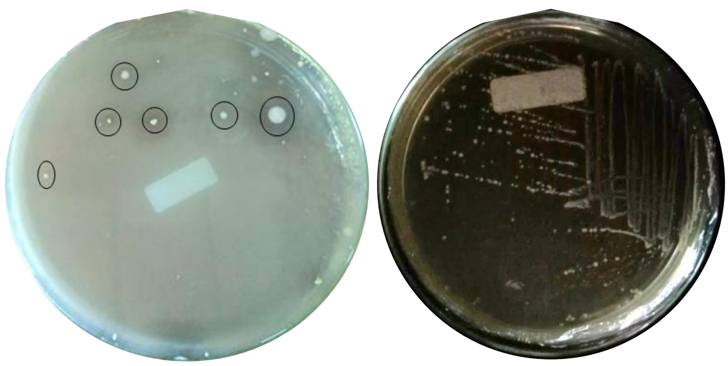

**Figure 1**. Morphology of probiotic isolates (left) and purification results of probiotic isolates (right)

**Table 1**. Microscopic observation of probiotic bacteria isolates of genetically unselected Indonesian local chicken after Gram staining

| Bacterial Isolate Code | Cell Shape | Gram Type |
|---|---|---|
| J1 | Bacilli | Negative |
| J2 | Bacilli | Positive |
| J3 | Bacilli | Positive |
| J4 | Bacilli | Positive |

Gram staining was performed to identify the Gram type of bacterial isolates, based on differences in cell wall structure. Crystal violet initially stains all bacteria purple, but during the alcohol decolorization step, Gram-positive bacteria retain the stain due to their thicker peptidoglycan layer, while Gram-negative bacteria lose it. Safranin is then applied as a counterstain, which colors Gram-negative bacteria pink, while Gram-positive bacteria remain purple (Becerra et al. 2016). As shown in Table 1, three bacterial isolates were Gram-positive, and one was Gram-negative. The J1 isolate was a Gram-negative bacillus, while the J2, J3, and J4 isolates were Gram-positive bacilli.

**Probiotic test**

Probiotics are typically administered orally, so they must survive the digestive tract, particularly the acidic environment of the stomach, and reach the small intestine alive (Shehata et al. 2016). To assess this, resistance tests were conducted at pH 3 for gastric acidity and with 5% bile salts. The results are presented in Table 2 below. Based on the observations in Table 2, the LAB isolates grew in a medium with a pH of 3 and in a medium containing 5% synthetic bile salts; this is evident from the turbidity and growth of bacterial colonies in the test tubes, indicating that these LAB isolates can survive the acidic conditions of the stomach and reach the small intestine, making them suitable as probiotic bacteria.

According to Jannah et al. (2018), lactic acid bacteria can regulate their cytoplasmic or intracellular pH to maintain near-neutral conditions even when exposed to low extracellular pH (Jannah et al. 2018). The ability of lactic acid bacteria to tolerate bile salts is linked to the activity of the bile salt hydrolase (BSH), which hydrolyzes conjugated bile acids, thereby reducing their bactericidal effect (Cho et al. 2006). Additionally, other studies suggest that the resistance of lactic acid bacteria to low pH and bile salts in the digestive tract may also be due to the role of extracellular polysaccharides (EPSs) (Boke et al. 2010; Jurášková et al. 2022).

**Biochemical characterization test**

Based on the biochemical characterization results shown in Table 3, isolates J1, J2, and J3 tested positive for the Methyl Red (MR) test, indicating their ability to produce stable acid end-products from glucose fermentation. However, only J1 and J3 tested positive for the Voges-Proskauer (VP) test, while J2 showed a negative result, suggesting that J2 did not produce the fermentation byproduct. All three isolates were negative in the catalase and motility tests, indicating they did not produce catalase and were non-motile. Further analysis using the Triple Sugar Iron Agar (TSIA) test revealed differences in sugar fermentation capabilities among the isolates. Isolates J3 and J4 could ferment all three sugars—glucose, sucrose, and lactose—whereas isolated J2 was only able to ferment glucose. This variability in sugar fermentation profiles among the isolates could affect their metabolic versatility and potential as probiotics.

**Inhibitory activity of probiotic bacteria against pathogenic bacteria**

The ability of LAB isolates to inhibit the growth of pathogenic bacteria can be determined through an antimicrobial susceptibility test. The presence of clear or inhibitory zones indicates the inhibitory effect on pathogenic bacteria. Inhibitory test results of probiotic bacteria isolated against pathogenic bacteria are also presented in Table 4.

The observations in Table 4 indicate that all four bacterial isolates inhibited the pathogenic bacteria *E. coli* and *S. typhi*, as evidenced by clear inhibitory zones around the paper disks. This inhibitory activity is a key characteristic of probiotic bacteria, which are known to produce extracellular antimicrobial compounds such as organic acids (e.g., lactic acid, acetate, propionate, and formate), hydrogen peroxide, diacetyl, and bacteriocins, which collectively inhibit the growth of pathogenic bacteria (Shehata et al. 2016; Haghshenas et al. 2023; Husain et al. 2023).

The specific mechanism by which bacteriocins exert their inhibitory effects involves direct interaction with the bacterial cell membrane. Bacteriocin molecules disrupt the proton motive force (PMF) by creating pores in the bacterial cell membrane, leading to impaired cell growth and eventual cell death (Farha et al. 2013; Arfani et al. 2017). This pore formation is particulary effective

**Table 2.** The resistance of probiotic bacteria isolates to gastric acidity at pH 3 and resistance to bile salt at a concentration of 5%

| Bacterial Isolates Code | pH Test | Bile Salt Test |
|---|---|---|
| J1 | + + | + + |
| J2 | + + | + + + |
| J3 | + + | + + + |
| J4 | + | + + |

+= Slight sediment, less turbid; + += Moderate sediment, slightly turbid; + + += Significant sediment, turbid

**Table 3**. Biochemical characterization test of probiotic bacteria isolates

| Biochemical Characterization Test | | Probiotic Bacteria Isolates | | | |
|---|---|---|---|---|---|
| | | J1 | J2 | J3 | J4 |
| MR Test | | + | + | + | + |
| VP Test | | - | + | + | + |
| TSIA Test | *Slant* | Red | Yellow | Yellow | Yellow |
| | *Butt* | Yellow | Yellow | Yellow | Yellow |
| | $H_2S$ | - | - | - | - |
| | Gas | - | - | - | - |
| Catalase Test | | - | - | - | - |
| Motility Test | | - | - | - | - |

(-)= Negative result; (+)= Positive result

**Table 4**. The result of the inhibitory activity of probiotic bacteria

| Bacterial Isolates Code | Diameter of Inhibiting Zone (mm) | | | |
|---|---|---|---|---|
| | *Escherichia coli* | | *Salmonella typhi* | |
| | 1x24 hour | 2x24 hour | 1x24 hour | 2x24 hour |
| J1 | 4 | 4 | 6 | 6 |
| J2 | 11.5 | 11.5 | 15.5 | 15.5 |
| J3 | 12 | 12 | 13,5 | 13,5 |
| J4 | 6 | 6 | 11 | 11 |
| Ciprofloxaxin | 18 | 18 | 22 | 22 |

in preventing the proliferation of harmful bacteria. The results in Table 4 also highlight variability in the size of the inhibitory zones formed by each probiotic isolate. Among the isolates, J3 exhibited the largest inhibition zone against *E. coli* (12 mm), while J2 showed the most significant inhibition against *S. typhi* (15.5 mm). For comparison, the positive control, which utilized tetracycline, produced inhibition zones of 18 mm for *E. coli* and 22 mm for *S. typhi*. The size of the inhibition zone is a direct measure of the isolate's ability to inhibit pathogenic bacteria. According to Li et al. (2016), inhibition zones between 20-25 mm are considered very strong, 15-20 mm as strong, 10-15 mm as moderate, and 5-10 mm as weak (Li et al. 2016).

Overall, the findings suggest that J2, isolated from the intestines of probiotic bacteria obtained from genetically unselected Indonesian local chickens, was the most effective probiotic in inhibiting the growth of both *E. coli* and *S. typhi*. Although the inhibition zones produced by the J2 and J3 isolates were classified as moderate, their ability to inhibit these pathogenic bacteria demonstrates their potential as effective probiotic agents. The J2 isolate showed the most significant overall inhibitory effect, indicating its promise for further development and application in probiotic formulations.

**Meat quality**

Given that the J2 isolate demonstrated strong inhibitory effects, we conducted a further evaluation to assess its impact as a feed additive on meat quality, as seen in Table 5. The result was aligned with Mohammed et al. (2021), who found that probiotic-supplemented broilers have a lower value in lightness, resulting in less pale meat, redness, and yellowness compared to control ($P<0.05$) (Mohammed et al. 2021). The color quality of the samples remained strong and had not degraded, and the presence of probiotics maintained the pigment's quality. Previous studies have reported that the ultimate pH range in broiler meat (breast) is 5.9 to 6.2 at 15 min after slaughter, while values at ≤5.8 are considered pate, soft, and exudative meat, and ≥6.3 is for dark, firm, and dry meat (Ristic & Damme 2013). In this study, the meat pH values of broilers that were given probiotics were higher, around ultimate pH 5.9, while the meat pH values of broilers that were given BP-11 (control) were lower than 5.7, which was defined as PSE (pate, soft, and exudative) meat. Similarly, Cramer et al. (2018) also showed that dietary supplementation with *B. subtilis* increased the $pH_{24h}$ of breast muscle (Cramer et al. 2018). The probiotics' roles in maintaining meat pH and color could account for the findings in the current studies.

The ionic strength and pH of meat significantly influenced the WHC value of probiotic-supplemented broilers, which was higher than that of BP-11-supplemented broilers. Mohammed et al. (2021) conducted a previous study that yielded a similar result: the probiotic diet increases WHC%, thereby improving meat moisture retention (Mohammed et al. 2021). However, the impact of probiotic supplementation on meat WHC is not always consistent. Zhang et al. (2012) found in another study that supplementing broilers with *Bacillus subtilis* did not affect their water-holding capacity (Zhang et al. 2012).

pH and water-holding capacity correlate with cooking loss. Higher pH and WHC values result in reduced meat cooking loss. However, the current findings showed that probiotic-supplemented broilers had higher cooking losses than BP-11-supplemented broilers. Factors that influence meat cooking loss include pH, relative humidity, time, and temperature (Devi et al. 2019). Other factors that affect cooking loss include genetics and the feeding system (Barbanti & Pasquini 2005).

Cholesterol and fat values in probiotic-supplemented broilers were lower than those in BP-11-supplemented broilers. Abdurrahman et al. (2016) conducted a previous study that revealed that the addition of probiotics to broilers' feed significantly reduced the cholesterol value of meat (Abdurrahman et al. 2016). Bacterial growth cells can achieve this phenomenon through assimilation or incorporation into the probiotic microorganism's cell surface, thereby inhibiting the body's absorption of cholesterol (Aluwong et al. 2013).

**Table 5.** Meat quality result of modern broiler chicken, fed a diet supplemented with J2 probiotic bacteria

| Meat Quality | Treatment | Control |
|---|---|---|
| Color (L*) lightness | 44.24±0.34 | 45.24±0.20 |
| Color (a*) redness | 4.46±0.04 | 4.56±0.04 |
| Color (b*) yellowness | 4.14±0.10 | 4.49±0.08 |
| pH | 5.96±0.01 | 5.55±0.05 |
| Water holding capacity (WHC) | 54.12±0.27 | 52.35±0.84 |
| Cooking losses (%) | 25.25±0.39 | 21.75±1.02 |
| Cholesterol (mmol/L) | 3.07±0.06 | 3.20±0.01 |
| Fat Rate (%) | 1.65±0.01 | 1.79±0.03 |

Data represented as value±STD; J2= Bacilli shape – gram-positive type of probiotic bacteria, which had the most significant inhibitory effect on *E. coli* and *S. typhi*

**Figure 3.** Broiler chicken meat of the chicken fed diet, A) with probiotic (Treatment), B) without probiotic (control)

## CONCLUSION

The study conducted on the isolation of probiotic bacteria from genetically unselected Indonesian local male chickens in Malakaji Village, Gowa Regency, has yielded four distinct isolates, labeled J1, J2, J3, and J4, each demonstrating notable probiotic characteristics. Among these, the J2 isolate emerged as particularly promising, showing the most significant inhibitory effects against *S. typhi* and *E. coli*, with inhibition zones measuring 15.5 mm and 11.5 mm, respectively. All isolates demonstrated resilience in gastrointestinal conditions, including resistance to gastric acidity at pH 3 and tolerance to 5% bile salts, characteristics essential for probiotic efficacy. The isolates were identified as Gram-positive (J2, J3, J4) and Gram-negative (J1), all of which were rod-shaped, catalase-negative, and non-motile. Moreover, the application of J2 as a probiotic feed additive was found to enhance meat quality by maintaining optimal pH, improving water-holding capacity, and reducing cholesterol and fat content. However, it also led to increased cooking losses, underscoring the intricate balance required in dietary supplementation. These findings highlight the potential of probiotics in enhancing meat quality and underscore the need for further research to refine their application in poultry nutrition.

## CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported. All authors have agreed with the contents of the manuscript.

## AUTHOR'S CONTRIBUTION

The authors confirm their contribution to the paper as follows: study conception and design: DRH; data collection: DS, RW, FG; analysis and interpretation of results: DRH, DS, RW, UA; draft manuscript preparation: RW, UA, AJF, FG, JL.

## ETHICS APPROVAL

This research was conducted at the Microbiology Laboratory, Universitas Hasanuddin, Indonesia. The procedure in this study followed the guidelines for the use of animals (Buchanan et al., 2012).

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# Pathological Changes and Virus Detection in Chickens Infected with Fowl Adenovirus 8b

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## ABSTRAK

Sohaimi NM, Hamsa NN, Bejo MH, Mazlan M, Clifford UC. 2025. Perubahan patologi dan deteksi virus pada ayam yang terinfeksi oleh *fowl adenovirus* 8b. JITV 30(3):159-169. DOI:http://dx.doi.org/10.14334/3502.

*Fowl adenovirus* (FAdV) serotipe 8b adalah agen virus patogen dari hepatitis badan inklusi (IBH) pada ayam dengan mortalitas tinggi di peternakan yang terkena dampak. Tujuan dari penelitian ini adalah untuk menentukan perubahan patologis dan untuk mendeteksi FAdV dengan metode molekuler pada organ ayam bebas patogen spesifik (SPF) yang terinfeksi FAdV serotipe 8b. Isolat FAdV, UPM1901, diinokulasi pada anak ayam umur sehari melalui *rute oral* diikuti dengan pengambilan sampel pada jam ke-0 (h) untuk kelompok kontrol dan selanjutnya pada jam ke-12, 24, hari ke-3 dan ke-7 pasca inokulasi (pi). Berat badan, hati dan bursa Fabricius diukur dan dilanjutkan dengan pengumpulan organ untuk pemeriksaan histopatologi dan deteksi virus menggunakan reaksi berantai polimerase (PCR). Hasil pengamatan menunjukkan bahwa ayam dalam kelompok yang terinfeksi mengalami depresi dan tidak nafsu makan sebelum kematian mendadak dengan total mortalitas 48% dalam hari ke-5pi. Berat badan rata-rata secara signifikan lebih rendah (P<0,05) dibandingkan kelompok kontrol pada hari ke-3 dan ke-7 pi. Pembengkakan, perdarahan, dan nekrosis hati terekam dengan banyaknya badan inklusi intranuklear basofilik di dalam hepatosit bersama dengan perdarahan limpa dan ginjal pada hari ke-3 dan seterusnya. Asam nukleat FAdV terdeteksi di sumsum tulang pada 12 jam pi diikuti oleh organ lain pada 24 jam pi di limpa, hati, dan tonsil sekum. Pada hari ke-3 dan ke-7 pi, DNA virus terdeteksi di semua organ yang dipilih. Studi ini membuktikan bahwa FAdV patogen serotipe 8b menyebabkan kematian anak ayam, perubahan patologis dengan keberadaan DNA virus pada 12 jam pi di organ limfoid sebelum didistribusikan ke organ lain dan memerlukan strategi pengendalian yang efektif terhadap penyakit tersebut.

**Key Words:** *Fowl Adenovirus* (FAdV) serotip 8b, Hepatitis Badan Inklusi (IBH), Organ Limpoid, Reaksi Polimerase Berantai (PCR)

## ABSTRACT

Sohaimi NM, Hamsa NN, Bejo MH, Mazlan M, Clifford UC. 2025. Pathological changes and virus detection in chickens infected with fowl adenovirus 8b. JITV 30(3):159-169. DOI:http://dx.doi.org/10.14334/3502.

Fowl adenovirus (FAdV) serotype 8b is a pathogenic viral agent of inclusion body hepatitis (IBH) in chickens with high mortality in affected farms. The objective of this study is to determine the pathological changes and to detect FAdV by molecular method in organs of specific-pathogen-free (SPF) chickens infected with FAdV serotype 8b. FAdV isolate, UPM1901, was inoculated into day-old chicks via oral route followed by sampling at 0 hour (h) for control group and subsequently at 12h, 24h, days 3 and 7 post-inoculation (pi). Body weight, liver and bursa of Fabricius weights were measured followed by organs collection for histopathological examination and virus detection using polymerase chain reaction (PCR). It showed that chickens in the infected group were depressed and inappetence prior sudden death with 48% total mortality within day 5pi. Mean body weight was significantly low ($p$<0.05) than control group at days 3 and 7pi. Swollen, haemorrhages and necrosis of liver were recorded with numerous basophilic intranuclear inclusion bodies in the hepatocytes along with haemorrhages spleen and kidney at 3 dpi onward. FAdV nucleic acid detected in bone marrow at 12h pi followed by the other organs at 24h pi in spleen, liver and cecal tonsils. At days 3 and 7pi, viral DNA was detected in all the selected organs. This study proves that pathogenic FAdV serotype 8b caused chick`s mortality, pathological changes with existence of viral DNA at 12h pi in lymphoid organ prior distributed to other organs and necessitates effective control strategies against the disease.

**Key Words:** Fowl Adenovirus (FAdV) serotype 8b, Inclusion Body Hepatitis (IBH), Lymphoid Organs, Polymerase Chain Reaction (PCR)

## INTRODUCTION

Fowl adenovirus (FAdV) is a causative agent of inclusion body hepatitis (IBH), hepatitis hydropericardium syndrome (HHS), adenovirus gizzard erosion (AGE), respiratory disease and necrotizing pancreatitis (Kiss et al. 2021; Islam et al. 2023). FAdV belongs to genus of Aviadenovirus of family *Adenoviridae*. It is classified into five species (FAdV-A to FAdV-E) and has 12 serotypes (Harrach & Kajan 2011). FAdV is a non-enveloped icosahedral virion with a linear double-stranded DNA genome.

Virus can be detected by viral isolation and molecular detection using Polymerase Change Reaction (PCR). Vertical transmission of the virus from the hens to the eggs and horizontal transmission from the infected bird to another by direct contact with faeces, respiratory discharges and fomites (Pereira et al. 2014; El-Shall et al. 2022). FAdVs are distributed globally and are recognized as economically significant pathogens in many countries. They can infect all domestic avian species across various age groups, with young broiler chickens being the most commonly affected. However, FAdVs can also be isolated from clinically healthy birds (Adel et al. 2021). Clinical disease of IBH in chickens occurs worldwide and is caused by 12 serotypes of FAdV, with mortality rates ranging from 10% to as high as 30% (Song et al. 2024).

The first case of IBH in Malaysia was reported in 2005 by Hair-Bejo. Additionally, FAdV serotype 8b was reported to be the primary agent of IBH that is highly pathogenic in SPF chickens (Sohaimi et al. 2019). Infected chickens with IBH will show clinical signs such as depression, inappetence, ruffled feathers and sudden onset death (Cizmecigil et al. 2020). The liver and kidney are primarily impacted organs in IBH. Liver appeared pale, yellow, swollen, and friable with petechial hemorrhages while the kidneys were pale and swollen (Cizmecigil et al. 2020; Norina et al. 2016). Histopathologically showed eosinophilic intranuclear inclusion body in the hepatocytes and infiltration of mononuclear inflammatory cells indicated hepatitis (Hair-Bejo 2005; Norina et al. 2016).

The IBH outbreak in chickens caused a high mortality rate and poor performance leading to serious economic losses to the affected farm. To date, the study on the effect of pathogenic FAdV serotype 8b at specific period following infection is scanty and necessitate further investigation for effective control and prevention strategies. It is important to know the impact of the disease on lymphoid organs and other tissues for better understanding of the virus tropism in chicken. The objectives of this study were to determine the pathological changes and to detect FAdV by molecular method in organs of specific-pathogen-free (SPF) chickens infected with FAdV serotype 8b.

## MATERIALS AND METHODS

### Virus isolate

FAdV isolate namely, UPM1901, was obtained from Johore in 2019 from 25-day-old commercial broiler chickens with a history of 1.22% mortality. Liver was swollen, necrotized and haemorrhages upon necropsy from the dead chickens. The isolate was characterized as FAdV species E serotype 8b, showing 98% nucleotide sequence identity in the L1 loop regions of the hexon gene to reference serotype 8b strains in the GenBank database (Sohaimi et al. 2022).

### Preparation of virus inoculum

Liver samples were processed by subjecting them to three cycles of freezing and thawing, then pooled and macerated in a sterilized mortar and pestle to prepare a 1:2 (w/v) suspension in sterile phosphate-buffered saline (PBS, pH 7.4). The suspension was centrifuged at 381 × g for 30 minutes for clarification. The supernatant was collected, filtered through a 0.45 µm membrane, and treated with Penicillin-Streptomycin-Amphotericin B solution (Gibco®, USA) at a 1:10 dilution. It was then incubated at 4°C for 1 hour prior to inoculation onto the chorioallantoic membrane (CAM) of 9-day-old SPF embryonated chicken eggs (Alemnesh et al., 2012). The eggs were incubated at 37°C until day 10 post-inoculation (pi). All embryos died, exhibiting necrotized and swollen livers within 7 to 9 days pi. Subsequently, the livers were harvested, homogenized, and processed for inoculation into SPF CEE via the CAM route as the second passage. This process was repeated for three passages to increase the volume of homogenate liver embryos. The virus inoculum from liver embryos at the third passage, with a virus titer of $10^{7.1}$ $TCID_{50}$/ml, was used in this study (Reed & Muench 1938).

### Experimental design and sampling protocol

The study protocols described were undertaken in accordance with criteria approved by Universiti Putra Malaysia (UPM), Institutional Animal Care and Use Committee (IACUC): UPM/IACUC/AUP-U019/2024. Thirty-six (36) day-old chicks were divided into 2 major group namely, FAdV infected group and control group. Sixteen (16) chicks were assigned in FAdV infected group and twenty (20) chicks in the control group. All chicks in FAdV infected group were inoculated with 1ml FAdV isolate, UPM1901, at virus titer $10^{7.1}$$TCID_{50}$/ml via oral route at day old. All chicks in the control group were uninoculated and used as the control group in this study. All chickens were monitored throughout the trial with feed and water were given *ad libitum.* At 0 hour (h) post-inoculation (pi), body weight was recorded, and blood samples were collected via wing vein from four chicks in control group prior sacrifice by cervical dislocation. Sample of liver, thymus, bursa of Fabricius, spleen, bone marrow, caecal tonsils and kidney were harvested from chicks for virus detection by PCR test and histological examination. Sampling was performed subsequently in both groups at 12 hours (h), 24 h, day 3 and 7 pi.

### Clinical signs and mortality observation

All chicks were monitor for any clinical signs associated with FAdV infection throughout the trial such as changes in behavior, physical appearance and feed or water consumption. Any mortality was recorded until the end of the study (Sohaimi & Bejo 2021).

### Measurement of liver and bursa of Fabricius weight

Liver and bursa of Fabricius were weight as gram (g) unit and were calculated its ratio to body weight (Sohaimi & Bejo 2021).

### Gross and histopathological examinations

All chicks were observed for any abnormalities associated with FAdV upon necropsy. Any gross lesions were recorded throughout the trial. Sample of liver, thymus, bursa of Fabricius, spleen, bone marrow, caecal tonsils and kidney were harvested and fixed in 10% buffer formalin for histological examination. All samples were stained with Haematoxylin and Eosin (H&E) according to method by Gurina & Simms (2023). All slides were viewed, evaluated and described based on the severity of the lesions.

### Statistical data analysis

Mean data for body weight, liver weight, liver to body weight ratio and bursa of Fabricius to body weight ratio were compared between infected and control groups using independent T test based on sampling day using SPSS software version 27**.** Significant differences were measured at alpha $P<0.05$ value (Sohaimi & Bejo 2021).

### Molecular detection (DNA extraction, conventional PCR and gel electrophoresis)

All the tissue samples from chickens were extracted using DNA extraction kit (Kylt® RNA/DNA purification, Germany) according to the manufacturer`s protocol. The eluted DNA was measured for concentration and DNA purity by using a biophotometer (Eppendorf, Germany). Extracted DNA sample was used as template for amplifying hexon gene using MyTaq™ HS Mix (Bioline, UK). Published primer was used namely Hexon A (forward) and Hexon B (reverse) with expected PCR size product at 897 base pairs (bp) (Sohaimi et al. 2022). Conventional PCR was performed according to the protocol published by Sohaimi et al. (2022). Subsequently, all PCR products were separated in a 1.5% agarose gel electrophoresis using RedSafe™ Nuclei Acid Staining solution (iNtRON, Korea) and 1kb DNA Marker (GeneDirex, USA). Electrophoresis was conducted at 110 volts for 25 minutes prior visualization of DNA fragment band under U.V. transillumination.

