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(Short Communication)

Comparative Study of Dehydration Methods in Plastination of Piglet and Kid Cadavers as Anatomy Specimens

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Abstract

Background: In the field of Veterinary anatomy in the Philippines, the traditional method of using embalmed specimens in teaching anatomy is still widely used, as plastination requires intensive labor, high costs, and a lengthy process. Given current research exploring alternative plastination techniques that use locally available resources, this study compared acetone and ethyl alcohol as dehydration solutions for room-temperature and passive plastination. **Methodology:** Using the modified Elnady technique of plastination, one (1) piglet and one (1) kid were subjected to acetone as a dehydration solution (Treatment 1), while one (1) piglet and one (1) kid were subjected to ethyl alcohol dehydration (Treatment 2). The plastination procedure lasted for approximately 25 weeks. The weight and color of the specimens were recorded pre- and post-plastination and were analyzed descriptively. **Results:** Preliminary results show that there is a 29.21% (Kid) and 23.81% (Piglet) decrease in the weight of specimens dehydrated with acetone (Treatment 1), while 35.19% (Kid) and 33.85% (Piglet) decrease in the weight of specimens dehydrated with ethyl alcohol series. The color of the organs was slightly darker after the impregnation, but lightened after the curing process. Although a significant weight reduction was noted, the morphological features of the specimens were well-preserved and identifiable; also, the specimens exhibited flexibility, firmness, no odor, and a natural appearance, with some color variations observed between the two dehydration methods. **Conclusion:** The preliminary results suggest that ethyl alcohol can serve as an alternative dehydration solution, yielding preserved specimens that can serve as useful models as a supplement to dissection studies.

Keywords

Alternative plastination, Ethyl alcohol dehydration, Room-temperature plastination, Elnady technique

1. Introduction

Anatomy is an essential course in veterinary medicine, the backbone of veterinary medical education [1]. The dissection of animal cadavers is commonly studied in this subject. Dissection is a crucial learning experience that enhances manual dexterity, complements theoretical knowledge with practical application, highlights individual anatomical differences, fosters collaboration, and encourages personal reflection [2].

The coronavirus disease 2019 (COVID-19) has had a profound impact on anatomy education [3]. During the pandemic, students had no access to cadavers, which have been the primary way to learn anatomy since the 17th century. The outbreak prompted anatomy educators to revisit all possible teaching methods to develop innovations. A research study proposed using various techniques for anatomy education: living and radiological anatomy teaching and computer-

based learning, including virtual reality (VR), augmented reality (AR), and three-dimensional printing (3DP) digital models [4]. Plastinated specimens have been used to expand the quality of teaching and learning anatomy [5,6]. Further, many studies have concluded that students are motivated and interested in using such technologies as augmented reality (AR) and virtual reality (VR) [7,8]. Despite the availability of digital models and computer-based learning, not all students can access such resources, while plastinated specimens may offer these students an alternative way to study structures without the need to carry embalmed specimens for independent study.

Plastination is a technique developed for the preservation of biological specimens as dry and odorless, which can facilitate 'out of the dissection hall' teaching and overcome the disadvantage of formalin-fixed specimens, which are wet and irritating to the eyes and the airways. It has four steps: fixation, dehydration, forceful impregnation, and tissue curing. Compared to organs preserved in formalin, plastinated organs exhibit superior morphology and structure preservation, enabling the demonstration of intricate anatomical structures. Although not a replacement for traditional dissections, plastination provides an additional learning tool for long-term preservation and for teaching complex anatomy [9]. However, standard plastination is an expensive technique because of the equipment and chemical materials required; the materials and equipment can be difficult to obtain in some areas [10].

Hence, several studies have been conducted to develop a cost-effective, non-patented, less complicated plastination technique that utilizes locally available chemicals, and the process is performed at room temperature [10,11,13-18]. Exploration on the use of other dehydration solutions was also conducted by utilizing isopropyl alcohol instead of acetone for dehydration following the standard plastination technique [11-13]. These studies utilized isopropyl alcohol and absolute alcohol, while this current study explores the use of ethyl alcohol in increasing concentrations for dehydration. Modification of the impregnation solution was also conducted, like the use of xylene with silicone [14] for lightweight plastination and the use of alkyd resin [10]. Plastinates produced were lightweight specimens that are easy to carry and can easily be used for teaching, odorless, completely biosafe, robust, dry, flexible, durable, natural in color and texture, and can be used for a long time without decay [10,11,13-18].

Various modifications were also explored, like the use of room temperature plastination [15], the passive plastination method [16,17,18], and a combination of forced and passive impregnation [15,19] during the embedding process. The Elnady Technique (Elnady, 2016) combines the utilization of room temperature and passive plastination and uses locally inexpensive, non-patented chemicals. The study resulted in specimens that are realistic, durable, dry, soft, flexible, have no odor, and maintain good coloration. This research paved the way for developing countries with limited resources to create plastinated specimens that are easily developed using local resources and processed at room temperature.

Plastination in veterinary medicine in the Philippines is limited due to its intensive labor, high cost, and long duration of the process when compared with the utilization of traditional preservation methods. Therefore, the widespread use of the traditional method of embalming specimens in anatomy teaching persists. However, a multitude of studies have been undertaken to assess the risks associated with the use of formaldehyde as a fixative and individuals' exposure levels in laboratory settings. Belmonte *et al.* (2012) conducted a study that found that formaldehyde concentrations in laboratories exceeded the 0.75 ppm threshold, with a general trend of increasing over time. Health risks to laboratory personnel and students may arise when formaldehyde concentrations in the air exceed the established threshold [20]. This limits the appreciation of students for animal specimens during dissection due to the unpleasant and detrimental effects of formalin exposure on students. Therefore, this research was conducted to compare and evaluate the use of room temperature plastination with the use of acetone and increasing ethyl alcohol concentration for dehydration in the plastination of whole body plastinates of kids and piglets. This whole-body plastinate can offer relational studies among organs relative to their position in the body.

2. Methodology

Specimens

Specimens used in the study were preserved specimens. The two piglets, approximately three months of age, died naturally, and the two kids were stillborn from a doe that died from an accident. The specimens were collected and fixed with 10% formalin solution and displayed as neonate specimens at the anatomy laboratory. The piglets were fixed for eight months, while the kids were fixed for 26 months before they were subjected to washing for 72 hours (24 hours in running water and 48 hours soaked in tap water). After washing, the specimens were pre-dissected. The left and right lateral sides were dissected, and the limbs were separated to expose the thoracic and abdominal cavities, allowing exposure of the visceral organs of the piglets in Treatments 1 and 2, respectively. While a transverse incision between the thoracic and abdominal regions of the kids in Treatments 1 and 2 was conducted to expose the visceral organs. The purpose of the transection is to prepare the specimens in their pre-dissected form before subjecting them to the plastination procedure. After which, the specimens were weighed using a digital weighing scale.

Plastination Procedure

The plastination procedure was modified from the Elnady technique (Elnady, 2016). The Dye injection, muscle dissection, and bone drilling were not followed in the procedure. The dehydration protocol was modified by utilizing an increasing ethyl alcohol concentration as a dehydration solution in comparison with the standard acetone solution for dehydration. Since the specimens used in this preliminary study were fixed and stored over different time periods, the plastination procedure timeframe excluded the fixation time of the specimens used. Both the dehydration and impregnation procedure was conducted at room temperature and a passive process. Purposive distribution of the piglets and kids into two treatment groups was conducted before subjecting the specimens to the dehydration process.

Dehydration

The dehydration process was performed at room temperature. Treatment 1 (one piglet and one kid) specimens were submerged in three changes of a 100% acetone bath for one month each bath. In Treatment 2, the specimens (one piglet and one kid) were submerged in increasing concentrations of ethyl alcohol (80% and 90%) for one month each, with two changes to absolute ethyl alcohol occurring every two weeks during the third month. The entire dehydration process lasted for three months. Although Elnady (2016) dehydrated the specimens for a duration of three weeks, the researcher opted to prolong the duration of dehydration since the specimens were whole-body preparations, allowing penetration of the solution in deeper parts of tissues. The concentrations of acetone and ethyl alcohol used were measured using a hydrometer. The concentration of acetone and ethyl alcohol is re-measured before changing it to a new solution. When the concentration remains at 98-99%, the specimens are considered dehydrated, which was achieved after three changes in acetone and four changes in ethyl alcohol. This whole process of dehydrating the specimens was done in stainless steel airtight containers. The piglet and the kid were placed in the same container with 22 liters of acetone and 20 liters of ethyl alcohol in each change.

Glycerin Impregnation

The specimens were removed from the dehydration solutions and were allowed to drain excess acetone/alcohol in a draining tray. After draining excess acetone/alcohol, the specimens from Treatment 1 and Treatment 2 were submerged in glycerin in a plastic container with a lid. The volume ratio was five times the size of the specimen [16], and the total volume used for Treatment 1 was 22 liters, and 20 liters for Treatment 2. Passive impregnation allows glycerin to penetrate the specimen slowly, replacing acetone and ethyl alcohol in the specimens. The impregnation process was done for 12 weeks on both treatment groups. After impregnation, the specimens from Treatment 1 and 2 were removed from the glycerin bath and were drained for five days in a draining rack.

Curing with Corn Starch

Once the impregnation was completed, the specimens were cleaned of excess glycerin with adsorbent paper. After which, specimens were completely covered with cornstarch powder in plastic containers with a cover for 10 days. During

the curing process, the specimens were turned once daily. The cornstarch was replaced when it was saturated with glycerin, as shown by the clumping on most of the specimens' surfaces. Two changes of cornstarch were done during the 10-day curing process. The specimens were taken out of the container, and cornstarch residues were removed with the use of a paintbrush.

Data Collection and Analysis

The weight of specimens after washing was obtained as pre-plastination weight and was then measured after curing for its post-plastination weight using a digital weighing scale. Data were presented in frequencies and percentages and analyzed descriptively. The percentage reduction can be calculated using the formula:

$$\%\ reduction = \frac{Pre\text{-}plastination - Post\text{-}plastionation}{Pre\ \ plastination} x100$$

The researcher noted data on color, texture, flexibility, and morphological changes through keen observations and analyzed them descriptively. Flexibility observation was based on the ability of the researcher to be able to manipulate the organs or parts of the body with ease, and the organs being able to return to their normal form after manipulation. Morphological changes were also noted based on the pre-plastination features of the specimen. The shrinkage of specimens was based on a reduction in their weight after plastination.

3. Results and Discussion

A comparative dehydration study on piglet and kid cadavers as platinates through a room temperature and passive process was conducted. Preliminary results of the study show that the weight of kids as specimens after plastination shows that there is a 29.21% decrease in the weight of the kid dehydrated with acetone at room temperature and a 35.19% decrease in the weight of the kid dehydrated with increasing concentration of ethyl alcohol post-plastination. Similarly, there is a marked decrease of 23.81% and a 33.85% in the weight of piglets subjected to the room temperature acetone and ethyl alcohol post-plastination, as shown in Table 1. Note that ethyl alcohol dehydration significantly reduced weight compared to acetone. Similar results were also observed by Brown *et al.* (2002), where the average shrinkage of room temperature acetone-dehydrated specimens was 20.2%, while for room temperature methanol-dehydrated specimens, it was 22.6% [21]. Furthermore, specimens in Treatment were 5.98% more dehydrated (kid) and 10.04% more dehydrated (piglet) when compared to specimens in Treatment 1. This can be attributed to the fact that neonates have more body water, and since the piglets were approximately three months of age, their significant growth also reduced their water composition to 90%. According to Cherian (2019), water makes up one-half to two-thirds of the body mass of adult animals and more than 90% of the body mass of newborn animals [22].

Table 1. Pre- and post- plastination weight (g) of kids and piglets subjected to two different dehydration solutions.

Treatment	Pre-Plastination Weight	Post-Plastination Weight	Percent Difference
Kids Cadaver			
Treatment 1	825	584	29.21%
Treatment 2	1089	753	35.19%
Piglets Cadaver			
Treatment 1	3578	2726	23.81%
Treatment 2	2839	1878	33.85%

Note: Treatment 1: acetone dehydration; Treatment 2: ascending ethyl alcohol dehydration

In both treatment groups, plastination did not change the specimens' color. For the color changes, the skin of the kid specimen in Treatment 1 had a light brown color post-dehydration, then became slightly darker post-impregnation; however, the color of the skin became lighter post-curing (Fig. 1, A1-3), while no significant change in color was noted in specimens in Treatment 2 (Fig. 1, B1-3). The specimens in both treatment groups were flexible, and signs of shrinkage were evident as shown by the grooves of the thoracic cage post-curing (Fig. 1 A3, B3), but the morphological conformation of the animal was not compromised and remained intact (Fig. 1 A4, B4). The eyes had obviously shrunk; this was corrected by the researcher by infiltration of the eyeball with silicon sealant to fill in the space where the aqueous and vitreous humor are originally located to return the shape of the eyeball using a syringe.