## RESULTS AND DISCUSSION

### Clinical signs and mortality

It was demonstrated that pathogenic FAdV serotype 8b isolate UPM1901 into day old SPF chicks via oral route induced IBH similarly as seen in the natural outbreak throughout the trial. The virus isolate caused several clinical signs associated with FAdV infection such as depression, diarrhoea, ruffled feathers and inappetence in chickens prior to sudden death at day 3pi onwards. 48% mortality rate was recorded in infected chickens during the experiment. Three chickens (9%) died at day 3pi, followed by seven chickens (23%) died at day 4pi and five chickens (16%) died at day 5pi (Figure 1). All chickens in the control group were clinically healthy and normal throughout the trial. This finding was consistent with previous works shown in SPF chickens due to FAdV serotype 8b infection using intramuscular route at 1 day old (Sabarudin et al. 2021).

### Mean body weight of chickens

For control group, mean body weight at 0h pi was $47\pm2.04$ g and increasing to $60.33\pm1.20$ g at day 7 pi. Similarly, body weight for the infected group was increased from $46.8\pm1.39$ g at day 3 pi to $57.4\pm2.0$ g at day 7 pi. However, the mean body weight of the infected group was significantly lower ($P<0.05$) than the control group at day 3 and 7 pi (Figure 2) due to inappetence and reduced feed consumption which similarly showed in the field IBH disease in chickens (Tsiouris et al. 2022).

### Liver weight and liver-to-body weight ratio

Mean liver weight of the control group was $2.8\pm0.5$ g, $2.2\pm0.2$ g, $2.9\pm0.3$ g, $3.1\pm0.2$ g and $3.8\pm0.3$ at 0 h, 12 h, 24 h, day 3 and 7 pi, respectively. For FAdV infected group, mean liver weight was 3.6±0.1 g, 3.1±0.3 g, 4.1±0.2 g and 5±0.3 g at 12 h, 24 h, day 3 and 7 pi, respectively. There is high liver weight significantly (P<0.05) at 12 h, day 3 and 7 pi in the infected group than control group (Figure 3). Analysis on liver-to-body weight ratio revealed infected group has high ratio significantly (P<0.05) than control group at 12h, days 3 and 7pi (Figure 4). It indicated that the increased liver weight and liver-to-body weight ratio observed in infected chickens as early as 12 h pi, and consistently at day 3 and 7 pi, reflect liver damage caused by FAdV replication, characterized by liver enlargement and swelling due to the virus's primary tropism. This finding consistent with previous report after inoculated via subcutenous route (Sun et al. 2024).

**Mean bursa of Fabricius weight**

Mean bursa weight for control group was 0.01±0 g at three consecutive periods, 0 h, 12 h and 24 h pi. At day 3 and 7 pi, it was 0.02±0 g and 0.05±0 g, respectively. In infected group, the means was 0.01±0 g at 12 h and 24 h pi followed by 0.02±0 g at day 3 pi and 0.03±0 g at day 7 pi. There is no significant difference (P>0.05) in mean bursa weight between groups at 12 h, 24 h and day 3 pi, however, the mean was low significantly (P<0.05) in the infected group (0.03±0 g) compared to the control group (0.05±0 g) (Figure 5). Low bursa of Fabricius weight suggested due to atrophy and compatible with previous work after infected with FAdV serotype 4 isolates in the immunosuppressed birds (Naeem et al. 1995; (Rashid et al. 2024).

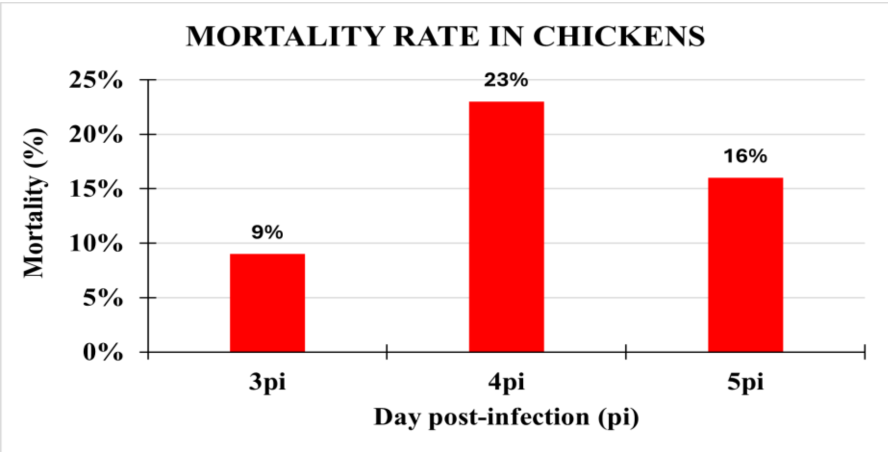


**Figure 1.** Mortality rate in specific pathogen free (SPF) chickens following infection with FAdV serotype 8b isolate UPM1901 via oral route at day-1-old

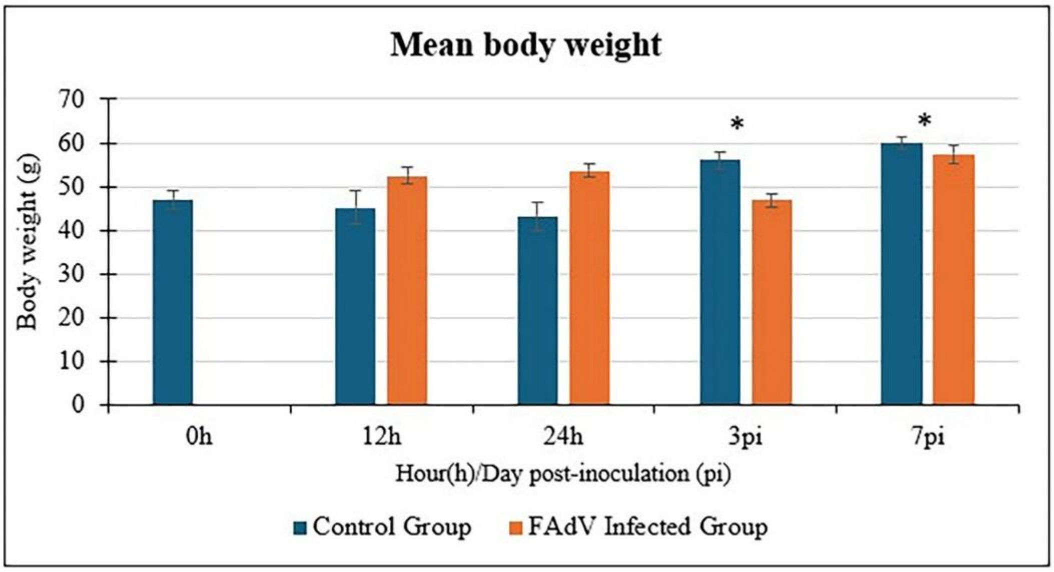


**Figure 2.** Mean body weight in chickens between control and infected group following inoculation with FAdV-8b isolate UPM1901 at day old. The body weight of the infected group was significantly lower (P<0.05) than the control group on days 3 and 7 pi. Asterisk * indicates significant difference between group at alpha value P<0.05

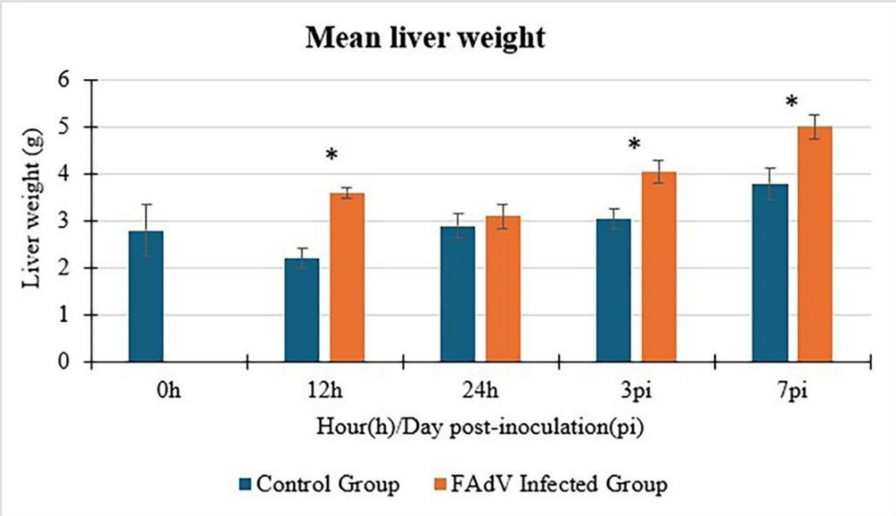


**Figure 3.** Mean liver weight of chicken between control and infected group with FAdV-8b isolate UPM1901. *= indicates significant difference between group at alpha value P<0.05

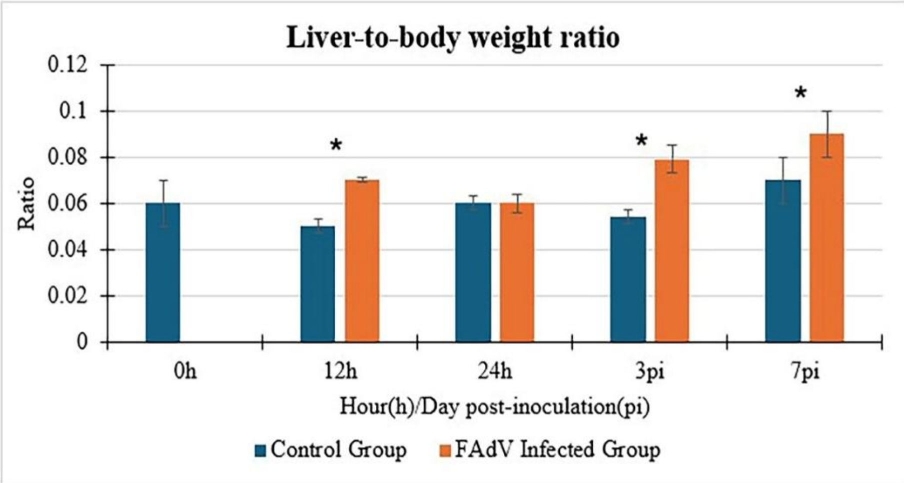


**Figure 4.** Liver-to-body weight ratio in chicken between control chickens and infected group with FAdV-8b isolate UPM1901. *= indicates significant difference between group at alpha value P<0.05

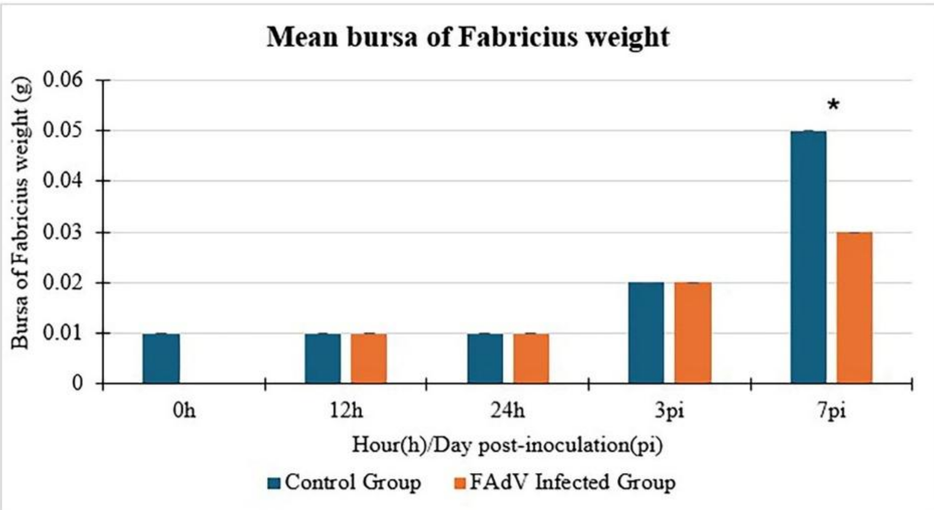


**Figure 5.** Mean bursa of Fabricius weight in chicken between control and FAdV infected group inoculated with FAdV-8b isolate UPM1901. *= indicates significant difference between group at alpha value P<0.05

## Gross lesions

Liver was normal in control chickens with brownish coloration, glistening surface and smooth edges at 0 h, 12 h, 24 h, day 3 and 7 pi. For FAdV infected group, swollen, pale and haemorrhages liver with sharp edges was observed from day 3 and 7 pi (Figure 6). The isolate induced lesions to other organs in infected chickens with mild petechial haemorrhages of the caecal tonsils as well as swollen and haemorrhagic spleen and kidney at day 3 and 7 pi. Based on the gross lesion findings, the changes were pronounced during acute infection phase after day 3 pi following virus distribution throughout gastrointestinal tract (GIT) from oral route. These findings indicates that the disease impact of IBH in chicken which also similarly found in the natural outbreak regardless FAdV serotype in worldwide (Sun et al. 2024; Qiao et al. 2024; Zhang et al. 2023). Additionally, the severity of disease manifestations is influenced by both the route of inoculation and the viral serotypes. Compared to chickens infected via the intramuscular route, as reported in previous studies, the lesions observed in this study are more extensive, showing greater congestion and necrosis in organs such as the pancreas and proventriculus (Lou et al. 2024). In contrast to FAdV serotype 4 strains, the virus caused hydropericardium with excessive accumulation of straw-colored fluid in the pericardial sac surrounding the flabby heart (Rashid et al. 2024; Zhao et al. 2015). It has become a major cause of death in meat-producing chickens due to heart failure, with mortality rates reaching up to 80% as reported in India and China (Chen et al. 2019; Asthana et al. 2013).

## Histopathological lesions

Based on the current study, serotype 8b virus isolate induced histopathological lesions in liver, kidney and lymphoid organs particularly at day 3 pi onwards. Histopathological findings revealed normal liver architecture in control chicken (Figure 7A), while numerous basophilic intranuclear inclusion bodies (INIB) in the nucleus of the hepatocytes were detected in infected groups at day 3 and day 7 pi (Figure 7B and 7C) (Table 1). Liver is the primary tropism for FAdV with evidence of numerous basophilic INIB in the hepatocytes as shown at day 3 and 7 pi under microscopic observation.

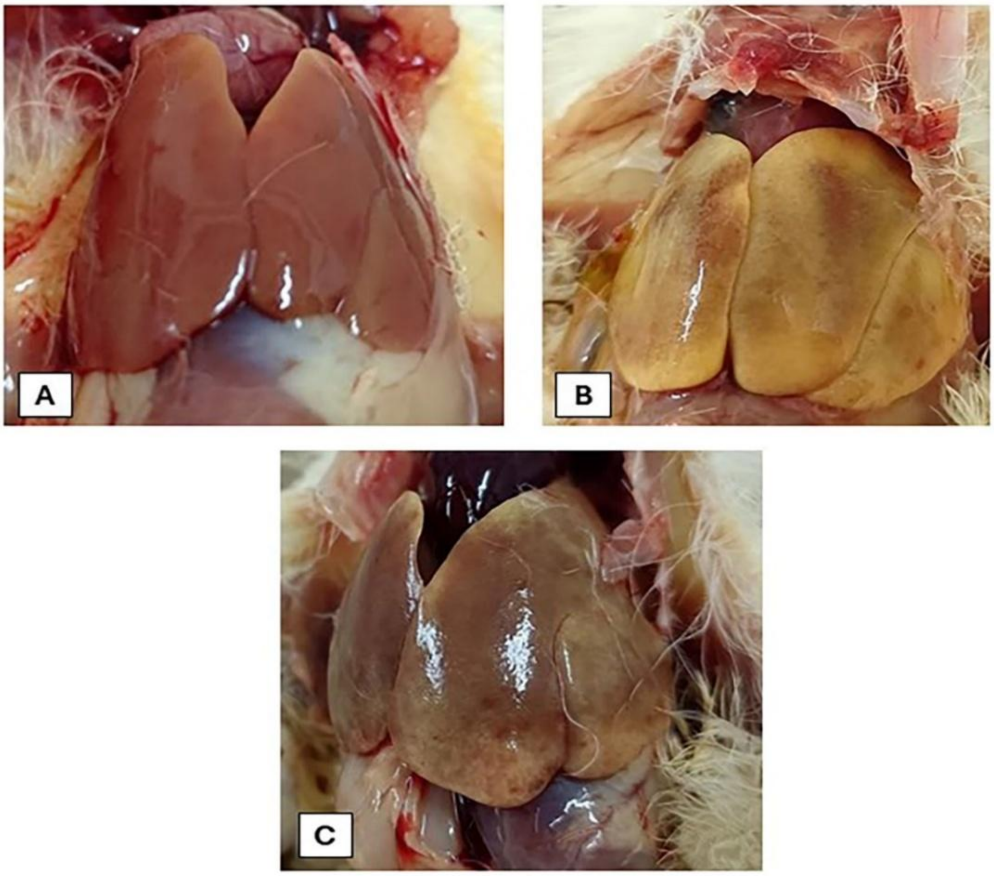


**Figure 6.** Necropsy findings in the liver of chicken for control and FAdV infected group following inoculation with FAdV-8b isolate UPM1901 at day 7 pi. (A): Normal liver appeared glistening surface, smooth edges and brownish coloration in control chicken, (B): Swollen, pale and petechial haemorrhages with sharp edges liver in the infected chicken. (C): Pale, necrotised liver with sharp edges and petechial haemorrhages in dead chicken at day 3 pi

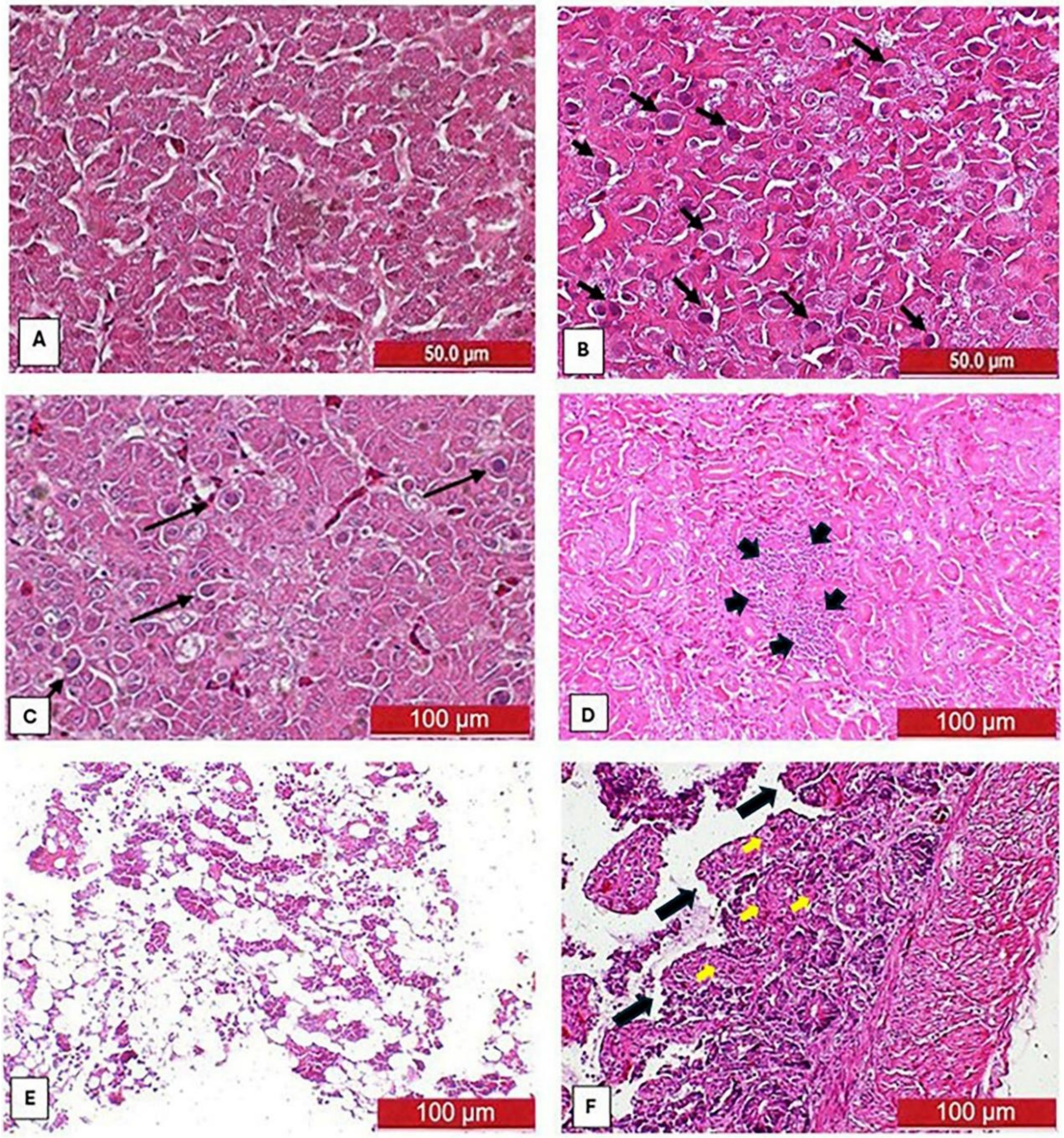


**Figure 7:** Histopathological findings of the collected samples from infected chicken with FAdV-8b isolate UPM1901. (A): Normal liver architecture in the control chicken, (B): liver of infected chicks at day pi with presence of numerous basophilic intranuclear inclusion bodies (INIB) (arrow), (H&E, 200X), (C): liver from dead chickens at day 5 pi with numerous basophilic INIB (arrow), (H&E, 400X), (D): infiltration of inflammatory cells in kidney tubule (arrows) at day 3 pi (H & E, 400X), (E): low bone marrow cellularity at 12 h pi (H & E, 400X), (F): necrosis (yellow arrows) and degeneration (black arrows) of villi in ceca tonsil at day 3 pi (H & E, 400X).

It showed that this INIB primarily a big, spherical, irregular shape and basophilic with clear and pale halo which contain virus particles in those intranuclear bodies. These findings are in agreement with previous paper studies, INIB in the liver is a hallmark of FAdV infection and has been reported in both IBH and HHS worldwide (Nakamura et al., 2011; Yuan et al. 2021); El-Shall et al. 2022). In addition, the formation of INIB was believed that the inflammatory cells, Kupffer cells phagocytose circulating viruses and eventually multiply in the hepatic cells lead to liver injury (Saifuddin & Wilks 1992).

Examination to other organs revealed swollen and haemorrhagic kidney with presence of infiltration of inflammatory cells in the at day 3 and 7 pi (Figure 7D). There was a depletion of lymphocytes in both spleen and bursa of Fabricius on day 3 pi. Thinning of the cortex was also found in the thymus at day 3 pi and reduced bone marrow cellularity at 12 h pi (Figure 7E). In caecal tonsil, lesion was only found in the dead chicks at day 3 pi, with degeneration and necrosis of the villi (Figure 7F). The lesions were noticed in kidney and lymphoid organs from day 3pi as disease continued progressed due to virus distribution in circulation. These findings were consistent with previous works as reported by Cizmecigil et al. (2020) and Islam et al. (2023). According to Saifuddin & Wilks (1992), the disease outbreak caused severe lesions in the lymphoid organs without involvement of other immunosuppressive viruses such as infectious bursal disease virus (IBDV) or chicken anaemia virus (CAV). Infected SPF chicks in this study showed lymphocytic depletion in spleen, bursa of Fabricius and thymus in histopathological changes which suggested that the immune system was compromised by the FAdV infection which led to weakness and depression chicks.

**Table 1.** Summary of histopathological findings of the collected samples from infected SPF chicken with FAdV-8b isolate UPM1901

| Day post-inoculation (pi) | Sample | Histopathological findings |
|---|---|---|
| 12 hour (h) pi | Bone Marrow | Reduced bone marrow cellularity |
| 24h pi | Bone Marrow | Reduced bone marrow cellularity |
| Day 3pi | Liver | Presence of numerous basophilic intranuclear inclusion bodies (INIB) in the nucleus of the hepatocytes |
| | Kidney | Swollen and haemorrhages kidney with presence of infiltration of inflammatory cells |
| | Spleen | Depletion of lymphocytes |
| | Bursa of Fabricius | Depletion of lymphocytes |
| | Thymus | Thinning of the cortex |
| | Caecal tonsil | Degeneration and necrosis of the villi in the dead chicks |
| Day 7pi | Liver | Presence of numerous basophilic intranuclear inclusion bodies (INIB) in the nucleus of the hepatocytes |
| | Kidney | Swollen and haemorrhages with the presence of infiltration of inflammatory cells |

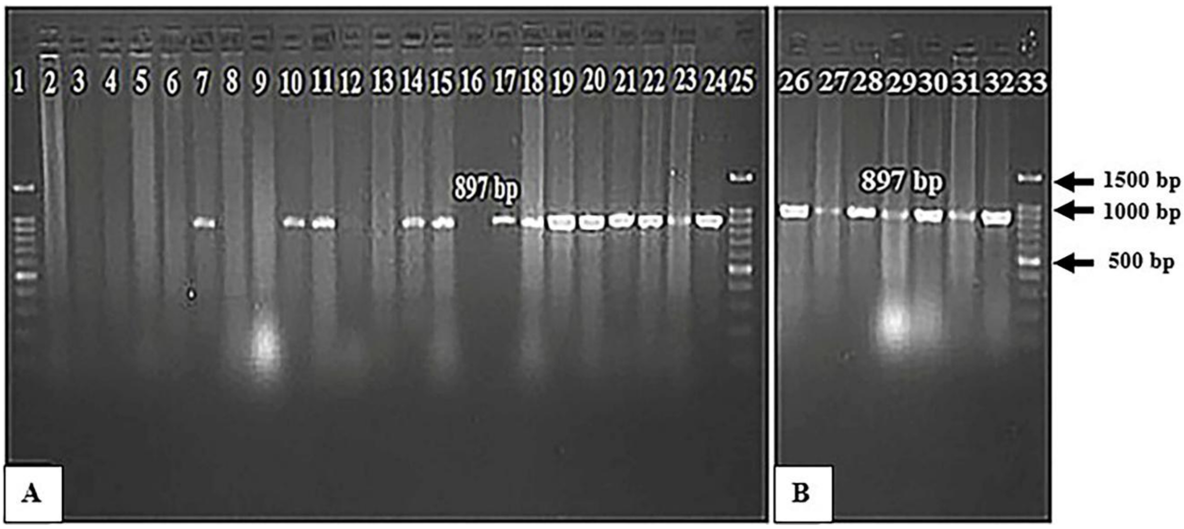


**Figure 8.** Agarose gel electrophoresis on amplification of PCR product with fragment size 897 base pairs (bp) using hexon gene primer of fowl adenovirus. (A). Lane 1 & 25: 100bp DNA ladder, Lane 16: Positive control (UPM1137), Lane 17: Negative control, Lane 2 – 8: Organs samples at 12 hour(h) post-inoculation (pi), Lane 2: Thymus, Lane 3: Spleen, Lane 4: Liver, Lane 5: Kidney, Lane 6: Bursa of Fabricius, Lane 7: Bone Marrow, Lane 8: Cecal Tonsil, Lane 9-15: organ samples at 24h pi, Lane 9: Thymus, Lane 10: Spleen, Lane 11: Liver, Lane 12: Kidney, Lane 13: Bursa of Fabricius, Lane 14: Bone Marrow, Lane 15: Cecal Tonsil, Lane 18-24: Organ samples of chicken at day 3pi, Lane 18: Thymus, Lane 19: Spleen, Lane 20: Liver, Lane 21: Kidney, Lane 22: Bursa of Fabricius, Lane 23: Bone Marrow; Lane 24: Cecal Tonsil. (B). Lane 26-32: Organ samples of chicken at day 7pi were positive for FAdV. Lane 33: 100bp DNA ladder