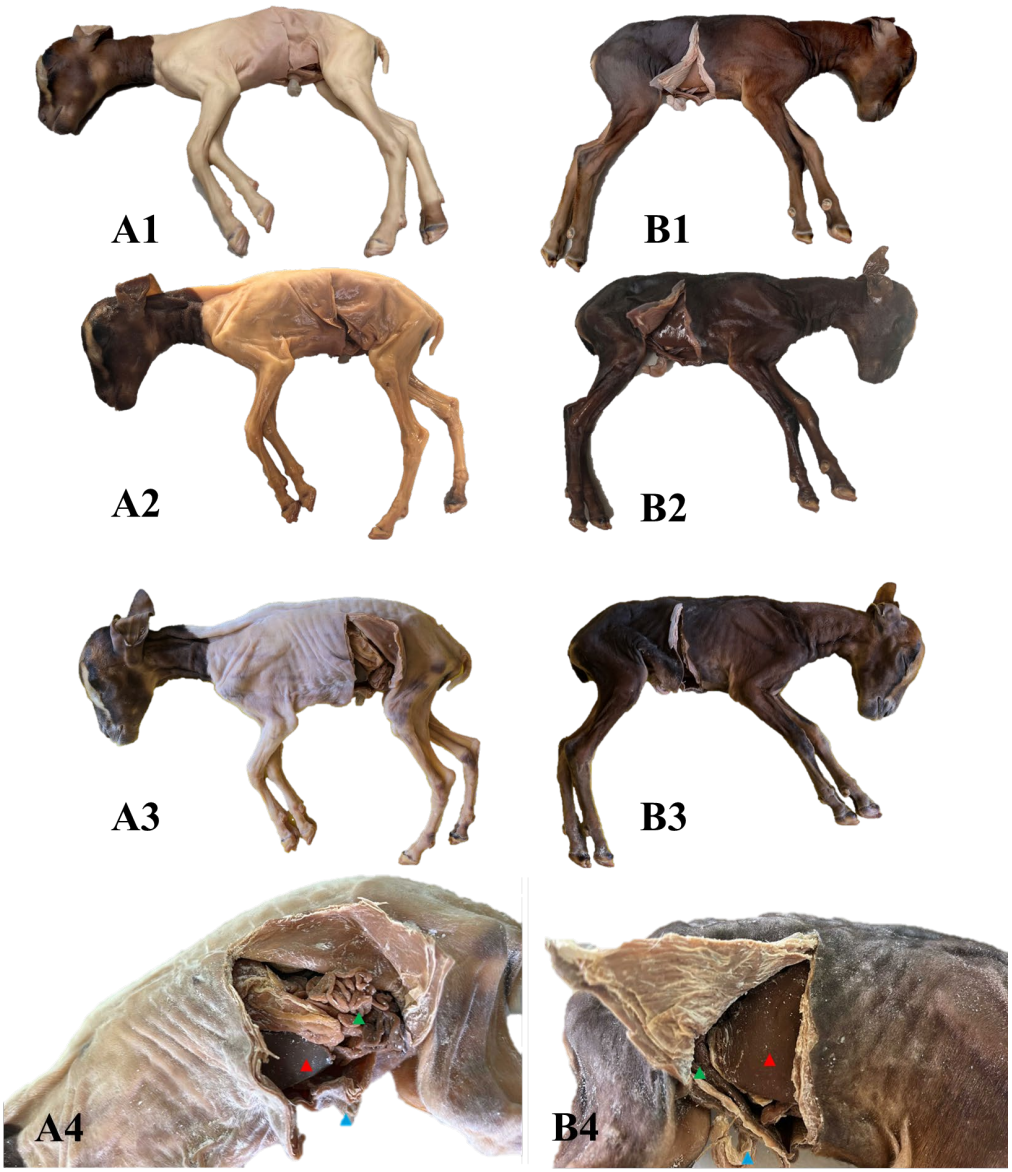

Figure 1. Photographs of kid plastinates showing the differences in color between treatment groups (A1-4: Treatment 1, B1-4: Treatment 2) post-dehydration (A1, B1), post-impregnation (A2, B2), and post-curing (A3, B3). Enlarged photograph of the visceral organs (A4, B4). In arrowhead: liver (red), jejunum (green), and umbilical cord (blue).

During the post-dehydration, there were no significant color changes noted in both specimens (Fig. 2 A1, B1). The muscles and visceral organs appear light to dark brown in color, while the adipose tissues appear white. Yellowish discoloration can be seen on the body of the piglet in Treatment 1 after it was subjected to impregnation. Furthermore, the color of the visceral organs varies from dark brown, like the spleen, to light brown in muscles and lungs. The visceral organs and muscles of the specimen in Treatment 2 exhibit a deeper brown color compared to those in Treatment 1. The lungs of the piglet in Treatment 2 appear darker when compared to the lungs of the piglet in Treatment 1 (Fig. 2 A2, B2; Fig. 3B). However, the color of organs in both treatment groups lightens post-curing with cornstarch (Fig. 2 A3, B3; Fig. 3B). It can be noted that the liver and diaphragm appeared dark brown after the impregnation process and changed to a lighter brown color in both treatment groups post-curing. The organs were firm to the touch and had no unpleasant odor from the plastinated specimens, even after 4 months post-plastination. This result is similar to that of Srisuwatanasagul *et al.* (2010), where dehydration by acetone gives a more natural color with no shrinkage of the heart specimens, while dehydration by ethyl alcohol gives a darker color and more shrinkage [13].

Shrinkage starts during the dehydration process; hence, it is an important consideration during plastination studies. Although a marked shrinkage, as shown by the marked reduction of weight of specimens post-plastination, was observed, the morphological features of the specimens showed no signs of degradation, and

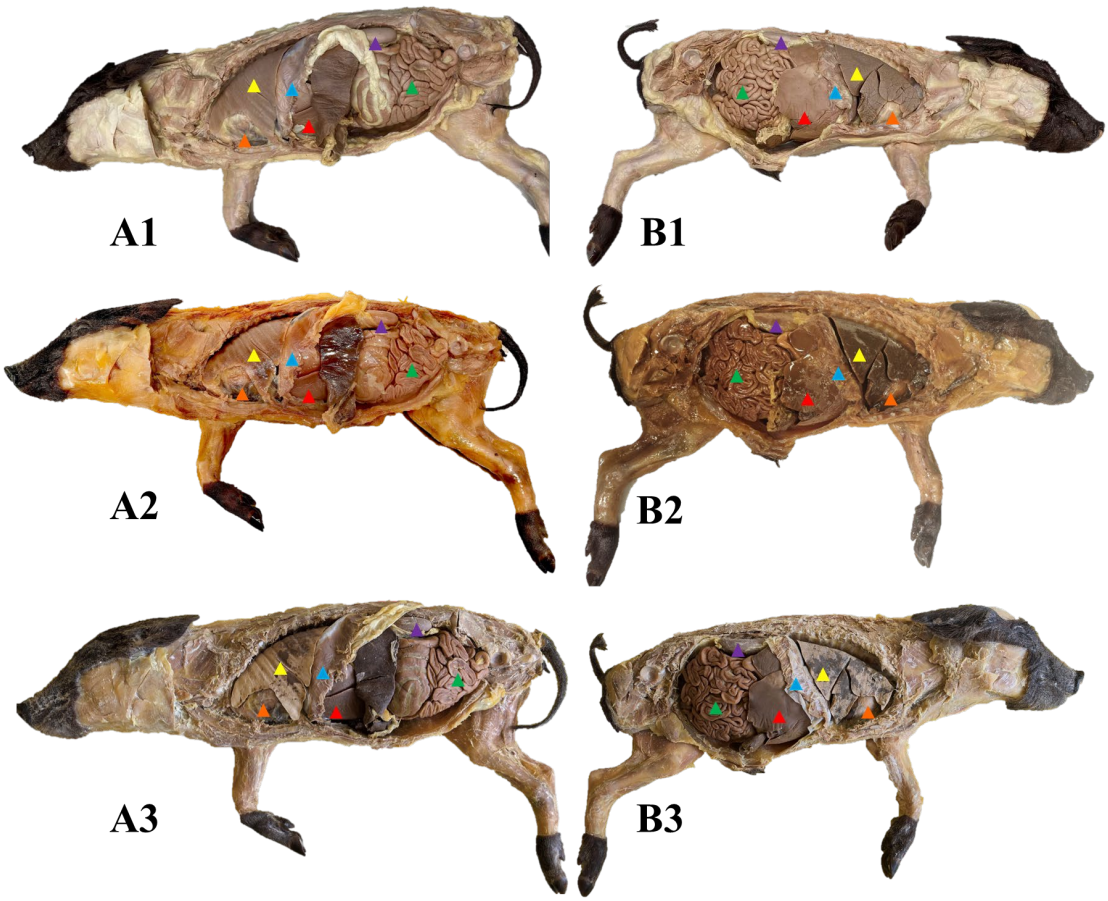

Figure 2. Photographs of piglet plastinates showing the comparison of specimen color between treatment groups (A-Treatment 1, B-Treatment 2) post-dehydration, post-impregnation, and post-curing with corn starch. In arrowhead: lungs (yellow), diaphragm (blue), liver (red), jejunum (green), kidney (purple), and heart (orange).

organs remained discernible, were dry, and had no odor even after 4 months of post-plastination. Similar results were observed by various studies utilizing locally available resources and the room plastination technique, where organ plastinates are odorless, completely biosafe, robust, dry, flexible, durable, natural in color and texture, can be used for a long time without decay, are lightweight specimens that are easy to carry, can easily be used for teaching, and demand significantly less maintenance than conventional cadavers. [10-19,26]

Currently, most anatomical specimens are preserved and stored in formalin solution. Although these formalin-immersed specimens are cost-efficient and can be maintained for long-term storage, they are typically stored in bulk containers or jars, which are difficult to handle in practical anatomy teaching courses [24] and carry a higher risk of exposure to formalin fumes, which can cause conjunctival and respiratory irritation. [23] Formalin-immersed specimens limit students to a hands-on learning experience, a valuable attribute of using plastinates as a learning resource. [20,23] Students' and professors' exposure to health hazards is decreased by the specimens' durability, odorlessness, and being free from dangerous substances. [16,25]

Since its introduction, this technique has gained considerable recognition in both medical and veterinary institutions for offering highly realistic representations of anatomical structures. [25,27] Thus far, Plastination has proven to be an almost ideal technique for long-term preservation of biological tissues, which can serve as specimens for practicing surgical techniques. [28] Plastination can provide a supplementary method to demonstrate anatomical differences and is an ideal method for long-term preservation of the most valuable preparations. In addition, plastinates are essential to complement the traditional dissection courses and contribute to a better preparation of postgraduates and clinicians [24].

5. Conclusions

In conclusion, this study demonstrates the effectiveness of room temperature plastination, comparing acetone and ethyl alcohol as dehydration solutions for preserving piglet and kid specimens. Both acetone and ethyl alcohol dehydration methods resulted in remarkable weight reduction, but the morphological features of the specimens were well-preserved. The plastinated specimens exhibited flexibility, firmness, no odor, and a natural appearance, with some color variations observed between the two dehydration methods. Moreover, ethyl alcohol can also be used as an alternative dehydration solution in place of acetone. Especially, acetone is classified as a controlled chemical, which makes it difficult to purchase. The results suggest that this alternative plastination method can be a valuable method for long-term preservation of anatomical specimens, could provide a hands-on learning experience for students, and reduce exposure to formalin. The preserved specimens can serve as potentially useful models for comparative anatomy studies, supplementing traditional dissection methods. Overall, this study highlights the potential of plastination as a reliable and effective technique for preserving biological specimens.

Author Contributions

Conceptualization, J.D.C.; Methodology, J.D.C.; Investigation, J.D.C.; Writing – Original Draft, J.D.C.; Writing – Review & Editing, J.D.C.; Funding Acquisition, J.D.C.; Resources, J.D.C.; Supervision, J.D.C.

Ethics Approval and Consent to Participate

Not applicable

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Conflict of Interest

The author declares no conflict of interest.