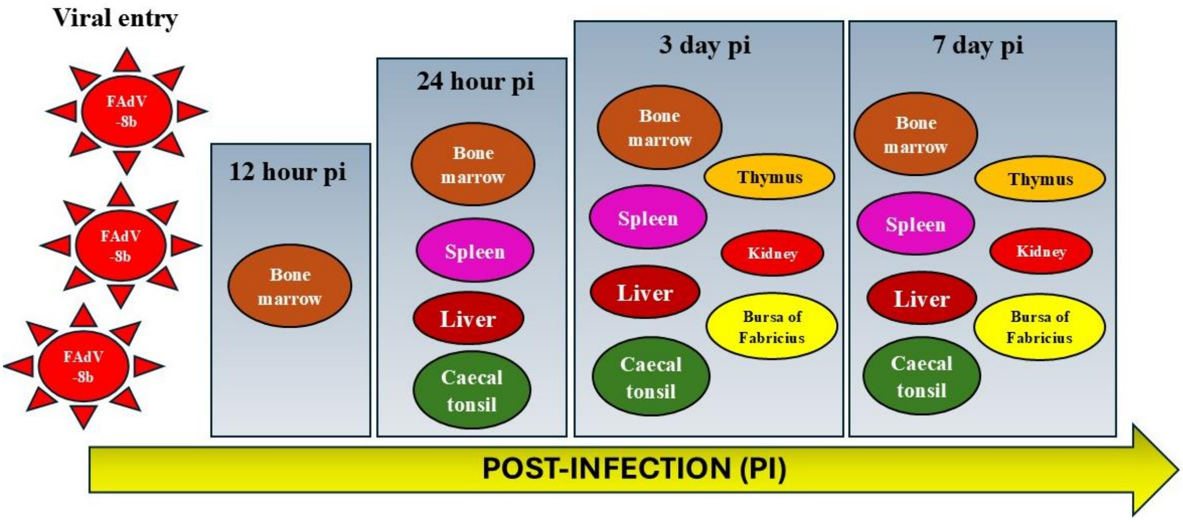


**Figure 9.** Schematic illustration of viral distribution kinetics across the organs over time in SPF chickens after infected by fowl adenovirus serotype 8b isolate UPM1901

**Virus detection in organs**

At 12h pi, only bone marrow was positive to FAdV with 897 bp as shown in Figure 8A. Subsequently, at 24 h pi, samples of spleen, liver, bone marrow and ceca tonsils were positive to FAdV. Nevertheless, at day 3 and day 7 pi, all the collected organs which are bone marrow, spleen, liver, kidney, thymus, ceca tonsils and bursa of Fabricius was positive to FAdV infection (Figure 8B). This study used PCR as the molecular detection by amplifying the hexon genes, which enables broad-range detection of the FAdV nucleic acid regardless the serotype (Adel et al. 2021). Based on the current findings, FAdV was first detected in bone marrow as early as 12h pi, followed by other organs in spleen, liver and caecal tonsils at 24 h pi. It indicates that FAdV was replicated primarily in lymphoid organs prior distributed to other organs as shown at day 3 and 7 pi, which all organs include thymus, bursa of Fabricius and kidney were positive to FAdV (Figure 9). Furthermore, the PCR found in this study showed that the targeted organ which is liver in infected chickens was positive to FAdV started from 24 h pi and persistent until day 7 pi. These findings were consistent with liver weight, gross and histological lesions predominantly in the liver organ which suggested that the incubation period was 2 days for this study. It also found that the viral nucleic acid was detected in all infected chickens, although few studies states that FAdVs may isolated from both healthy and sick chicks (Schachner et al. 2018; Niczyporuk 2016).

## CONCLUSION

It was concluded that pathogenic FAdV serotype 8b induced pathological changes in the liver and lymphoid organs in SPF chickens following oral or natural route with evidence of viral nucleic acid as early as 12 h pi in the bone marrow and existed in various organs up to day 7 pi. Therefore, effective control strategies such vaccination programs against FAdV infection are needed to overcome disease and immunosuppressive effects in the poultry flock.

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# Assessing Greenhouse Gas Emissions from Urban Dairy Farming in Jakarta as an Indicator of Environmental

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## ABSTRAK

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Penelitian ini bertujuan untuk mengevaluasi potensi emisi gas rumah kaca (GRK) dari peternakan sapi perah sebagai salah satu indikator keberlanjutan lingkungan di wilayah perkotaan DKI Jakarta. Kajian dilakukan terhadap 59 peternak aktif yang tersebar di Jakarta Timur, Selatan, dan Pusat, dengan pendekatan Tier 2 the Intergovernmental Panel on Climate Change (IPCC). Hasil menunjukkan bahwa emisi rata-rata per ekor sapi per tahun berada pada kisaran 1,67 ton CO2-eq yang sebanding dengan standar negara berkembang. Jakarta Timur menyumbang emisi total terbesar, sementara Jakarta Pusat mencatat emisi per ekor tertinggi meski jumlah ternaknya paling sedikit. Variabilitas emisi antar wilayah mencerminkan pengaruh faktor manajerial, spasial, serta ketersediaan infrastruktur pengelolaan limbah. Temuan ini menyoroti perlunya kebijakan terpadu yang tidak hanya meningkatkan produksi susu, tetapi juga menekan dampak lingkungan melalui pengelolaan peternakan yang adaptif dan berkelanjutan.

**Kata Kunci:** Sapi Perah, Keberlanjutan Lingkungan, Emisi Gas Rumah Kaca, Peternakan Urban, Jakarta

## ABSTRACT

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This study assesses greenhouse gas (GHG) emissions from urban dairy farming as a key indicator of environmental sustainability in Jakarta, Indonesia. Using the IPCC Tier 2 methodology, data were collected from 59 dairy farmers across East, South, and Central Jakarta. The findings reveal an average annual emission of 1.67 tons $CO_2$-eq per cow, aligning with estimates for developing countries. East Jakarta accounted for the highest total emissions, while Central Jakarta recorded the highest per-cow emission despite having the fewest animals. Emission disparities reflect variations in manure management, spatial conditions, and resource access. These results highlight the need for integrated policies that balance nutritional demands with sustainable farming practices in urban settings.

**Key Words:** Dairy Farming, Environmental Sustainability, Greenhouse Gas Emissions, Jakarta, Urban Agriculture

## INTRODUCTION

Dairy farming is a significant contributor to greenhouse gas (GHG) emissions within the agricultural sector, primarily through enteric fermentation, manure management, and feed production (Rotz 2018; Congio et al. 2022; Knapp et al. 2014). The two main GHGs emitted by this sector—methane ($CH_4$) and nitrous oxide ($N_2O$)—have global warming potentials approximately 28 and 265 times greater than carbon dioxide ($CO_2$) over a 100-year time horizon (IPCC 2006; Baceninaite et al. 2022; Díaz de Otálora et al. 2024).

Emission levels from dairy farms are influenced by a range of factors, including production systems, feed types, manure handling practices, animal health, and local climatic conditions (Munidasa et al. 2023; González-Quintero et al. 2025). Pasture-based and confined-housing systems exhibit varying emission intensities, as demonstrated by several studies (Ouatahar et al. 2025; Arndt et al. 2020). Feed composition, particularly fiber and protein content, plays a crucial role in determining methane emissions from enteric fermentation (Holtshausen et al. 2021; Shetty et al. 2017).

To improve the accuracy of GHG assessments, the Intergovernmental Panel on Climate Change (IPCC) has developed a tiered methodology. The Tier 1 method uses generalized emission factors and yields broad estimates that may overlook local farm characteristics (Penman et al. 2006; EMEP/EEA 2019). In contrast, the Tier 2 approach incorporates farm-specific data, such as milk production, live weight, and feed intake, resulting in more precise, context-relevant estimates (Opio et al. 2013; Arndt et al. 2020). As such, Tier 2 methods are increasingly adopted in North America, South America, and Europe (Aguirre-Villegas et al. 2024; Cashman et al. 2025; Ferraz et al. 2024). In Indonesia, Tier 2 has also been applied in several regions (Ishak et al. 2019; Widiawati et al. 2016; Amriana et al. 2024), but its use remains limited, particularly in urban areas.

Dairy farming in urban areas like Jakarta faces distinct challenges, mainly due to land scarcity, which necessitates intensive or semi-intensive housing systems. These systems influence feed strategies and manure management practices (Kumar et al. 2019; Herawati 2012). Nevertheless, the use of Tier 2 methodologies in urban livestock systems, particularly in developing countries such as Indonesia, remains limited. In Jakarta and similar urban areas, GHG emission estimates from dairy farms are typically based on Tier 1 methods, which do not fully capture the complexity of local farming conditions (Sukmono et al. 2024; Ishak et al. 2019).

Several GHG mitigation strategies have been proposed for the dairy sector, including improving feed quality, enhancing manure management, and implementing genetic selection (Pelton et al. 2024; Ni et al. 2025; Guðmannsdóttir et al. 2024). However, the effectiveness of such strategies in Jakarta remains poorly understood due to constraints on infrastructure, access to technology, and policy frameworks that differ from those in high-income countries.

Evaluating GHG emissions from urban dairy farming in Jakarta is essential to support Indonesia's Nationally Determined Contribution (NDC) under the Paris Agreement and to provide a foundation for sustainable urban agriculture policies (FAO 2018; Pramono 2016). Moreover, this study aims to inform broader discussions on sustainable livestock production in Southeast Asian cities and contribute to the global discourse on sustainable food systems (Dillon et al. 2021; Olthof et al. 2025; Horrillo et al. 2024).

This study, therefore, seeks to evaluate the potential GHG emissions from small-scale urban dairy farms in Jakarta using the IPCC Tier 2 method and primary data collected from local farmers. The findings are expected to offer more accurate emission estimates and guide the development of policy and mitigation strategies to promote environmentally sustainable dairy farming in urban Indonesia.

## MATERIAL AND METHODS

### Material

This study involved 59 smallholder dairy farmers representing the entire population of small-scale dairy farms in the Province of DKI Jakarta. The farmers were located across three administrative regions: 32 in East Jakarta, 26 in South Jakarta, and only 1 in Central Jakarta. The data indicate that currently, only one active smallholder dairy farmer remains in Central Jakarta. DKI Jakarta was selected as a representative urban area where dairy farming is still practiced by residents, despite the city's highly urbanized characteristics.

### Methods

The study was conducted from October to November 2024. A survey method was employed to collect comprehensive data on dairy farming practices, including herd physiological structure, management systems, and feed types. To estimate greenhouse gas (GHG) emissions—specifically methane ($CH_4$), nitrous oxide ($N_2O$), and carbon dioxide ($CO_2$)—this research adopted the Tier 2 approach as recommended by the 2006 IPCC Guidelines.

The analysis focused on quantifying emissions from two primary sources: enteric fermentation and manure management. The Tier 2 method was selected for its ability to provide more accurate estimates by incorporating detailed animal-specific data, such as body weight, feed intake, and milk production. This approach enables a more representative assessment of GHG emissions from smallholder dairy farming activities in urban settings.