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(Original Research)

Prevalence of Gastrointestinal Parasites in Cattle: Influence of Age in Lamongan, East Java, Indonesia

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Abstract

Background: Cattle play a crucial role in Indonesia's agricultural and economic sectors; however, gastrointestinal parasite infections remain a significant constraint on their productivity. This study aimed to determine the prevalence, diversity, and infection patterns of gastrointestinal parasites in cattle in Lamongan Regency, East Java. **Methods:** A total of 120 fecal samples were collected from smallholder farms and examined using standard flotation and McMaster techniques. **Results:** The overall prevalence of gastrointestinal parasites was 65% (78/120). Identified helminths included *Oesophagostomum* sp. (22.5%), *Strongylus* sp. (16.66%), *Trichuris* sp. (10.83%), *Moniezia* sp. (10%), *Toxocara vitulorum* (9.16%), *Haemonchus* sp. (5.83%), and *Capillaria* sp. (4.16%). The only protozoan detected was *Eimeria* sp., which had the highest prevalence (30%). Males exhibited slightly higher infection rates (33.33%) than females (31.66%), and the highest prevalence was observed in calves aged less than 6 months (45.83%). Single infections (79.49%) were more common than mixed infections (20.51%). Although the prevalence was high, most infections were of mild intensity (1–500 eggs per gram of feces). **Conclusion:** These findings underscore the importance of routine parasitological monitoring and integrated parasite control strategies, particularly for young cattle and farms with poor management practices, to reduce productivity losses and improve overall herd health.

Keywords

Cattle, Disease, Gastrointestinal parasites, Parasitic infection, Prevalence

1. Introduction

Fulfilling the dietary protein needs, cattle play a vital role in Indonesia's agricultural system, serving as an essential source of food, income, and rural livelihood [1]. The livestock sector significantly contributes to national food security, community welfare, and employment, while addressing the increasing demand for animal protein driven by population growth and urbanization [2,3]. In Indonesia, approximately 90% of beef production originates from smallholder farms, with the remaining 10% supplied by private enterprises and government-supported programs [4]. Despite its importance, the sustainability of

the cattle industry faces numerous challenges, including fluctuating feed availability, limited access to modern farming infrastructure, and the burden of infectious diseases, particularly gastrointestinal parasitic infections [5,6].

Gastrointestinal parasitic infections are a significant constraint in tropical livestock production systems, partly due to the predominance of extensive and free-range farming practices. In such systems, cattle graze in open pastures where they are frequently exposed to contaminated feed, water, and grazing areas, increasing their risk of infection [2,4]. These infections cause considerable economic losses, including reduced live weight gains, which in turn lower the market value of livestock, along with decreased milk yield, increased veterinary costs, and, in severe cases, animal mortality [7]. However, accurate and up-to-date epidemiological data on gastrointestinal parasitic infections, especially in Indonesia, remain scarce. In regions such as East Java, where cattle farming is widespread, little is known about how parasitic infections correlate with husbandry practices, environmental conditions, and host factors such as age and sex [4]. This lack of data hampers the implementation of evidence-based strategies for parasite prevention, control, and treatment.

In Indonesia, common gastrointestinal parasites include protozoa and helminths. Protozoan parasites, such as *Eimeria* spp., are highly pathogenic and often resistant to standard treatments [8,9]. Helminths, including strongyles (*Haemonchus*, *Oesophagostomum*, *Trichostrongylus*) and *Toxocara vitulorum*, are well-adapted to tropical climates and may cause both localized gastrointestinal damage and systemic effects, such as anemia and immune suppression [10]. Animals raised under traditional and semi-intensive systems are particularly vulnerable due to poor sanitation, irregular deworming programs, and limited access to veterinary services [11]. While light infections often go unnoticed, moderate to heavy infestations can result in diarrhea, weight loss, stunted growth, reduced fertility, and even mortality [12,13].

The economic implications of gastrointestinal parasitic infections are considerable. In Indonesia, gastrointestinal nematode infections alone are estimated to cost farmers over four billion rupiah annually in lost productivity [14]. Globally, the burden is even more substantial, with countries like Mexico reporting annual losses of USD 1.4 billion due to parasitic diseases [15], Brazil estimating losses of USD 65.5 per infected cow [16], and a worldwide loss of USD 400 million attributed to coccidiosis caused by *Eimeria* species [9]. Contributing risk factors include infrequent manure removal, poor drainage systems, high humidity, overcrowded conditions, and insufficient sunlight exposure, all of which create favorable environments for parasite development and transmission [17–20]. These conditions align with the classical epidemiological triad of host, pathogen, and environment, emphasizing the complexity of parasite-host-environment interactions in cattle farming [21,22].

Gastrointestinal helminth infections continue to be a persistent concern in cattle production, with varying prevalence influenced by factors such as animal age, environmental hygiene, and management practices [5]. A previous study in Laren District reported a 37% prevalence of gastrointestinal helminth infections in beef cattle, identifying seven nematode species: *Oesophagostomum* sp., *Mecistocirrus* sp., *Bunostomum* sp., *Trichostrongylus* sp., *Cooperia* sp., and *Trichuris* sp., with the highest infection rates observed in cattle aged 7–24 months and no significant differences between Peranakan Ongole and Simmental breeds [23]. In contrast, another study conducted in Tikung District found a substantially lower prevalence, with only 6% of samples testing positive for nematode eggs and 2% for cestode (*Taenia saginata*) eggs, attributed to better livestock management and higher standards of hygiene [24]. These findings emphasize the need for updated, region-specific data on the prevalence and risk factors of gastrointestinal parasitic infections in Indonesia. Therefore, this study was designed to determine the prevalence and intensity of gastrointestinal parasite infections in cattle in Lamongan Regency, East Java, and to explore associations with host age, sex, and farm management practices. The results are expected to inform evidence-based control strategies aimed at reducing parasitic burdens, enhancing cattle health, and improving overall livestock productivity.

2. Materials and Methods

2.1 Study Area

The study was conducted in Lamongan Regency, East Java, Indonesia (Fig. 1), on a small-scale cattle farm, a region with a tropical climate and extensive cattle farming. The study period was from October to November 2023, coinciding with the onset of the rainy season. Laboratory examinations were performed at the Protozoology Laboratory, Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga.

the data, as well as to anticipate possible sample loss or laboratory errors. Each sample was obtained from a different animal and collected directly from the rectum using disposable anal gloves. Samples were stored in labeled plastic containers containing 10% formalin for preservation and transported in a refrigerated container to the laboratory.

Sampling design considered three age groups namely: Group 1: 40 samples from cattle aged 0–1 year (20 males, 20 females), Group 2: 40 samples from cattle aged 1–2 years (20 males, 20 females), and Group 3: 40 samples from cattle older than 2 years (20 males, 20 females)

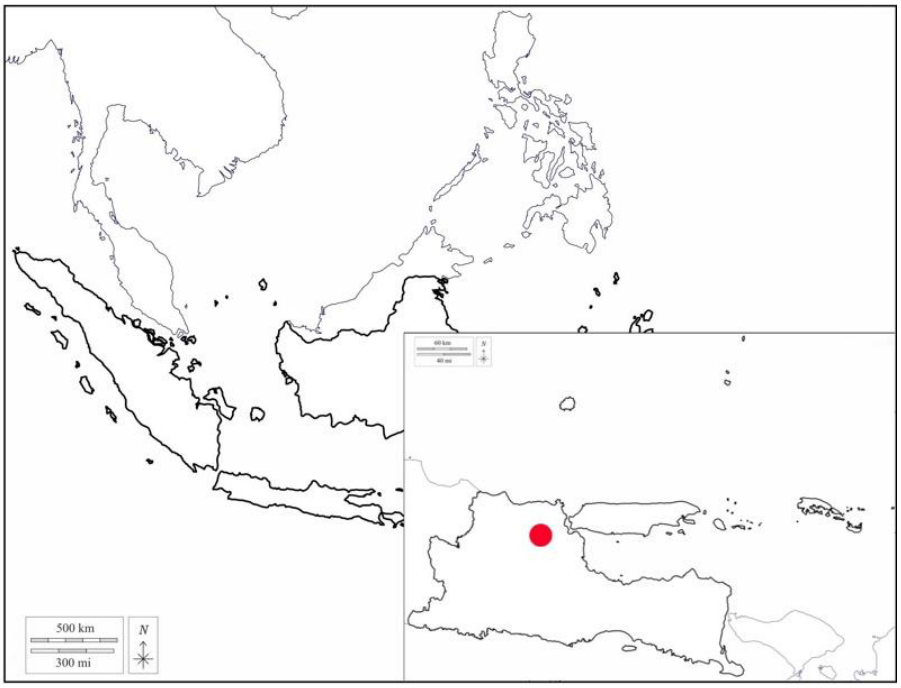

Figure 1. Lamongan Regency.

2. 2 Samples Collection

The study protocol was reviewed and approved by the Ethics Committee of the Faculty of Veterinary Medicine, Airlangga University (Ethical Clearance Certificate No. 1.KEH.167.12.2022) before the commencement of the research. A total of 120 fresh fecal samples were collected from smallholder cattle farms in Lamongan Regency. Although the minimum sample size calculation indicated only 100 samples, additional samples were collected to increase the robustness and representativeness of

At the time of sampling, all cattle appeared clinically healthy. Sample size was determined using the following formula [23]:

$$n = \frac{4P(1-P)}{L^2} = \frac{4(0.5)(1-0.5)}{(0.1)^2} = 100$$

Explanation:

n = Required sample size
P = Estimated prevalence (50%)
L = Accepted error level (10%)

2.3 Parasitological Examination

2.3.1 Flotation Method

Fecal samples were first examined using the simple flotation technique with saturated sugar solution. Approximately two grams of feces were placed in a centrifuge tube, mixed with distilled water, and centrifuged at 1500 rpm for five minutes. After discarding the supernatant, saturated sugar solution was added to fill three-fourths of the tube and centrifuged again. The solution was added until a convex meniscus formed, and a cover slip was placed on top. After five minutes, the cover slip was transferred to a glass slide and examined microscopically under 400× magnification. Parasites were identified morphologically based on published identification keys [24,25].

2.3.2 McMaster Method

Samples positive for gastrointestinal parasites were quantified using the McMaster technique to estimate eggs or oocysts per gram of feces (EPG or OPG). An amount of approximately 2–3 g of fecal material was homogenized by vortex with 58 ml of saturated sugar flotation solution. The suspension was then filtered, and the filtrate was used to fill the McMaster counting chamber. The chamber was examined under 100× magnification, and only parasite eggs or oocysts within the grids were counted [26–28]. The final count was calculated using the formula [29]:

$$OPG = Number\ of\ oocysts\ counted\ x\ 100$$

Explanation:

OPG = oocysts per gram of feces
The multiplication factor of 100 is derived from the volume ratio and dilution used in the McMaster method: (0.3 ml counted × 60 ml total volume) ÷ 2 g feces = 100

2.4 Data on Host and Farm Management

Data on cattle age, sex, and farm management practices, including housing, feeding, and sanitation, were collected through direct farmer interviews and field observations. This data was used to assess potential risk factors related to parasite infection.

2.5 Data Analysis

Prevalence was calculated as the proportion of infected animals out of the total sample size. Descriptive statistics were used to summarize data based on age group, sex, and farm management practices. No inferential statistical tests were performed due to the descriptive design of the study.

3. Results

3.1 Parasite Identification and Prevalence

Out of 120 fecal samples examined, 78 (65%) tested positive for gastrointestinal parasites. The identified parasites comprised nematodes (*Strongylus* sp., *Haemonchus* sp., *Toxocara vitulorum*, *Trichuris* sp., *Capillaria* sp., *Oesophagostomum* sp.), cestodes (*Moniezia* sp.), and protozoa (*Eimeria* sp.). Table 1 summarizes the identification of gastrointestinal parasites with an overall infection rate of 65% (78/120). Morphological identification of parasite eggs was conducted using light microscopy at 400× magnification, as illustrated in Figures 2 and 3. These figures provide a clear visualization of key morphological characteristics, including shape and size, which are critical for accurate parasite identification and diagnosis. Accurate morphological diagnosis is essential for implementing effective control strategies.

Table 1. Identity and prevalence of gastrointestinal parasites in cattle from Lamongan Regency, East Java, Indonesia (n = 120).

Type	Parasites	Percentage (%)
Helminth	*Capillaria* sp.	4.16
	Haemonchus sp.	5.83
	Moniezia sp.	10.00
	Oesophagostomum sp.	22.5
	Strongylus sp.	16.66
	Toxocara vitulorum	9.16
	Trichuris sp.	10.83
Protozoa	*Eimeria* sp.	30.00

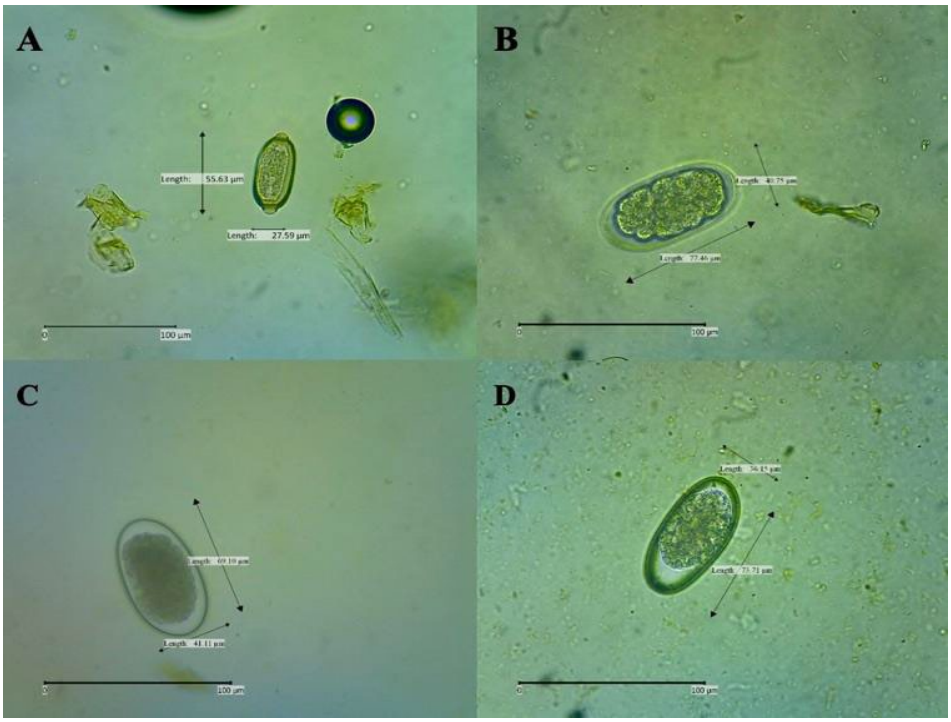

Figure 2. *Capillaria* sp. (A), *Haemonchus* sp. (B), *Oesophagostomum* sp. (C), *Strongylus* sp. (D), (400x magnification).

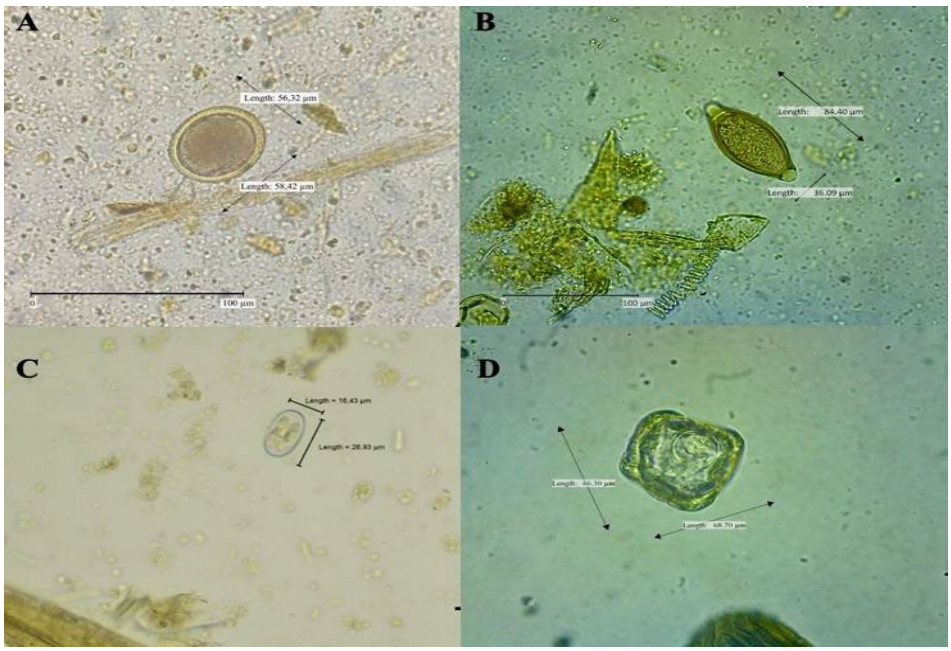

Figure 3. *Toxocara vitulorum* (A)*, Trichuris* sp. (B), *Eimeria* sp. (C), *Moniezia* sp. (D), (400x magnification).

Further analysis of parasite prevalence based on host factors, namely, sex and age, is presented in Tables 2 and 3. The data reveal a slightly higher prevalence in male cattle (33.33%) compared to females (31.66%). Regarding age, the highest infection rate was found in the youngest group (0–1 year) at 26.66%, followed by the 1–2 years group (25%), and the lowest prevalence was observed in cattle over two years old (13.33%). These findings indicate that younger cattle are more susceptible to parasitic infections, likely due to

Table 2. Prevalence of gastrointestinal parasites by age group of cattle in Lamongan Regency, East Java, Indonesia (n = 120).

Age	Prevalence (%) - middle
0-1 year (n= 40)	80.00
1-2 year (n= 40)	75.00
> 2 year (n= 40)	40.00

Note: The difference in prevalence among age groups was not statistically significant ($p > 0.05$).

Table 3. Single and multiple infections of gastrointestinal parasites in cattle in Lamongan Regency, East Java, Indonesia (n = 120).

Infection	Parasites	Number Infected	Percentage of Positive Samples (%)	Average TCPGT (EPG/OPG) ± SD
Single Infection (62/78; 79.49%)	*Capillaria* sp.	4	6.45	120.00 ± 38.73
	Haemonchus sp.	5	8.06	120.00 ± 38.73
	Moniezia sp.	5	8.06	216.00 ± 201.15
	Oesophagostomum sp.	11	17.74	138.00 ± 56.09
	Strongylus sp.	12	19.35	111.00 ± 50.13
	Toxocara vitulorum	6	9.67	197.00 ± 173.90
	Trichuris sp.	7	11.29	75.00 ± 36.74
	Eimeria sp.	12	19.35	275.00 ± 185.05
Multiple Infections (16/78; 20.51%)	*Toxocara vitulorum* *Oesophagostomum* sp. *Eimeria* sp.	2	1.67	153.00 ± 83.44
	Toxocara vitulorum *Strongylus* sp.	1	0.83	105.00 ± 36.06
	Oesophagostomum sp. *Trichuris* sp., *Eimeria* sp.	4	3.33	102.00 ± 41.55
	Haemonchus sp. *Moniezia* sp., *Eimeria* sp.	1	0.83	150.00 ± 70.71
	Oesophagostomum sp. *Moniezia* sp. *Eimeria* sp.	6	5.00	170.00 ± 67.82
	Strongylus sp. *Oesophagostomum* sp.	2	1.67	90.00 ± 84.85

underdeveloped immune systems and increased exposure to contaminated environments. Additionally, the study investigated the occurrence of single and multiple parasitic infections, as summarized in Table 4. The most common mixed infections involved *Oesophagostomum* sp., *Moniezia* sp., and *Eimeria* sp., underscoring the complexity of parasitic infestation patterns in the study area. Environmental and management factors contributing to parasite prevalence were also examined, including housing conditions, feeding

Table 4. Cattle farm management practices in Lamongan, East Java (n = 120).

Farm Management Practice Variables	Yes (n, %)	No (n, %)
Permanent housing system (cement floor, ventilation)	65 (54.2%)	55 (45.8%)
Daily cleaning of cattle housing	72 (60.0%)	48 (40.0%)
Functional drainage system in the pen	58 (48.3%)	62 (51.7%)
Clean water source (well/piped)	80 (66.7%)	40 (33.3%)
Routine provision of feed supplement (concentrate)	68 (56.7%)	52 (43.3%)
Deworming at least twice a year	44 (36.7%)	76 (63.3%)
Proper manure disposal	70 (58.3%)	50 (41.7%)
Isolation of sick cattle from healthy ones	40 (33.3%)	80 (66.7%)

practices, and hygiene standards. The findings emphasize the need for improved husbandry practices to reduce parasite transmission and mitigate the negative impacts of infection on cattle health and productivity. Overall, this study provides important epidemiological data on gastrointestinal parasites in cattle in Lamongan Regency, offering a basis for enhanced control strategies and further research.

The representative micrograph of the specimens identified in the stool samples in Figure 2 shows microscopic examination of cattle fecal samples at 400× magnification revealed the presence of various gastrointestinal nematode eggs with distinct morphological characteristics. *Capillaria* sp., eggs appeared as elongated ovals measuring 27.59–55.81 µm, with thick walls and prominent bipolar plugs resembling a cork-like structure. *Haemonchus* sp., eggs were oval-shaped (40.75–77.46 µm), possessing thin shells and multiple blastomeres, indicating an advanced embryonic stage. *Oesophagostomum* sp., eggs, ranging from 40.11–69.10 µm, were also oval and surrounded by a thin, single-layer membrane enclosing homogeneous internal contents. Meanwhile, *Strongylus* sp., eggs were elliptical (36.15–73.71 µm), with thin walls and well-organized embryonic cell clusters. These morphological features provide a reliable basis for microscopic identification of gastrointestinal parasite species in cattle.

Examination of cattle fecal samples at 400× magnification revealed a diverse range of gastrointestinal parasite eggs with distinct morphological features. *Toxocara vitulorum* eggs (Panel A) are nearly spherical, measuring 56.32–58.42 µm, with thick brownish walls and a characteristic albumin layer, and are commonly found in young calves. *Trichuris* sp., eggs (Panel B) are elongated ovals ranging from 36.09 to 88.40 µm, with thick shells and prominent bipolar plugs, serving as a distinctive trait of this genus. *Eimeria* sp., oocysts (Panel C) are round to oval, measuring 16.43–26.93 µm, with smooth, transparent walls and a color range from yellowish to colorless; these protozoa are the causative agents of coccidiosis in cattle. *Moniezia* sp., eggs (Panel D) are nearly square (66.30–68.70 µm), featuring a well-developed pyriform apparatus and a complex embryonic structure, which are characteristic of cestodes from this genus. Morphological characteristics such as shape, size, wall thickness, and internal structures are essential diagnostic criteria in differentiating parasite species. Accurate identification based on these microscopic features is crucial for developing targeted and effective parasite control strategies, thereby minimizing the health and productivity losses in cattle due to gastrointestinal parasitic infections.

Identification of gastrointestinal parasites in cattle from Lamongan Regency was carried out using the flotation and McMaster techniques. Table 1 presents only the results obtained from the flotation technique. The overall prevalence of gastrointestinal parasitic infections reached 65% (78/120), indicating a considerable burden in the sampled population. Within the helminth group, *Oesophagostomum* sp., showed the highest prevalence at 22.50% (27/120), followed by *Strongylus* sp., at 16.66% (20/120), *Trichuris* sp., at 10.83% (13/120), *Moniezia* sp., at 10.00% (12/120), *Toxocara vitulorum* at 9.16% (11/120), *Haemonchus* sp., at 5.83% (7/120), and *Capillaria* sp., at 4.16% (5/120). In the protozoan group, only *Eimeria* sp., was identified, but with a notably high prevalence of 30.00% (36/120), making it the most common gastrointestinal parasite found in this study. These findings highlight the persistent and widespread nature of gastrointestinal parasitism in cattle in the region, particularly infections caused by *Eimeria* sp., and *Oesophagostomum* sp., the high prevalence emphasizes the importance of regular monitoring and targeted antiparasitic control strategies to improve cattle health, enhance productivity, and minimize economic losses at the farm level.

The results of the analysis of gastrointestinal parasite prevalence based on sex are shown in Figure 4. Male cattle showed a slightly higher infection rate compared to females. Among the male cattle examined, 33.33% (40/120) tested positive for gastrointestinal parasites, while 16.66% (20/120) tested negative. In contrast, 31.66% (38/120) of the female cattle were positive, while 18.33% (22/120) tested negative. These findings suggest that sex-based differences, potentially related to physiological, hormonal, metabolic, and stress factors, may influence susceptibility to gastrointestinal parasitism in cattle.