Greenhouse gas (GHG) emissions were calculated using the Tier 2 methodology as recommended by the IPCC (2006). The equations used in this study are presented below:

$$\text{Enteric CH4 emission} = \left(\frac{FE_{enteric(T)}\ x\ N_T}{10^6}\right)$$

$$\text{Enteric CH4 emission from manure management} = \left(\frac{FE_{manure(T)}\ x\ N_T}{10^6}\right)$$

$$N_2O_{D(mm)} = \left[\sum_s\left[\sum_T(N_T \cdot Fex_{(h)} \cdot BB_{(t)}\right]FE_{t(s)}\right]\frac{44}{28} \cdot 365c$$

where $FE_{enteric(T)}$ is enteric $CH_4$ emission factor for livestock type $T$ based on IPCC 2006 default values (kg $CH_4$/head/year), $FE_{manure(T)}$ is manure $CH_4$ emission factor for livestock type $T$ based on IPCC 2006 default values (kg $CH_4$/head/year), $N_{(T)}$ is livestock population of type $T$ (head/year), $10^6$ is conversion factor from kilograms to gigagrams, $N_2O_{D(mm)}$ is direct $N_2O$ emissions from manure management (kg $N_2O$/year), $Fex_{(h)}$ is nitrogen excretion factor per 1000 kg of live animal weight per day (kg N/1000 kg live weight/day); value used: 0.47 (IPCC 2006 default), $BB_{(t)}$ is average live weight of the animal (kg/head), $FE_{t(s)}$ is $N_2O$ emission factor for manure under intensive management systems (kg $N_2O$-N/kg N); value used: 96 (IPCC 2006 default), 365 is Number of days in a year, and 44/28 is molecular weight ratio of $N_2O$ to $N_2$.

## RESULT AND DISCUSSION

This study estimates the magnitude of greenhouse gas (GHG) emissions from dairy farming in the urban area of Jakarta, focusing on key sources: enteric methane ($CH_4$), methane from manure management, and nitrous oxide ($N_2O$). The following figure presents boxplots for each GHG emission component: enteric $CH_4$, manure $CH_4$, $N_2O$, and total emissions. These visualizations illustrate the distribution of data, variability among farmers, and the presence of outliers in the emission values.

The distribution of greenhouse gas (GHG) emissions from enteric methane ($CH_4$) produced by 59 small-scale dairy farmers in DKI Jakarta is presented in a boxplot (Figure 1). Emissions are expressed in gigagrams of $CO_2$-equivalent (Gg $CO_2$-eq), based on the Global Warming Potential (GWP) of methane as outlined by the IPCC methodology. The visualization shows substantial variation in emission levels among farmers, which is directly linked to the environmental sustainability performance of each farming system.

### Enteric $CH_4$ emissions

The boxplot of enteric methane shows a skewed distribution with a long right tail and several outliers, suggesting that some farms have significantly higher emissions than the majority. The mean emission was 0.0275 Gg $CO_2$-eq, exceeding the median of 0.0199 Gg $CO_2$-eq, suggesting that a small number of farms disproportionately contribute to the total emissions. The interquartile range (IQR) spanned from 0.0086 to 0.0338 Gg $CO_2$-eq, with the maximum value reaching 0.1452 Gg $CO_2$-eq—more than five times the third quartile (Q3). Such a distribution underlines that the variability in emissions is primarily driven by a few farms with extreme values well above the average.

Further analysis revealed that the three farms with the highest emission levels accounted for approximately 22.5% of the total enteric methane emissions, despite representing only a small fraction of the sample. These outliers consistently originate from farms with larger herd sizes, particularly those dominated by lactating cows and breeding cows.

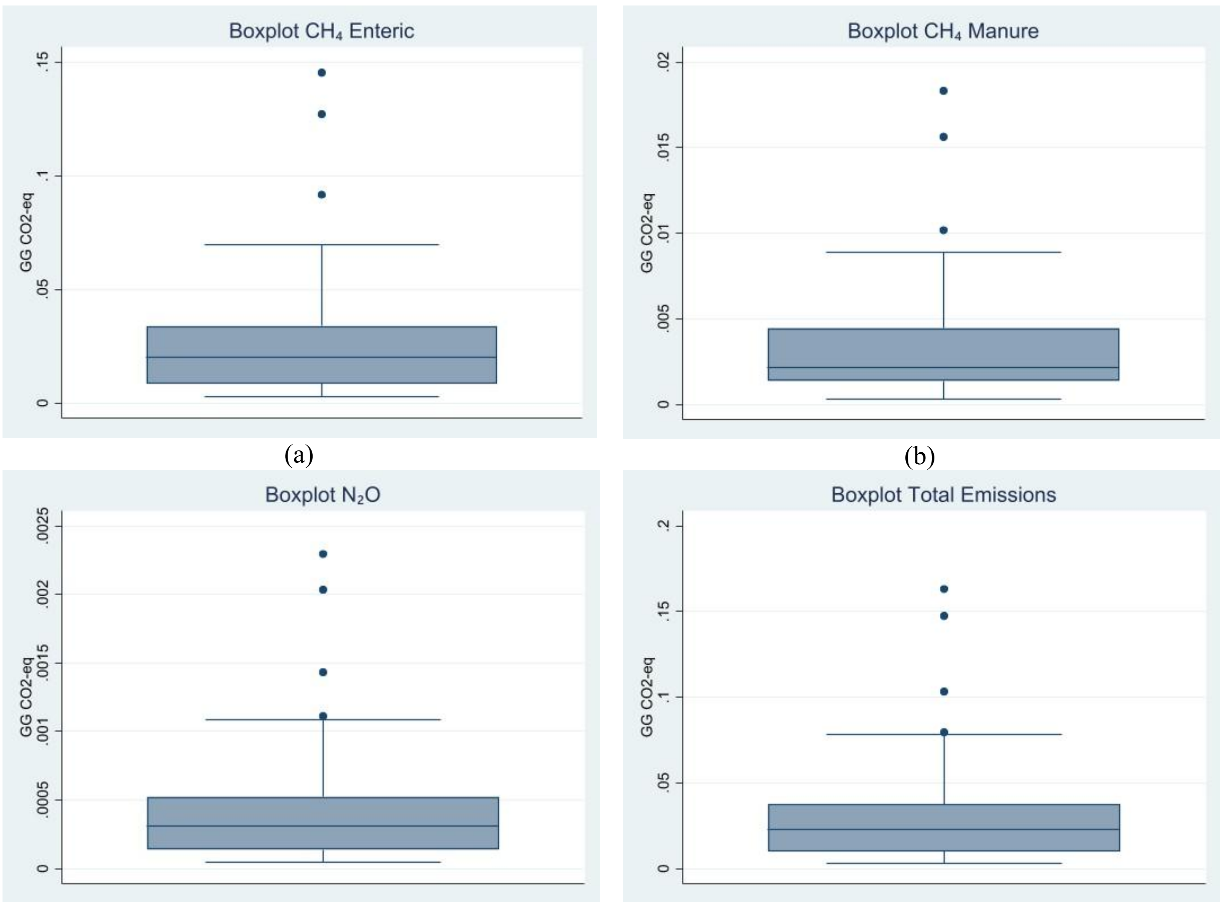


**Figure 1.** GHG emissions results (in $CO_2$ equivalents) from livestock sources: (a) $CH_4$ emissions from enteric fermentation, (b) $CH_4$ emissions from manure management, (c) $N_2O$ emissions, and (d) total GHG emissions. The plots indicate the median, interquartile range, and the presence of extreme values (outliers) across sampled production units.

The proportion of CH4 emissions from enteric fermentation generated by dairy cows also showed a relatively consistent pattern compared with the total cattle population (Marklein et al., 2021), reinforcing the evidence that lactating and breeding groups account for a substantial share of overall emissions. These categories of cattle require higher feed intake and exhibit more intensive rumen fermentation, thereby producing greater methane emissions. These findings align with those of Ibidhi et al. (2021), who emphasized that herd size and structure are critical determinants of emission variability across dairy farms.

The result carries important implications for emission mitigation strategies in smallholder dairy systems in Jakarta. Mitigation efforts should not rely solely on reducing average emissions, as farms with larger numbers of lactating and breeding cows make the most substantial contribution to aggregate enteric methane output. Targeted interventions focusing on these high-intensity farms are therefore more likely to yield significant reductions in total emissions, while simultaneously enhancing the environmental benefits of urban dairy production systems.

**Manure-derived $CH_4$ emissions**

Methane emissions from manure showed a narrower distribution, with a median of approximately 0.005 Gg $CO_2$-eq. Although smaller than enteric emissions, some outliers were still observed, particularly in farms with larger or more productive animals. These differences are primarily shaped by herd structure—such as age, physiological status, and body weight—that influences excretion rates. Widiawati et al. (2016) found that manure emission factors for beef cattle in Indonesia differ across animal categories. Similarly, Amriana et al. (2024) stressed the importance of including animal age and body weight data to explain category-specific variability better. Consistent with this, the IPCC Tier-2 guidelines also derive manure emission factors from animal categories rather than manure management practices.

**$N_2O$ emissions**

The boxplot of $N_2O$ emissions shows a median of approximately 0.0005 Gg $CO_2$-eq, which is substantially lower than 0.002 Gg $CO_2$-eq and represents the smallest contribution among the three emission sources analyzed: enteric $CH_4$, manure $CH_4$, and $N_2O$. The relatively narrow distribution indicates that most farms consistently generated low emissions. Nevertheless, a few outliers above 0.001 Gg $CO_2$-eq were observed, suggesting variation among farmers.

According to the IPCC Tier 2 guidelines, this variability is primarily due to differences in nitrogen excretion, which are calculated based on body weight, milk productivity, feeding practices, and environmental factors influencing the transformation of nitrogen into N2O(IPCC 2006; Bougouin et al. 2022; Reed et al. 2015). With the Tier 2 approach, nitrogen excretion is estimated individually for each animal, so differences in animal characteristics and feed management are directly reflected in the $N_2O$ emission estimates, including the appearance of outliers in the boxplot.

**Total GHG emissions**

The boxplot for total GHG emissions reflects the cumulative contribution of all three sources (enteric $CH_4$, manure $CH_4$, and $N_2O$). The median total emission was approximately 0.03 Gg $CO_2$-eq, though outliers approached 0.20 Gg $CO_2$-eq. This wide distribution underscores the disparity in emissions across farms and highlights the potential for targeted mitigation strategies. These findings support the prioritization approach advocated by Ouatahar et al. (2025) and González-Quintero et al. (2025), who recommend focusing mitigation efforts on high-emission farms through incentive schemes and environmental education.

Among the four emission types analyzed, the environmental sustainability of small-scale dairy systems in DKI Jakarta appears highly dependent on feed and waste management practices. The uneven emission patterns reflect structural challenges in standardizing farming practices across urban settings. GHG emissions not only indicate climate impacts but also serve as proxies for system efficiency.

In the Indonesian context, Sukmono et al. (2024) stressed the importance of location-specific and socio-economic considerations in evaluating and reducing the livestock sector's carbon footprint. Strategies such as improved feeding management, the introduction of micro-scale waste-treatment technologies, and farmer training in low-carbon practices are critical steps toward a more sustainable dairy sector.

Understanding the potential contribution of GHG emissions from dairy farms also requires analysis by livestock age group and administrative region; this is important because physiological characteristics influence emission levels and feed consumption, both of which vary with age. For instance, adult cows typically emit more greenhouse gases than calves due to higher metabolic activity and milk production. Furthermore, differences in farm management practices across Jakarta's administrative regions—such as herd density, feeding routines, and waste handling systems—also affect the magnitude of emissions.

Greenhouse gas (GHG) emissions from the dairy farming sector are a crucial indicator for evaluating environmental sustainability, particularly in urban areas such as DKI Jakarta, where land pressure, spatial conflict,

**Table 1.** Estimated emissions by age group and administrative region in Jakarta

| Area | East Jakarta | | South Jakarta | | Central Jakarta | | Total | | Estimated Emissions Average (GG $CO_2$-eq / year/head) |
|---|---|---|---|---|---|---|---|---|---|
| Range Age of Cattle | Total (head) | Estimated Emissions (GG $CO_2$-eq /year) | Total (head) | Estimated Emissions (GG $CO_2$-eq /year) | Total (head) | Estimated Emissions (GG $CO_2$-eq /year) | Total (head) | Estimated Emissions (GG $CO_2$-eq /year) | |
| Calves (<1 year) | 122 | 0.0588 | 57 | 0.0275 | 1 | 0.0005 | 180 | 0.0867 | 0.0005 |
| Growers (1–2 years) | 158 | 0.1689 | 53 | 0.0567 | 0 | 0 | 211 | 0.2256 | 0.0011 |
| Young Adults (2–4 years) | 177 | 0.2891 | 136 | 0.2221 | 0 | 0 | 313 | 0.5112 | 0.0016 |
| Mature Adults (>4 years) | 298 | 0.7556 | 98 | 0.2485 | 9 | 0.0233 | 405 | 1.0274 | 0.0025 |
| Total | 755 | 1.2724 | 344 | 0.5548 | 10 | 0.023 | 1109 | 1.8509 | |

and resource constraints present significant challenges. Based on estimations from 59 dairy farmers across three administrative regions (East Jakarta, South Jakarta, and Central Jakarta), the total annual emissions were calculated at 1.8509 GG $CO_2$-equivalent ($CO_2$-eq), with an average emission of 1.67 tons $CO_2$-eq per cow per year.