The analysis of gastrointestinal parasite prevalence by age group, as shown in Table 2, reveals that the highest infection rate occurred in the youngest cattle (0–1 year), with 80% (32/40) testing positive. The 1–2 year age group exhibited

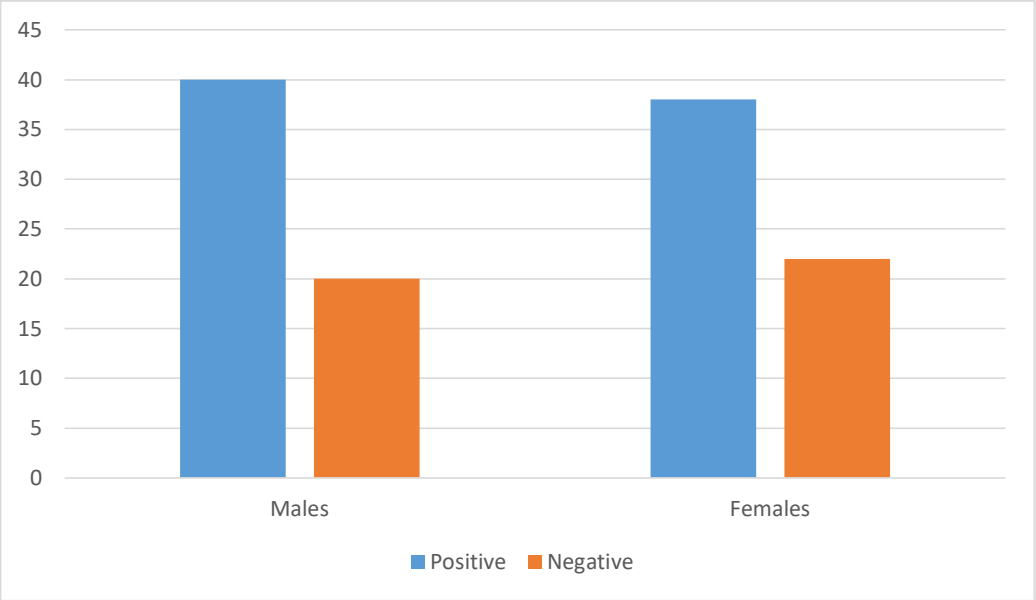

Figure 4. Prevalence of gastrointestinal parasites in cattle by sex in Lamongan Regency, East Java, Indonesia. Out of 60 males examined, 40 (66.7%) were positive and 20 (33.3%) were negative. Out of 60 females reviewed, 38 (63.3%) were positive and 22 (36.7%) were negative.

a slightly lower prevalence, with 75% (30/40) positive cases. In contrast, cattle over two years of age showed a much lower prevalence, with only 40% (16/40) infected. This decreasing trend with age may be attributed to the maturation of the immune system, which enhances the host's resistance to parasitic infections. Younger cattle are particularly vulnerable due to their underdeveloped immune responses, increased environmental exposure, and possibly suboptimal hygiene and nutrition. These findings highlight the importance of implementing targeted parasite control strategies, especially in younger cattle, to reduce infection rates and mitigate the negative effects on growth, health, and productivity.

The quantitative analysis of gastrointestinal parasitic infections using the Total Count Per Gram of Feces (TCPGT) method provided detailed insights into the infection intensity in cattle. TCPGT values, expressed as eggs per gram (EPG) for helminths and oocysts per gram (OPG) for protozoa, served as reliable indicators of parasitic load and potential health impacts. Among single infections (62/78; 79.49%), TCPGT values ranged from 75 ± 36.74 EPG for *Trichuris* sp., to 275 ± 185.05 OPG for *Eimeria* sp., The lowest count in *Trichuris* sp., reflects a relatively low egg output and likely reduced pathogenicity. In contrast, the highest value in *Eimeria* sp., indicates a considerable protozoal burden, which may predispose young cattle to subclinical or clinical coccidiosis. Other parasites demonstrated intermediate egg counts, including *Toxocara vitulorum* (197 ± 173.90 EPG), *Moniezia* sp., (216 ± 201.15 EPG), *Oesophagostomum* sp., (138 ± 56.09 EPG), *Haemonchus* sp., (120 ± 38.73 EPG), *Capillaria* sp., (120 ± 38.73 EPG), and *Strongylus* sp., (111 ± 50.13 EPG).

In mixed infections (16/78; 20.51%), average TCPGT values varied between 90 ± 84.85 and 170 ± 67.82 EPG/OPG, depending on the parasite combinations. The most frequent co-infections were *Oesophagostomum* sp., *Moniezia* sp., and *Eimeria* sp. (6/120; 5.00%), with a relatively high mean TCPGT of 170 ± 67.82, suggesting possible additive or synergistic effects. Other notable combinations included *Oesophagostomum* sp., *Trichuris* sp., and *Eimeria* sp., (3.33%; 102 ± 41.55), and *Toxocara vitulorum* with *Oesophagostomum* sp., and *Eimeria* sp. (1.67%; 153 ± 83.44). Less common cases were *Strongylus* sp., with *Oesophagostomum* sp., (1.67%; 90 ± 84.85), *Toxocara vitulorum* with *Strongylus* sp., (0.83%; 105 ± 36.06), and a unique triple infection involving *Haemonchus* sp., *Moniezia* sp., and *Eimeria* sp., (0.83%; 150 ± 70.71).

Overall, 62 of the 78 positive cases represented single infections, with *Strongylus* sp., and *Eimeria* sp., being the most prevalent (each 19.35% of positives; 12/62). These were followed by *Oesophagostomum* sp., (17.74%), *Trichuris* sp., (11.29%), *Toxocara vitulorum* (9.67%), *Moniezia* sp., (8.06%), *Haemonchus* sp., (8.06%), and *Capillaria* sp., (6.45%). Based on standard classification (mild: 1–500 EPG/OPG, moderate: 501–1,000, heavy: >1,000), all observed infections were categorized as mild, with TCPGT averages below 500. Statistical analysis (Chi-square) further revealed no significant association ($p > 0.05$) between infection intensity and either sex ($p = 0.157$) or age ($p = 0.199$). These findings suggest that although the parasitic burden in cattle was generally low, the occurrence of mixed infections and the notable contribution of protozoal parasites, such as *Eimeria* sp., may still pose risks to health and productivity, particularly in young or immunocompromised cattle.

3.2. Cattle management practices

Interviews with 120 cattle farmers in Lamongan revealed considerable variation in farm management practices Table 4. More than half of the farmers (54.2%) used permanent housing with cement floors and adequate ventilation, while 45.8% still relied on traditional housing. The majority (60.0%) reported cleaning cattle housing daily, whereas 40.0% admitted that cleaning was not performed regularly. Only 48.3% of farmers maintained a functional drainage system, with the remaining 51.7% experiencing drainage problems.

In terms of resources, 66.7% of farmers provided clean drinking water from wells or piped systems, and 56.7% regularly supplemented cattle with concentrates. However, compliance with internal parasite control was low, as only 36.7% administered deworming at least twice per year. Proper manure disposal was practiced by 58.3% of respondents, while 41.7% had not adopted appropriate waste management. The isolation of sick cattle from healthy ones was rarely implemented, with only 33.3% of farmers reporting this practice.

Overall, these findings indicate that while basic management practices, such as housing, sanitation, and the provision of clean water, were generally adopted, critical aspects, including regular deworming, drainage systems, and the isolation of diseased animals, remained weak. Such deficiencies may contribute to the persistence of gastrointestinal parasitic infections in cattle farms in Lamongan. Consistent with parasitological results, the study confirmed a relatively high prevalence of gastrointestinal parasites, predominantly *Eimeria* sp., and *Oesophagostomum* sp., although infection intensities were mostly mild based on TCPGT values. Younger cattle were more susceptible than older ones, although no statistically significant differences in infection intensity were observed between age groups or sexes. Taken together, the parasitological data and management findings suggest that inadequate husbandry practices and feeble parasite control measures may sustain infection risks. This highlights the need for integrated control strategies addressing both parasitological and management factors to improve cattle health and productivity.

4. Discussion

In the present study, the overall prevalence of gastrointestinal parasitic infections was 65% (78/120), indicating a considerable parasitic burden in the examined cattle population. Among helminths, *Oesophagostomum* sp., was the most prevalent species (22.5%, 27/120), followed by *Strongylus* sp., (16.66%, 20/120), *Trichuris* sp. (10.83%, 13/120), *Moniezia* sp., (10%, 12/120), *Toxocara vitulorum* (9.16%, 11/120), *Haemonchus* sp., (5.83%, 7/120), and *Capillaria* sp., (4.16%, 5/120). For protozoa, only *Eimeria* sp., was identified, showing a relatively high prevalence of 30% (36/120). The prevalence reported in this study is higher than previous reports from Tukung District, Lamongan (59%, 59/100) [30], Sugio District (43%, 43/100) [31], and Leces District, Probolinggo (38%, 38/100) [32], but slightly lower than the findings of Hamid in Central Java (65.93% of 455 cattle infected, with single infections reaching 79.33%) [33].

The Total Count Per Gram of Feces (TCPGT) values obtained provide a quantitative description of the infection intensity [9]. All average TCPGT values fell within the mild category (1–500 EPG/OPG), both in single and mixed infections [8,34]. Despite being classified as mild, recurrent infections, particularly with *Eimeria* sp., can still cause subclinical impacts, such as growth retardation, reduced feed efficiency, and increased susceptibility to other diseases, especially in calves

[30,35]. The highest TCPGT values were observed for *Eimeria* sp., indicating strong coccidial infection pressure in the study area. Meanwhile, nematode infections such as *T. vitulorum*, *Moniezia* sp., and *Oesophagostomum* sp., showed moderate egg counts, which, although not yet causing severe clinical disease, warrant attention to prevent environmental buildup. TCPGT is therefore a valuable tool for monitoring, evaluating treatment efficacy, and guiding risk-based control programs at the farm level.

These findings are consistent with previous reports from Java and other regions. Sari documented infections with *Oesophagostomum* sp., *Bunostomum* sp., *Mecistocirrus* sp., *Trichostrongylus* sp., *Trichuris* sp., and *Moniezia benedeni* in PO and Limousin cattle in Lamongan [30]. Similar results were noted by Paramitha in Surabaya (*T. vitulorum*, *M. digitatus*) [36], Firdayana in Makassar (*Oesophagostomum* sp., *Cooperia* sp.,) [37], and Antara *et al*. in Bali cattle (*Strongylus*-type nematodes, *Capillaria bovis*, *Strongyloides papillosus*) [38]. Age also played a key role: prevalence was higher in younger cattle (26.66% at 0–1 year; 25% at 1–2 years) and declined in cattle older than 2 years (13.33%). This aligns with Paramitha and Sari [36], as well as Khozin [12], who emphasized greater resistance in older cattle due to improved mucus secretion and immune response.

The high prevalence observed in Lamongan is strongly associated with traditional housing and management practices. Risk factors include high stocking density, poor ventilation, inadequate drainage, insufficient lighting, low-quality water sources, and limited pen cleaning, typically performed only once per day [39-41]. Such conditions favor the accumulation and spread of infective stages, while rainfall further disperses them across wider areas. Other determinants such as age, physiological status, immune competence, and population density also affect infection dynamics [42,43].

The farmer interviews conducted in this study further support the role of management practices as critical risk factors for gastrointestinal parasitism Table 4. Although most farmers adopted basic measures, such as daily cleaning and providing clean drinking water, compliance with key preventive practices remained low. Only 36.7% of farmers reported deworming cattle at least twice per year, while less than half had functional drainage systems, and only one-third practiced the isolation of sick animals. These deficiencies are consistent with previous studies highlighting that poor anthelmintic usage, inadequate drainage, and the absence of biosecurity measures contribute to the persistence of parasitic infections in smallholder cattle farms [20,44]. Strengthening farmer awareness and promoting integrated husbandry improvements, including routine deworming and proper waste and drainage management, are therefore essential to reduce parasite transmission and sustain cattle productivity.

Proper housing systems are essential to reduce parasite transmission. Well-designed and well-maintained housing minimizes exposure to contaminated soil, water, and bedding, while improving animal health and productivity [21,26]. However, infections can persist in intensive systems if hygiene is neglected. For example, Moniezia infections have been reported in housed cattle due to oribatid mites surviving in moist bedding materials, which act as intermediate hosts [36]. Therefore, housing hygiene should be integrated with comprehensive and strategically timed deworming programs. Anthelmintic administration before the grazing season and after peak exposure can interrupt parasite life cycles and reduce environmental contamination, providing a sustainable approach to parasite control [2].

Environmental factors such as temperature, humidity, rainfall, and pH critically influence parasite survival and transmission dynamics [45,46]. In this study, sampling was conducted during the rainy season, when humidity and moisture levels were high, conditions that generally favor the survival and development of gastrointestinal parasite eggs and larvae. High moisture combined with moderate pH supports the proliferation of infective stages, while cooler and wetter conditions can prolong their viability [13,34]. Conversely, extremely high temperatures under dry conditions can reduce parasite survival through accelerated metabolic exhaustion. Understanding these environmental determinants is therefore essential for epidemiological surveillance and helminth management [47-49].

Even low-level infections require timely interventions. Persistent gastrointestinal

parasites are best addressed through integrated control strategies that combine improved housing hygiene, environmental management (e.g., drainage improvement and rotational grazing), and strategic anthelmintic treatment tailored to local parasite species [50-52]. Administering anthelmintics before the grazing season and after peak exposure periods can break parasite life cycles, reduce reinfection risks, and support sustainable livestock productivity.

Finally, Chi-square analysis revealed statistically significant associations, confirming the influence of the investigated variables. These findings are consistent with Sari [36], who also reported significant associations between age and gastrointestinal helminth infections in PO and Simmental cross cattle. Future research should incorporate molecular diagnostic techniques (e.g., PCR, qRT-PCR, LAMP) to enhance species-level identification and evaluate the pathogenic potential of these parasites. Furthermore, integrating farmers' knowledge, attitudes, and practices into research will provide deeper insights into behavioral factors that influence parasite control and overall cattle productivity.

4. Conclusions

This study provides an updated overview of the prevalence of gastrointestinal parasites in cattle from Lamongan Regency, East Java, Indonesia, with an infection rate of 65% (78/120). The identified parasites included *Capillaria* sp., *Haemonchus* sp., *Moniezia* sp., *Oesophagostomum* sp., *Strongylus* sp., *Toxocara vitulorum*, *Eimeria* sp., and *Trichuris* sp., with infection intensity generally classified as mild (TCPGT 1–500). The most frequently detected species were *Strongylus* sp., and *Eimeria* sp., Age was found to be a significant factor influencing prevalence, with younger cattle (0–1 year) showing higher infection rates compared to older cattle (>2 years). These findings emphasize the need for more effective parasite control strategies, particularly in young cattle, to prevent negative impacts on health and productivity. Practical recommendations include improved housing sanitation, regular fecal examinations, and targeted anthelmintic administration. Future studies should employ molecular diagnostic tools to strengthen species-level identification and monitor potential anthelmintic resistance. Additionally, integrating farmers' knowledge, attitudes, and practices (KAP) into research will support more effective interpretation and intervention planning.

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(Original Research)

A Study on Neoplastic and Non-neoplastic Masses in Companion Animal Patients in Malang Raya: Histological Classification and Case Proportion

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Abstract

Background: Understanding the case proportion of neoplastic and non-neoplastic disease in companion animals could reveal the trends; novel subtypes architectures, differential diagnoses and disease prognoses based on histopathological features for clinical purposes and future epidemiological study. This study aimed to explore trends, patterns, histopathological features and potential paraneoplastic syndromes associated with tumors in companion animals. **Methods:** Archived neoplastic tissue samples were collected over five years from an animal hospital, clinics, and veterinary practitioners in Malang Raya, Indonesia and processed through routine histopathologic examination. **Results:** A minimum of 30 feline and 40 canine patients with confirmed neoplastic lesions were identified, with glandular tumors being the most frequently reported in both species. Twenty-four non-neoplastic superficial masses were also reported including subcutaneous abscess, granulomatous dermatitis, pseudomycetoma/deep fungal mycosis, and panniculitis. Different regions worldwide may show varying prevalence rates and distinct patterns in the most frequently encountered abnormal superficial masses. **Conclusions:** Understanding these trends and considering the potential differential diagnoses can assist diagnosticians in systematically ruling out less likely conditions. The significant/notable proportion of neoplastic cases in companion animals underscores the importance of further investigations into possible environmental carcinogens, which may also pose risks to human health.

Keywords: Tumor, Animal, Non-neoplastic, Prevalence, Malang Raya

1. Introduction

Tumors – including abnormal nodules, lumps, and masses – are etymologically defined as abnormal

enlargement of tissues, which refer to either neoplastic or non-neoplastic growth [1]. Various tumor types have been reported worldwide presenting diverse consequences to the hosts due to their complex architectural features. Tumor statistics in companion animals could help clinicians to see the prevalence, forecast the pattern of occurrence and prognoses, as well as discriminate the neoplastic and non-neoplastic masses in terms of diagnostic pathology. Moreover, establishing curated animal cancer registry, such as the one developed by Australian Animal Cancer Registry (https://www.acarcinom.org.au/), could provide valuable epidemiological data to help correlating the occurrence of specific neoplastic cases with potential internal and external risk factors in the future [2]. Laboratory of veterinary anatomic pathology in the Faculty of Veterinary Medicine Universitas Brawijaya has been operating and serving for histopathology examination since 2021 for the veterinary hospital and clinics around Malang Raya area, East Java Province, Indonesia. Curation and analysis of our database and archival tissues are intended to serve and provide updated information of various neoplastic and non-neoplastic cases in submitted animal specimens. This study aimed to report the various neoplastic and non-neoplastic cases diagnosed in companion animals (pets and other domesticated animals e.g. ornamental birds and reptiles, domesticated rodents) in Malang Raya, Indonesia by analyzing medical records and archival tissue samples from various animal health centers. Curation of these data might also be a seed to the regional cancer registry establishment by compiling associating risk factors for epidemiological study. By analyzing the architectural characteristics of these tumors, we aim to gain insights into their malignancy, metastatic potential and other histological features. Processing tissues for histopathology examination spends more time, which requires clinical doctors to take initial treatment to support patients' life. Exploring the trend of neoplastic and non-neoplastic masses occurrence will assist clinicians to strategize the initial therapy based on common tumor cases.

2. Materials and Methods

2.1 Ethical Approval

This study was performed under approval of the research ethics committee (document number No: 041-KEP-UB-2023).

2.2 Study Period and Location

This study was conducted from April 2023 to November 2024. All examinations and analyses were performed at the laboratory of veterinary anatomic pathology Universitas Brawijaya, Indonesia.

2.3 Medical Records and Archival Tissue Collection

Medical records and archived tissues were collected from animal health facilities in Malang Raya including an animal hospital (67), animal clinics-veterinary practitioners (25), and direct submission to the laboratory (87). Most of the collected tissues were from externally visible masses in superficial organs (e.g. skin, mammary glands). Medical records, either digital or printed, were analyzed based on the species and final diagnosis. All medical records with a final diagnosis containing the keywords 'mass', 'tumor', 'growth', 'lump', 'nodule', and 'swelling' were selected. For all records with aforementioned keywords but no final diagnosis available or submitted tissues with no decisive diagnosis would be included in the 'unknown' category. We grouped the data based on the most common species recorded: canine, feline, avian. Other species such as exotic reptiles and rodents were included in the 'others' group. Samples with archived tissues and/or histopathology report of the masses were included in the final dataset. New submissions of masses without any conclusive clinical diagnosis underwent routine histopathological examination (paraffin embedding, haematoxylin-eosin staining-reviewed by ABH).

2.4 Tumor Classification and Histopathological Description

Each tumor was classified using standard classification systems as provided by Meuten *et al.*, in 2020 [3], World Health Organization (WHO) classification of tumors, SCC classification as described by Muller *et al.* in 2018 [4] and surgical pathology of tumors in domestic animals with reference to CL Davis-Thompson foundation [5,6,7,8]. All information gathered were summarized in a database, were visualized, and analyzed descriptively based on histological features.

3. Results

3.1 Case Proportion

A total of 179 medical records and specimens were initially collected, which were then narrowed down to 117 cases containing tumors. Of these, almost a quarter (24.17%) were identified as non-neoplastic masses. Among the remaining neoplastic cases, approximately one-third were classified as tumors with no specific characteristics and/or no further histological information available, leading to their categorization as unknown tumors (Fig. 1). The distribution of identified tumors in our database is presented in Table 1.

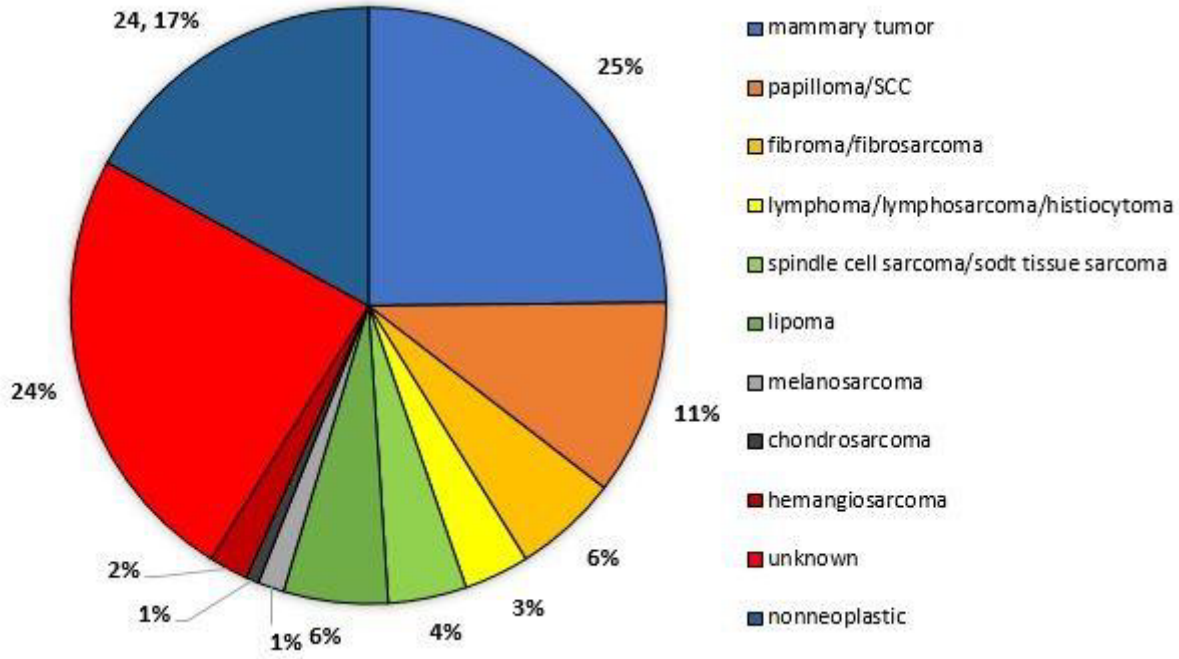

Figure 1. Proportion of neoplastic and non-neoplastic masses. Proportion of non-neoplastic masses included almost a quarter of total superficial masses cases collected.

Table 1. Summary of superficial masses collected in this study.

Diagnosis	Canine	Feline	Avian	Others	Total
Mammary tumor	21	12		2	35
Papilloma/SCC	1	8		6	15
Fibroma/fibrosarcoma	5	2		1	8
Lymphoma/lymphosarcoma/ histiocytoma	3	2			5
Spindle cell sarcoma/soft tissue sarcoma	2	1	1	2	6
Lipoma	2	3	2	1	8
Melanosarcoma (cutaneous)	1			1	2
Chondrosarcoma	1				1
Hemangiosarcoma	1		1	1	3
Unknown	22	12			34
Non-neoplastic					24

Sixty-two medical reports with a clinical diagnosis of suspected neoplasm were collected from the canine patient group; however, only 37 were histologically confirmed as neoplastic masses (Fig. 2). The most common tumor type in this group was mammary gland neoplasia (21 cases). Three of them were classified as atypical malignant tumors due to poor differentiation of cancer cells.

patients were diagnosed with mammary tumors at first examination. Information regarding potential surgeries or reproductive interventions was not available at the time of clinical examination. Three cases of gland-type tumors (adenoma and adenocarcinoma) were excluded from the category of mammary tumors due to lack of information about location of the mass and presence of atypical structural patterns.

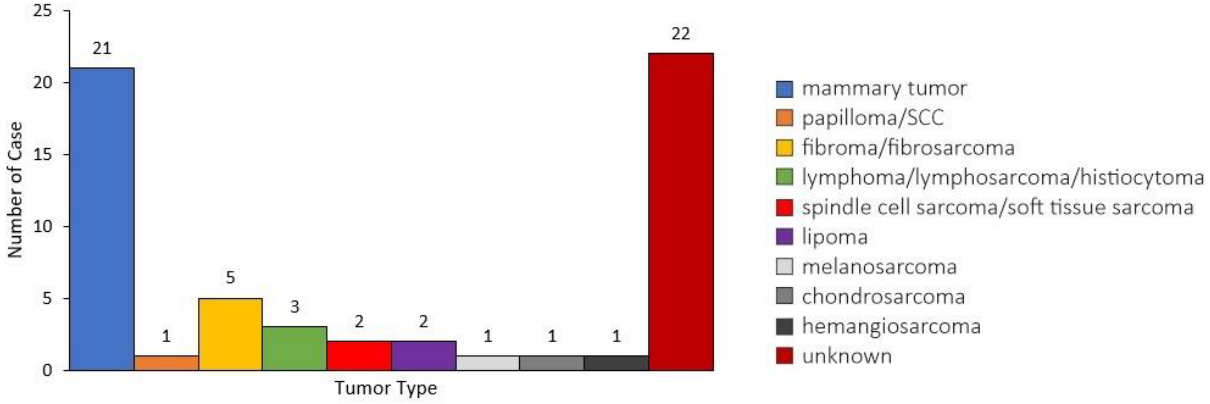

Figure 2. Case proportion of tumors in canine patients submitted in our facility from Malang Raya in the last five years. The most common specimen submitted for histopathologic examination was diagnosed as a mammary tumor with different subtypes. Other common tumors found in canine patients were skin tumors including fibroma/fibrosarcoma.