East Jakarta contributed the highest emissions, totaling 1.2724 GG $CO_2$-eq, followed by South Jakarta (0.5548 GG $CO_2$-eq) and Central Jakarta (0.023 GG $CO_2$-eq). When normalized by livestock population, regional variations in average emissions per cow were observed: 1.69 tons $CO_2$-eq in East Jakarta (755 head), 1.61 tons $CO_2$-eq in South Jakarta (344 head), and 2.30 tons $CO_2$-eq in Central Jakarta (10 head). The relatively high per-animal emissions in Central Jakarta likely reflect the small sample size, where statistical variation per individual becomes more pronounced.

These findings fall within the emission range reported by FAO (2018), which estimated 1.6–2.5 tons $CO_2$-eq per dairy cow annually in developing countries, depending on production systems, feed management, and manure handling. In Jakarta, most dairy farms operate at small, household scales, with heterogeneous management practices. According to Opio, C. et al. (2013), such systems tend to produce higher emissions when inputs are inefficient and manure management is suboptimal, particularly due to dominant methane emissions from enteric fermentation and nitrous oxide emissions from untreated manure.

An age-based analysis of the livestock showed that the largest share of emissions originated from adult cows (>4 years old), contributing 1.0274 tons $CO_2$-eq, or more than 55% of the total emissions. Cattle in this age group are generally in lactation and have higher feed intake, resulting in more intensive enteric fermentation. In contrast, calves (<1 year) contributed only 0.0867 tons $CO_2$-eq, consistent with lower metabolic activity and reduced feed consumption.

Spatial differences also revealed important dynamics. In East Jakarta, dairy farms are more clustered and benefit from government-supported collective manure management systems. These facilities process manure into liquid and solid fertilizers, reducing GHG emissions from waste and promoting circular economy practices in urban agriculture. Conversely, in South Jakarta, farms are scattered within densely populated residential areas and are often located near riverbanks, increasing the risk of pollution, social conflict, and poor waste management. As noted by Kumar et al. (2019), such conditions require adaptive policy approaches that address spatial planning challenges and the complex socio-human-animal interactions in urban contexts.

Meanwhile, the high per-cow emissions in Central Jakarta—reaching 2.30 tons $CO_2$-eq—may indicate managerial inefficiencies. Despite the tiny livestock population, this highlights that small scale does not guarantee low emissions; every farm unit, regardless of its size, must be supported with environmentally sound management practices.

Food policy contexts must also be taken into account. Government programs promoting free nutritious meals for school-aged children are expected to increase the demand for fresh milk. Without corresponding improvements in production systems and environmental management, the increased demand could lead to higher GHG emissions from urban dairy farms. Therefore, food and environmental policies must be developed in an integrated manner. As emphasized by Yuhendra et al. (2022), a sustainable food systems approach must balance environmental, social, and economic dimensions.

As a practical implication, GHG mitigation strategies for dairy farming in urban areas like Jakarta must be tailored to local conditions. These include providing technical training for farmers, offering incentives for using organic fertilizers derived from livestock waste, and implementing zoning policies that support the sustainability of small-scale farms. This study underscores the importance of integrating quantitative emission data with socio-economic understanding of local contexts as the foundation for evidence-based policymaking.

## CONCLUSION

Dairy farming in the DKI Jakarta area has been shown to contribute significantly to greenhouse gas (GHG) emissions, with an average emission of 1.67 tons $CO_2$-equivalent ($CO_2$-eq) per cow per year. Variations in emissions across farms and regions indicate that managerial practices, business capacity, and institutional support play a critical role in determining emission levels. The highest emissions were recorded from adult cows, highlighting a strong correlation between animal age and emission potential.

These findings underscore the importance of environmental management in urban livestock systems. Without implementing technology-based interventions and adaptive policy frameworks, increased milk production to meet urban food demand may exacerbate environmental pressures. Therefore, GHG emissions from dairy farming should be considered a key indicator of environmental sustainability in the planning of more integrated urban food systems.


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# Shelf Life of *Trichoderma* Mutant Inoculum with Dried Mud and Glutinous Rice as Carrier Materials


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## ABSTRAK

Mulyono AMW, Husein M, Ratriyanto A, Sukaryan S, Asmoro NW, Afriyanti. 2025. Umur simpan inokulum mutan *Trichoderma* dengan bahan pembawa lumpur kering dan beras ketan. JITV 30(3):178-184. DOI:http://dx.doi.org/10.13443/jitv.v30i3.3443.

Pembuatan inokulum berbentuk bubuk melalui proses pengeringan akan menyebabkan kerusakan sel spora Trichoderma dan menurunkan viabilitas spora. Penelitian ini mengkaji kapasitas penyimpanan inokulum mutan Trichoderma dengan bahan pembawa lumpur kering dan beras ketan. Spora mutan Trichoderma AA1 kering dicampur dengan media pembawa berupa campuran lumpur kering dan tepung ketan. Perbandingan lumpur kering dengan beras ketan sebanyak 6, yaitu: 0:10, 2:8, 4:6, 6:4, 8:2, dan 10:0. Campuran spora dan bahan pembawa disimpan dan dianalisis hingga 16 minggu. Ketersediaan inokulum pada seluruh kombinasi perlakuan diatas $3{,}19\times10^7$ CFU (Jumlah sel jamur). Fluktuasi ketersediaan terkecil ditunjukkan pada rasio 4:6 yang berkisar antara $3{,}54\times10^7$ dan $6{,}1\times10^7$ CFU. Aktivitas enzim selulase mengalami peningkatan selama penyimpanan 16 minggu dengan peningkatan sebesar 66,67%. Hasil penelitian menunjukkan bahwa Trichoderma dengan perbandingan lumpur kering:tepung ketan 4:6 mempunyai ketersediaan $3{,}54\times10^7$ CFU dan aktivitas pelepasan glukosa karboksimetil selulase (CMCase) 0,020 µmol/menit setelah penyimpanan 16 minggu. Temuan ini mengindikasikan bahwa inokulum dengan formulasi tersebut berpotensi digunakan dalam fermentasi hijauan pakan untuk meningkatkan kecernaan pakan unggas.



**Kata Kunci**: Lumpur Kering, Beras Ketan, Inokulum, Trichoderma Mutan.



## ABSTRACT

Mulyono AMW, Husein M, Ratriyanto A, Sukaryan S, Asmoro NW, Afriyanti. 2025. Shelf life of *Trichoderma* mutant inoculum with dried mud and glutinous rice as carrier materials. JITV 30(3):178-184. DOI:http://dx.doi.org/10.13443/jitv.v30i3.3443.

Drying to produce powdered inoculum can damage Trichoderma spores and reduce spore viability, a factor commonly affected by carrier materials. This study investigated the storage capacity of Trichoderma mutant inoculum with dried mud and glutinous rice as carrier materials. Dried Trichoderma AA1 mutant spores were mixed with the carrier in the form of a mixture of dried mud and glutinous rice flour. There were 6 ratios of dried mud: glutinous rice, including 0:10, 2:8, 4:6, 6:4, 8:2, and 10:0. The spore-carrier mixtures were stored and analyzed for an additional 16 weeks. The availability of inoculum in all combinations of treatment was above $3.19\times10^7$ CFU (Colony Forming Units). The smallest availability fluctuations were observed at a ratio of 4:6, ranging from $3.54\times10^7$ to $6.1\times10^7$ CFU. Cellulase enzyme activity increased by 66.67% during 16 weeks of storage. The research suggests that Trichoderma with a ratio of dried mud: glutinous rice flour of 4:6 had an availability of $3.54\times10^7$ CFU and 0.020 µmol/minute glucose-released carboxymethyl cellulase (CMCase) activity after 16 weeks of storage.



**Key Words**: Dried Mud, Glutinous Rice, Inoculum, Trichoderma Mutant


## INTRODUCTION

The use of microbes in feed technology is very challenging, especially compared to chemical and physical treatments. However, the use of chemicals in feed technology is more complicated, as it must be neutralized to a pH of 7.0. In comparison, physical treatment might not provide the optimal results. The use of microbes is considered natural, environmentally friendly, and safe for livestock.

Microbes are widely used to improve the nutritional quality of feed ingredients. Trichoderma is one of the fungi with high cellulolytic activity (Zayed et al. 2020; Iannaccone et al. 2022), and sit has been shown to improve the nutritive quality of cellulose-rich feed ingredients (Jayasekara & Ratnayake 2019; Taye & Etefa 2020). Trichoderma is characterized by rapid growth, mostly bright-green conidia, and a repeatedly branched conidiophore structure (Singh et al. 2020; Siti et al. 2021; Iqbal et al. 2022). Previous studies showed that Trichoderma produces cellulolytic enzymes that degrade cell wall substrates into glucose, which is readily absorbed by microbes as an energy source (Grujić et al. 2019). The degradation of substrate cell walls releases the substrate cell contents (Jha 2021), which can be digested by endogenous enzymes of animals, including poultry (Dimitrova 2020). Besides, Adebami & Adebayo-tayo (2020) and Gooruee et al. (*2*024) observed that Trichoderma produces complex cellulase enzymes, including cellobiohydrolase (1,4-β-glucan cellobiohydrolase, EC 3.2.1.91), endoglucanase (1,4-β-glucanohydrolase, EC 3.2.1.4), and cellobiose (β-glucosidase, EC 3.2.1.21).

The application of Trichoderma in feed biotechnology requires an appropriate inoculum. For that, the inoculum carrier must be able to carry sufficient quantities of Trichoderma spores under appropriate physiological conditions, ready for inoculation into the fermentation medium. Inoculum carrier quality can be determined by its ability to maintain the spore availability during storage. The shelf life and availability of inoculum are affected by storage temperature and nutrient content of carrier medium (Zope et al. 2019; Rimkus et al. 2023). Khan & Mohiddin (2018); Rajput et al. (2014) observed that Trichoderma pseudokoningii inoculum in substrates containing sorghum and rice showed higher availability than in wheat straw and rice hull.

Furthermore, dried mud has the potential to serve as an inoculum carrier for Trichoderma spores due to its naturally abundant availability (Munir et al. 2013; Pandya & Albert 2014; Singh et al. 2021). Moreover, adding glutinous rice to the inoculum might improve the nutrient content required by the microbes. Therefore, the study on the shelf life of Trichoderma inoculum with different carrier materials is essential as a preliminary step before its application in forage fermentation to improve feed quality. Thus, the objective of this study was to investigate the storage capacity of Trichoderma inoculum with a mixture of dried mud and glutinous rice as carrier materials.

## MATERIALS AND METHODS

### Experimental design

Carrier media consisted of dried mud and glutinous rice mixtures in the ratios 0:10, 2:8, 4:6, 6:4, 8:2, and 10:0. Each ratio was analyzed in five replicates. Inoculum availability was determined at weeks 0, 4, 8, 12, and 16 of storage.

### Preparation of Trichoderma spores, medium, and carrier

The mutant Trichoderma AA1 used in this study was initially isolated from oil palm bunches. The isolate was subjected to chemicals: EMS (Ethyl Methanesulfonate) and ETBr (Ethidium Bromide) mutagenesis as previously described by Mulyono (2008), and the selected mutant was maintained in the laboratory culture collection. The mutant Trichoderma AA1 was used in this study (Mulyono 2008). 5 mL of 0.05% Triton X-100 solution was mixed with the mutant Trichoderma AA1 spore culture in the agar medium, and the mixture was stirred until the spores dissolved, ready for inoculation. Potato dextrose agar (PDA) is used as a medium, and 100 ml of PDA solution is sterilized in an autoclave at 15 psi for 15 min. When the PDA solution reached about 50ºC, it was then poured onto plates, 10 ml per plate. The medium was stored at room temperature for 24 hours until it hardened into solid materials and was ready for inoculation (Sarhan 2015; Oeng et al. 2021).

Carrier media were prepared from dried mud and glutinous rice in ratios of 0:10, 2:8, 4:6, 6:4, 8:2, and 10:0. The dried mud was obtained from Gadjah Mungkur Reservoir in Wonogiri Regency, Indonesia. Each mixture was prepared to a maximum of 100 g, sterilized in the oven at 170ºC for 2 hours, and cooled to room temperature thereafter.

### Fermentation

The solid medium was inoculated with 0.1 ml of mutant Trichoderma AA1 spore solution in a petri dish, then incubated for 7 days. Microbial growth was observed daily. When the spores covered the entire surface of the medium, the fermentation process was terminated, and the spores were harvested.

Spore harvest was performed by drying the spore culture with media in the oven at 50°C for 24 hours. Dried cultures were then mixed with 100 g of carrier substrate and kept in the oven at 50°C for 24 hours until the moisture content was less than 10%. Dried cultures with carrier substrate were packed in aluminum foil, tightly sealed, and stored at room temperature for 16 weeks (Rajput et al. 2014).

### Determination of inoculum availability

One gram of dried culture was placed in a reaction tube and mixed with sterile physiological saline until the volume reached 10 ml. This culture solution was vortexed for 1 minute to form a spore suspension. The spore suspension was diluted $10^{-2}$, $10^{-3}$, and $10^{-4}$, and 0.1 ml of each dilution was inoculated onto a petri dish containing solid potato dextrose agar (PDA). The cultures were incubated for 48 hours, and several colonies per ml of spore suspension were counted to determine the number of colonies-forming units (CFU) (Patagundi et al. 2014).