In the feline cohort, 56 records and/or specimens of tumors were collected with 28 consisting of confirmed medical records and/or available archival tissue samples (Fig. 3). Twelve

The dataset also included 12 avian patients, although archival tissues were available for only four of them. Four reptilian patients were reported with masses on extremities and abdomen; however

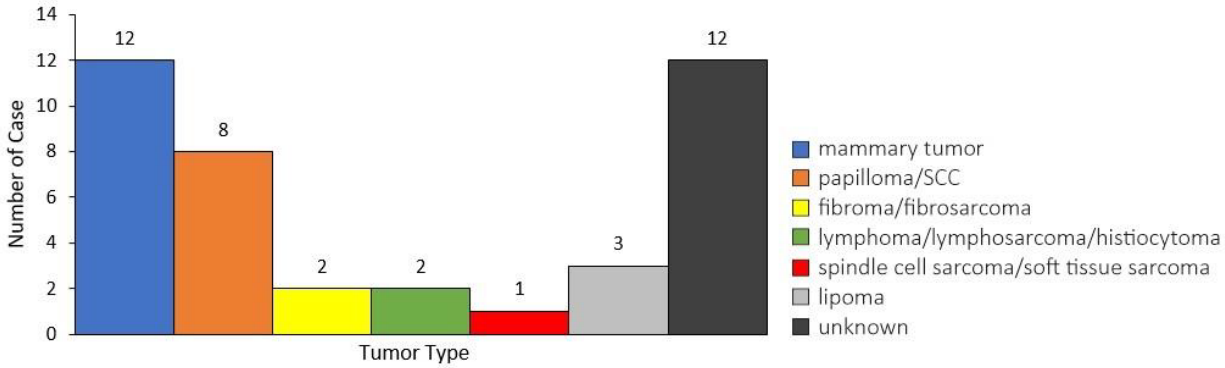

Figure 3. Case proportion of tumors in feline patients submitted in our facility from Malang Raya in the last five years. Similar to the trend observed in canine patients, mammary tumor was the most common reported tumor in feline patients.

no archival tissue was available. Two additional tumors were reported in a rabbit and a hamster and diagnosed as squamous cell carcinoma and adenocarcinoma, respectively.

3.2 Common Tumors and Subtypes

3.2.1 Squamous cell carcinoma (SCC)

Three cases of SCC were identified in canine, feline, and rodent patients, showing subtypes similar to human oral SCC. Histological variants were well-differentiated, moderately-differentiated, and papillary-type SCC (Fig. 4). Well-differentiated SCC consisted of islands and trabeculae of neoplastic epithelial cells invading the surrounding stromal tissue. Cells were round

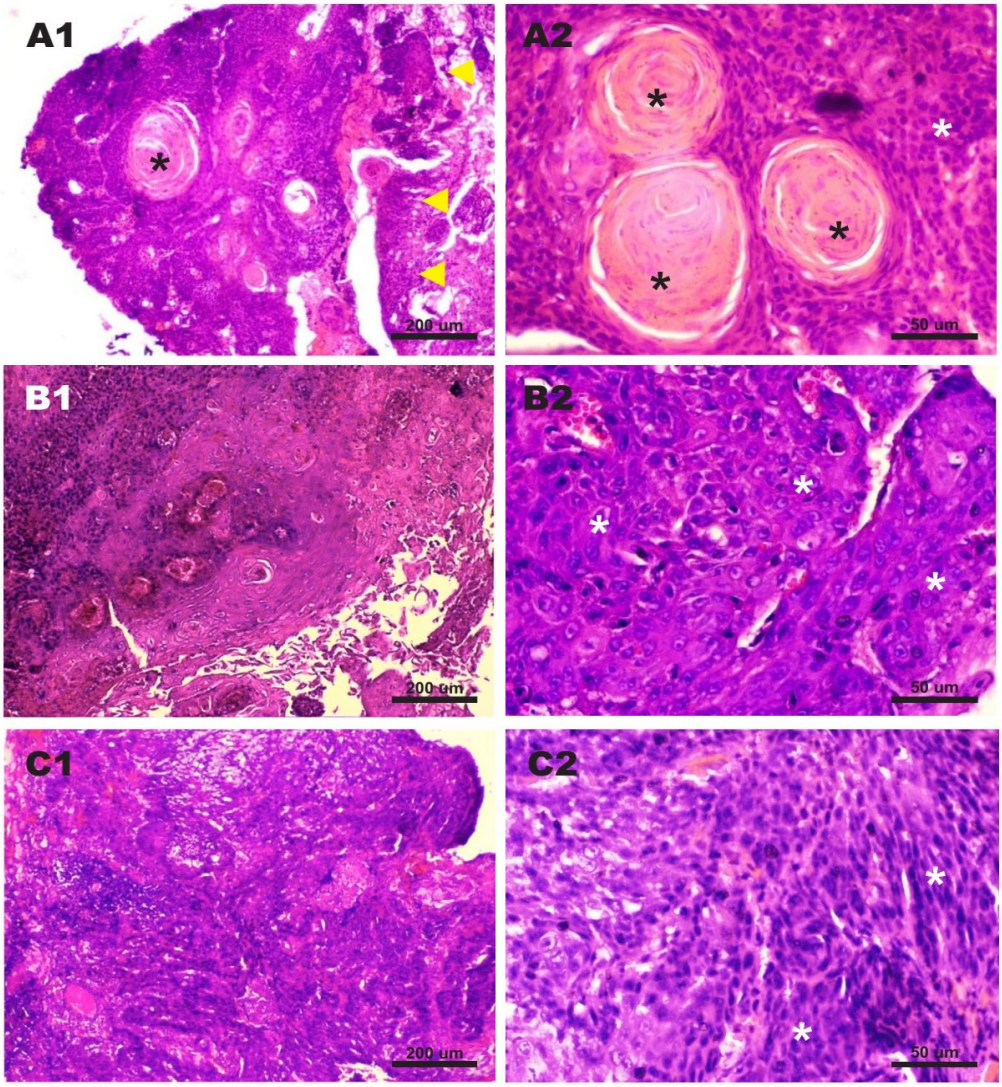

Figure 4. Histopathological architecture of putative subtype of squamous cell carcinoma in companion animal patients from Malang Raya. At least, three subtypes were reported as histopathological variation of SCC: well-differentiated (cat, A1-A2, 100x-400x magnification respectively), moderately-differentiated (hedgehog, B1-B2, 100x-400x magnification respectively), and papillary-type SCC (cat, C1-C2, 100x-400x magnification respectively). Black and white asterisks indicate keratin pearls and neoplastic epidermal cells consecutively. The structure is suspected of remaining epidermal layers (yellow arrowheads).

to polygonal with large vesicular nuclei, prominent nucleoli and moderate amount of eosinophilic cytoplasm. High anisocytosis and anisokaryosis were observed in the densely cellular areas, together with multifocal randomly scattered keratin pearls. Moderately-differentiated SCC presented similar characteristics to well-differentiated SCC, with minimal keratinization and lack of keratin pearls. Inverted papillary projections were present in papillary-type SCC. The papillary projections were formed by fibrovascular tissues with multiple layers of neoplastic epidermal cells.

3.2.2 Mammary adenocarcinoma

Adenocarcinoma was the most frequent tumor type in canine and feline species. Four subtypes were identified based on the classification system for canine and feline tumors from the Davis-Thompson Foundation: Solid type adenocarcinoma, mixed tumor with the proliferation of myoepithelial component/adenomyoepithelioma, and tubulopapillary carcinoma (Fig. 5). Solid-type adenocarcinoma (Fig. 5, A1-A2, C1-C2) consists of

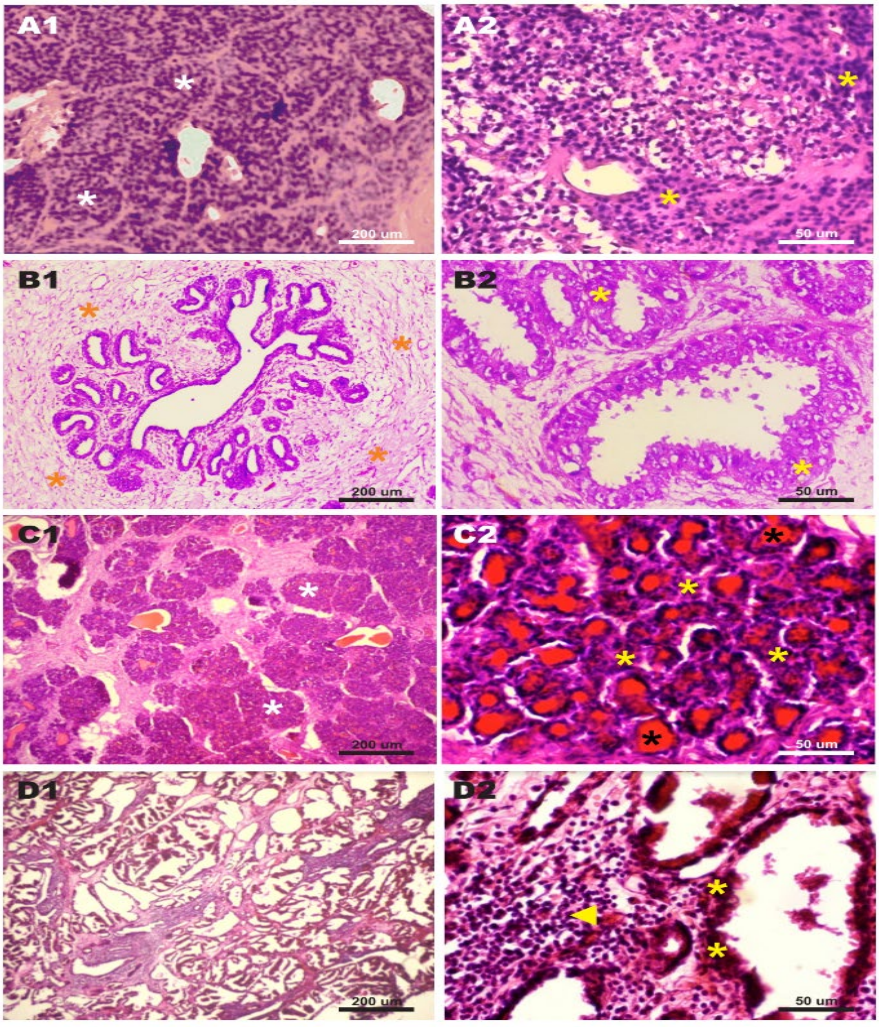

Figure 5. Histopathological architecture of putative subtype of mammary gland tumors in companion animal patients. Four subtypes were reported: solid type adenocarcinoma (dogs, A1-A2, C1-C2, 100x-400x magnification respectively) with some suspected secretory glands (black asterisks), mixed tumor (cat, B1-B2, 100x-400x magnification respectively) with proliferation of the myoepithelial component (orange asterisk), tubulopapillary carcinoma (dog, D1-D2, 100x-400x magnification respectively). Lower magnification shows nest formation (white asterisks) in solid type adenocarcinoma. Various epithelial cell proliferation patterns are present (yellow asterisks).

a dense population of epithelial cells supported by scant stroma, with a nest of invading cells that form solid masses with rare tubular formations. This form of tumor has polygonal to oval shaped cells with high number, exhibiting high anisokaryosis and anisocytosis. Nuclei are oval and often hyperchromatic with prominent nucleoli. Thin septa-like layers separated the hypercellular clusters and the outer margins showed irregular contours with subtle connective tissue capsulation (suggesting an *in-situ* malignancy). In some cases, a group of malignant cells were infiltrating adjacent tissues including fat tissues.

Mixed tumor with myoepithelial proliferation (Fig. 5, B1-B2) showed a mixture of more than two cell populations and variable amount of fibrous stroma. The first population consisted of tubules lined by cuboidal to columnar cells with moderate anisocytosis and anisokaryosis. The second population included spindle cells with poorly demarcated cell borders. Higher magnification revealed disorganized tubule formations, with pleomorphic arrangements of epithelial cells and projections extending into the luminal part of the tubules. Thin loose connective tissue was observed at the margins of the masses.

The last subtype of mammary carcinoma found in this study was tubulopapillary adenocarcinoma (Fig. 5, D1-D2), characterized by tubular and papillary structures. Higher magnification revealed adenomer-like structures with broad luminal space and simple cuboidal-columnar epithelial cells. The interstitial areas contained fibrovascular tissue and hypercellular clusters with moderate numbers of lymphocytes. Various thicknesses of connective tissues were present at the margin of the mass.

3.3 Non-neoplastic Superficial Masses

Records and specimens containing non-neoplastic superficial masses were assessed, which constituted 24.17% of the masses identified. Various types of non-neoplastic lesions were observed in including: nodular-granulomatous dermatitis with adnexal inflammation (n=14), pseudomycetoma - deep fungal mycosis (n=5), panniculitis (n=3), glandular hyperplasia (n=1), and cysts (n=1). Histopathological features of these non-neoplastic superficial masses are illustrated in Figure 6.