### Determination of cellulase activity

Cellulase activity was determined qualitatively by the Congo Red staining method (Grata 2020) and quantitatively by measuring carboxymethyl cellulase (CMCase) activity. The 1% CMC substrate was mixed with culture supernatant in a 50 mM citrate buffer at pH 4.8 for 60 minutes. The reducing sugar released was measured using the dinitrosalicylic acid method (Miller 1959).

### Data analysis

Statistical data were analyzed in Excel, and mean values were displayed in tables and graphs. Data on culture Availability and cellulase activity during storage were presented descriptively (Steel et al. 1996).

## RESULTS AND DISCUSSION

### Inoculum availability

The range of initial concentration of inoculum was between $5.18 \times 10^{\wedge 7}$ and $6.68{\times}10^7$ colony-forming units (CFU). After 16 weeks of storage, the inoculum concentrations mixed with up to 60% dried mud as a carrier remained above $3.61 \times 10^{\wedge 7}$ CFU (Table 1). This finding indicated that a combination of dried mud and glutinous rice can be utilized as a Trichoderma inoculum carrier with high spore availability. Modifying and manipulating media nutrients may increase spore availability (Abuhena et al. 2022). The presence of dried mud as a carrier material was similar to the natural habitat of *Trichoderma* spp., namely soil (Pandya & Albert 2014; Martinez et al. 2023). The inoculum grows in a suitable medium (Iqbal et al. 2017).

The CFU concentration after 16 weeks of storage showed that the treatment with dried mud at a proportion less than 60% resulted in a lower decrease in CFU value than 80% or 100% dried mud. The dried mud cannot be used in high proportions. The lowest decrease in CFU value (31.66%) was observed with a 4:6 ratio of dried mud:glutinous rice, indicating that this combination better maintains Trichoderma spore availability. Furthermore, spore availability fluctuates when the proportion of dried mud exceeds 40%. This result indicates that the shelf life of Trichoderma inoculum varied significantly across substrate media (Kaushal & Chandel 2017). A previous study showed that the shelf-life of Trichoderma pseudokoningii in different substrates attained the peak at 60-75 days and declined gradually thereafter (Rajput et al. 2014).

Substrates rich in nutrients promote a better shelf life than low-nutrient substrates. A combination of dried mud and glutinous rice as a carrier medium yielded better spore availability, likely due to the presence of dried mud as a nitrogen source and glutinous rice as a carbon source. Both nitrogen and carbon in the carrier medium of the inoculum promote conidial production, leading to more spore production. Following this result, Rajput et al. (2014) reported that storage ability and growth of T. pseudokoningii inoculum were affected by the presence of carbon and nitrogen in the carrier medium, and Rodrigo et al. (2020) reported the same in Trichoderma asperellum.

A 4:6 ratio of dried sludge to glutinous rice produced the most stable inoculum ($3.54{\times}10^7$ CFU) after 16 weeks of storage, serving as an effective dose to initiate microbial activity in the fermentation system. This finding indicates that the carrier combination maintains spore viability and ensures sufficient spores are available for potential use in feed fermentation.

### Quantitative CMC-ase

Quantitative CMCase is used to measure the activity of the cellulase enzyme, with activity expressed as µmol glucose released per minute (Vimal et al. 2017). The cellulase activity of Trichoderma inoculum with dried mud proportions up to 60% increased after 16 weeks of storage, although the increase varied depending on the dried mud-to-glutinous rice ratio. However, cellulase activity remained constant or decreased when proportions of dried mud were 20% or more (Table 2). This finding indicates that the Trichoderma inoculum requires nutrients supplied by the carrier medium. The high proportion of dried mud led to lower cellulase activity than the high proportion of glutinous rice. This result was in line with the finding on inoculum availability, where the 4:6 ratio of dried mud:glutinous rice yielded the best availability. Trichoderma is a cellulolytic organism that produces cellulolytic enzymes to hydrolyze cellulose (Singh et al. 2017; Strakowska et al. 2014). Cellulase enzymes are produced due to microbial activity in the growth medium. In this study, higher cellulase activity was observed in the medium containing a higher proportion of glutinous rice, consistent with the number of Trichoderma colonies.

The cellulase activity of Trichoderma inoculum with dried mud: glutinous rice at 4:6 was consistent (0.020 µmol/minute) after 16 weeks. Although this study did not directly test the inoculum on forage materials, maintaining active cellulase production during storage indicates that the inoculum can hydrolyse plant cell wall polysaccharides into glucose when applied in feed fermentation. Such enzymatic activity is essential to improve the digestibility of forages rich in cellulose.

**Qualitative CMC-ase**

The formation of a clear zone around colonies grown on a plate containing a single carbon source derived from cellulose indicates the secretion of cellulase by the growing colonies. The ratio of the apparent zone diameter to the colony diameter is an indicator of qualitative cellulase activity (Patagundi 2014; Dewiyanti et al. 2021). The changes in precise zone ratios after 16 weeks of inoculum storage were relatively small in all treatments. The use of single-carrier materials, both dried mud and glutinous rice, resulted in a decrease in the clear zone ratio. Whereas the use of a combination of carrier materials with the ratio of dried mud: glutinous rice 2:8, 4:6, and 8:2 increased the clear zone ratio (Table 3). The ratios of dried mud:glutinous rice 0:10 and 4:6 resulted in higher clear-zone averages than other ratios. The qualitative cellulase activity fluctuated in weeks 2 and 16, but was relatively constant in other weeks, with precise zone ratios of about 4-5. Extremely high values were observed in the treatment group for the 0:10 ratio at week 2 and the 8:2 ratio at week 16. In general, based on the comprehensive results of measuring inoculum availability and quantitative and qualitative cellulase activity, the use of dried mud:glutinous rice at a ratio of 4:6 yielded the best results.

The formation of a clear zone indicates the secretion of the cellulase enzyme from the Trichoderma inoculum, demonstrating the inoculum's consistent cellulolytic potential for breaking down cellulose. This stability is essential to ensure predictable performance when the inoculum is applied to forage fermentation systems.

**Table 1.** Number of colony-forming units (CFU, $\times 10^7$) per ml of inoculum spore suspension in various combination ratios of dry mud (DM) and glutinous rice (GR) as carrier medium during storage

| Ratio DM: GR | Storage Time (Weeks) | | | | | Changes (%) |
|---|---|---|---|---|---|---|
| | 0 | 4 | 8 | 12 | 16 | |
| 0:10 | 6.68 | 5.80 | 3.96 | 3.82 | 3.37 | -49.55 |
| 2:8 | 6.66 | 2.66 | 3.48 | 4.46 | 2.74 | -58.86 |
| 4:6 | 5.18 | 4.56 | 4.54 | 4.06 | 3.54 | -31.66 |
| 6:4 | 7.62 | 1.16 | 1.62 | 1.72 | 3.61 | -52.62 |
| 8:2 | 6.24 | 2.36 | 4.10 | 1.98 | 1.20 | -80.77 |
| 10:0 | 6.10 | 1.33 | 3.39 | 3.26 | 0.51 | -91.64 |

DM= Dry Matter, GR= Glutinous Rice

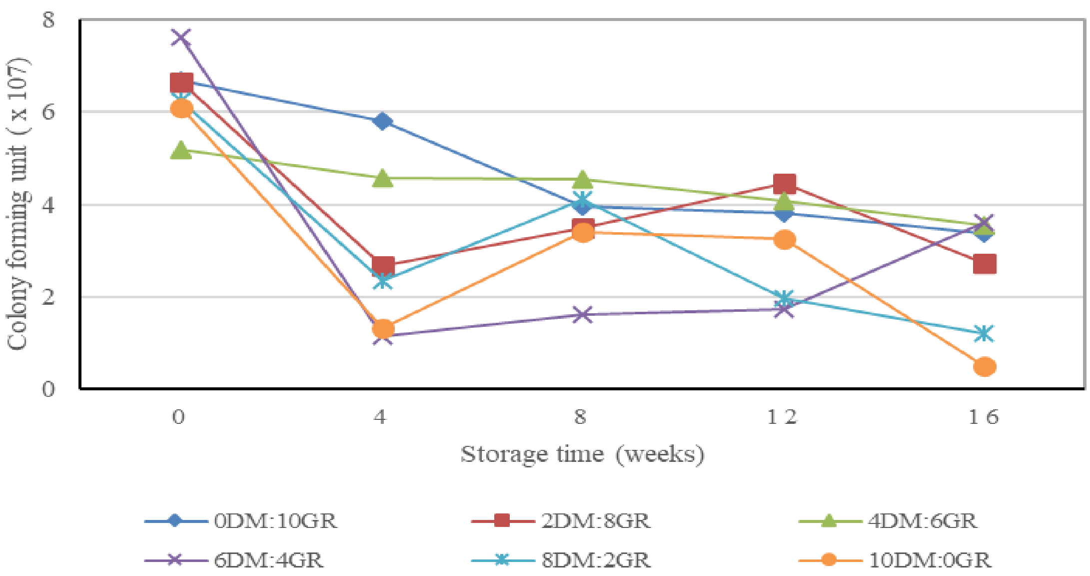


**Figure 1.** Availability of Trichoderma inocula in various combination ratios of dried mud (M) and glutinous rice (G) during storage

**Table 2.** Carboxymethyl cellulase activity (CMCase, µmol minute per minute glucose released) of Trichoderma inoculum in various combination ratios of dry mud (DM) and glutinous rice (GR) as carrier medium during storage

| Ratio DM: GR | Storage Time (Weeks) | | | | | | | Changes (%) |
|---|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 8 | 12 | 16 | |
| 0:10 | 0.014 | 0.047 | 0.038 | 0.014 | 0.040 | 0.003 | 0.040 | 185.71 |
| 2:8 | 0.015 | 0.013 | 0.031 | 0.034 | 0.016 | 0.014 | 0.026 | 73.33 |
| 4:6 | 0.012 | 0.020 | 0.018 | 0.014 | 0.029 | 0.025 | 0.020 | 66.67 |
| 6:4 | 0.025 | 0.030 | 0.027 | 0.019 | 0.033 | 0.018 | 0.045 | 80.00 |
| 8:2 | 0.021 | 0.031 | 0.031 | 0.028 | 0.041 | 0.031 | 0.036 | 71.42 |
| 10:0 | 0.015 | 0.017 | 0.016 | 0.004 | 0.017 | 0.012 | 0.011 | -26.67 |

DM= Dry Matter, GR= Glutinous Rice

**Table 3.** The qualitative activity of carboxymethyl cellulase (ratio of clear zone: colony diameters) of Trichoderma inoculum in various combination ratios of dry mud (DM) and glutinous rice (GR) as carrier medium during storage

| Ratio DM: GR | Storage Time (Weeks) | | | | | Average | Changes (%) |
|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 16 | | |
| 0:10 | 4.65 | 11.0 | 3.35 | 3.94 | 3.39 | 5.07 | -12 |
| 2:8 | 4.74 | 6.39 | 3.44 | 3.76 | 3.71 | 4.79 | 42 |
| 4:6 | 5.07 | 6.43 | 3.46 | 4.62 | 3.58 | 4.93 | 26 |
| 6:4 | 4.93 | 4.35 | 3.21 | 4.16 | 3.50 | 4.14 | -5 |
| 8:2 | 4.56 | 3.68 | 3.77 | 4.20 | 3.93 | 4.88 | 101 |
| 10:0 | 4.24 | 4.28 | 3.08 | 4.96 | 3.24 | 3.90 | -16 |

DM= Dry Matter, GR= Glutinous Rice

## CONCLUSION

The study concluded that the Trichoderma inoculum, using a combination of glutinous rice sludge as a carrier material, had a life force of 4.6, resulting in a glucose-releasing carboxymethyl cellulase (CMCase) activity of 0.020 µmol minute-1 after 16 weeks of storage. Although this study focused on the inoculum's shelf life and enzyme activity, the findings indicate that an inoculum with a 4:6 ratio of dried mud to glutinous rice can be used for forage fermentation. The combination of stable spore viability and consistent cellulase activity provides a strong basis for developing Trichoderma-based inoculum carriers in feed biotechnology to improve forage quality and animal nutrition.

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**Book:**

a. Alshelmani M, Abdalla E, Kaka U, Basit M. 2021. Advances in poultry nutrition research. In: Kumar Patra A, editor. Adv Poult Nutr Res. London (UK): IntechOpen; p. 19–32. DOI: 10.5772/intechopen.91547.
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**Thesis:**

Mwasame DB. 2020. Analysis of the socio-economic contribution of donkey ownership and use to houshold livelihoods in Kiambu country, Kenya (Thesis). Nairobi (KE). University of Nairobi

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**Institution:**

a. [PSA] Philippine Statistics Authority. 2016. Dairy Industry Performance Report, January – December 2015. Quezon City (Philiphine): Philippine Statistics Authority. P. 1-11
b. [FAO] Food and Agriculture Organization. 2021. Gateway to dairy production and products. Food Agric Organ United Nations. [accessed August 10, 2021]. https://www.fao.org/dairy-production-products/production/feed-resources/en/.

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