4. Discussion

Most submitted neoplastic masses in our histopathology service originated from superficial organ systems including skin and mammary glands. In all patient species, mammary gland tumor was the most common reported neoplasm followed by papilloma-SCC. However, this finding apparently is not reflecting the natural prevalence of neoplastic cases. There is a tendency that clients and or clinician will be more aware of visible superficial masses rather than profound or visceral masses contained in patients. According to our experience, visceral masses were more frequently encountered during necropsy in some deceased patients. Therefore, the natural-occurred (no experimental) animal tumor prevalence in Malang Raya is possibly greater than we expected.

Tumors can exhibit a wide range of structural and cellular variations depending on their location and pathogenesis. While many tumor subtypes are still under investigation, their clinical significance is increasingly in concern, with different subtypes leading to different outcomes and prognoses [9]. The cellular composition of a neoplastic mass plays a critical role in determining its aggressiveness, physical characteristics, functional behavior and prognosis, which are further influenced by cell communications through specific signaling proteins. As a result, molecular based identification methods for tumor subtypes are being proposed for integration into clinical practice. Katz *et al.* in 2018 [10] reported that sarcomas exhibit a wide range of responses to therapeutic agents. Most sarcomas are sensitive to chemotherapy, while others show better response to a combination of chemotherapy and anti-angiogenic agents, and others are resistant to chemotherapy but still responsive to anti-angiogenic agents or targeted therapies. Immunotherapy has also been developed to inhibit tumor development of poorly differentiated sarcoma types by targeting immune checkpoints.

Tumor subtype determination has become the foundation of protein-based treatments such

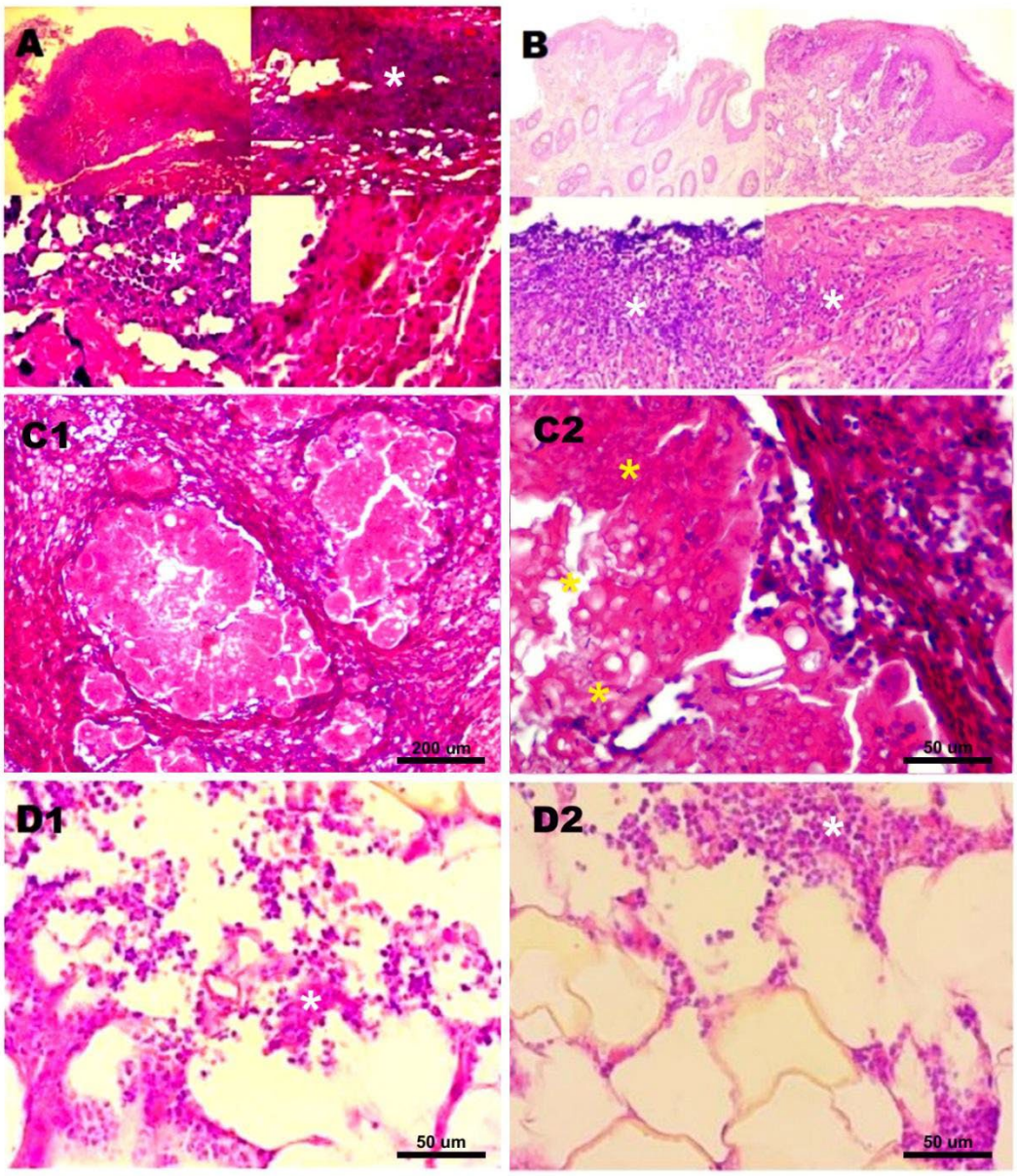

Figure 6. Most common non-neoplastic masses submitted: subcutaneous abscess, with granulomatous dermatitis (cats and dogs, A,B, various magnifications), pseudomycetoma (cat, fungal hyphae in yellow asterisks)/deep fungal mycosis (cat, C1-C2, 100x-400x magnification respectively), panniculitis (dog, D1-D2, 100x-400x magnification respectively). Hypercellularity containing clusters of inflammatory cells were present (white asterisks).

as hormonal therapy and immunotherapy. Ambs in 2010 [11] highlighted that biologically diverse cancers, such as those affecting the mammary gland, can lead to different outcomes. This has opened new avenues for developing cancer therapies tailored to specific tumor subtypes, as different subtypes exhibit distinct behaviors and structural characteristics [12, 13].

Currently, histopathology remains the most accessible diagnostic standard for identifying cancer subtypes, however clinical decision-making based solely on histopathology findings can sometimes be inconclusive. Histopathological assessment of tumor subtypes involved parameters such as cell shapes, pleomorphism, cellular composition, differentiation rate, and overall architecture. For example, SCC has numerous subtypes based on differentiation rates and histological features. Pereira *et al.* in 2007 [14] classified SCC into well, moderately, and poorly differentiated subtypes, while Cassarino *et al.* in

2006 [15] grouped SCC by risk potential into low, intermediate, high, and indeterminate malignant levels. Hemangiosarcomas (HSA) is classified into several subtypes based on histopathology (capillary, cavernous, and solid subtype) [16, 17]. In some cases, conventional haematoxylin-eosin staining is sufficient for clinical practice, especially for evaluating excision margins. Our contributors reported two recurrent cases of tumor, which were consistent with incomplete margin excision of the masses marked by absence of tissue transition between neoplastic mass and normal adjacent tissues or capsules.

Molecular profiling of tumor subtypes holds the potential to improve diagnostic accuracy and therapy recommendations. Studies exploring molecular alterations and signaling pathways within cancer cells and their microenvironment provide deeper insights into cancer types and their potential targets for treatment [11]. Retrospective studies on cancer prognosis, such as those on colorectal and gastric cancers, have already explored the implications of tumor subtypes and consensus molecular profiles [18, 19, 20].

Tumor molecular profiling involves studying signaling pathways, which play a key role in the pathogenesis of neoplastic events and influence prognosis. Certain proteins drive cell proliferation, affecting tumor size, vascularization, and metastatic potential. Perou *et al.* in 2000 [21] and Sorlie *et al.* in 2001 [22] identified five subtypes of mammary gland cancer according to gene expression profiles, including two estrogen receptor-positive (ER+) subtypes (luminal A and B), and three ER-negative subtypes (HER2-positive, and ER-negative subtypes). These subtypes are associated with different clinical outcomes and therapeutic options. Ambs in 2010 [11] emphasized that protein expression analysis by IHC can guide therapy selection, particularly for systemic adjuvant and gene repair strategies.

Genetic aberrations are strongly linked to certain tumors such as liposarcomas. Dei Tos, in 2014 [23] reported that specific liposarcoma subtypes, including well differentiated, de-differentiated, myxoid, and pleomorphic, are associated with distinct molecular alterations. Similarly, the WHO identified lymphoma as a type of tumor with the largest number of subtypes, each characterized by unique protein expressions. Valli *et al.* in 2011 [24] reported that several protein markers such as CD79a, CD20, CD3, anaplastic lymphoma kinase (ALK), and CD30 could effectively differentiate lymphoma subtypes in dogs.

Our finding also revealed a relatively high prevalence of non-neoplastic masses (24.17%) within our abnormal superficial masses cohort. These cases led to misdiagnosis, with practitioners initially suspecting neoplastic masses before submitting them for histopathology investigation. For example, deep fungal mycosis might mimic the metastatic feature of neoplastic mass due to recurrent mass growth after first excision. Thick connective tissue capsules in granulomas may have been falsely interpreted as tumors due to the hard consistency of mass margins. These findings highlight the potential for misdiagnosis when relying solely on external examination of masses. Histopathology remains the definitive method for determining the nature of these masses, guiding therapeutic actions and improving patient outcomes. However, diagnostic accuracy is not solely reliant on sophisticated facilities and testing.

5. Conclusion

A five-year analysis of medical records from companion animals diagnosed with tumors revealed mammary tumors as the most common neoplasia in the study population. Considering the various differential diagnoses of a merely superficial mass, it is crucial to adopt a comprehensive strategy and clinical reasoning approach for diagnosis determination. Developing a structured thinking algorithm can help ensure diagnostic accuracy. By consciously avoiding cognitive biases and maintaining a systematic approach to clinical decision-making, we can minimize the risk of misdiagnosis, ensuring more reliable outcomes and preventing malpractice in the process.

Ethics Approval and Consent to Participate

This study was performed under approval of the research ethics committee of Universitas Brawijaya Bioscience Institute (document number No: 041-KEP-UB-2023).

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

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(Original Research)

The Impact of Menhaden Fish Oil on Brain Tauopathy in Streptozotocin-Lipopolysaccharide-Induced Rodent Model of Alzheimer's Disease

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Abstract

Background: Tauopathies, including Alzheimer's disease (AD), are neurodegenerative conditions characterized by the accumulation of abnormally phosphorylated tau protein, often exacerbated by comorbid metabolic disorders such as diabetes mellitus. This study evaluated the neuroprotective effect of Menhaden Fish Oil (MFO), a rich source of preformed omega-3 fatty acids, on tau pathology in a streptozotocin-lipopolysaccharide (STZ-LPS)-induced rat model that mimics diabetes-associated AD. **Methods:** Thirty male Wistar rats were grouped into five distinct groups and subjected to treatment with either normal saline or MFO (1 g/kg or 3 g/kg) for 21 days, followed by biochemical, immunofluorescence microscopy, and RT-qPCR analyses. **Results:** MFO administration significantly reduced total tau protein levels and tau hyperphosphorylation at Ser202 in both the cortex and hippocampus. It also upregulated AKT-1 expression while downregulating GSK-3 and CASP-3, suggesting modulation of the AKT/GSK-3 signaling pathway. **Conclusions:** These findings demonstrate that MFO supplementation mitigates tau pathology and neuronal apoptosis in a metabolic-inflammatory model of AD, supporting its potential as a dietary neuroprotective strategy.

Keywords

Alzheimer's disease, Brain, Diabetes, Omega-3 fatty acid, Tau protein

1. Introduction

Tauopathies, particularly Alzheimer's disease (AD), are a major focus of neurodegenerative research due to their profound impact on cognition, memory, and quality of life. AD, the leading cause of dementia worldwide, is marked by abnormal protein aggregation and progressive neuronal loss [1]. Among the pathological hallmarks of AD, tau protein dysfunction plays a pivotal role. Under normal conditions, tau stabilizes neuronal microtubules and supports axonal transport [2], but in disease states, it becomes hyperphosphorylated and aggregates into neurofibrillary tangles (NFTs) that disrupt synaptic function and contribute to neuronal degeneration [3]. Tau-related pathology is also observed in other neurodegenerative disorders, including frontotemporal dementia and Parkinson's disease, indicating a broader relevance of tau dysfunction in brain health [4]. As AD prevalence continues to rise, understanding the mechanisms underlying tau pathology and developing interventions to counteract it remain crucial.

Mounting evidence suggests that metabolic dysfunction, particularly diabetes mellitus (DM), contributes significantly to AD development and progression. Insulin resistance and impaired insulin signaling in the brain have been shown to promote tau hyperphosphorylation, linking DM to tauopathy [5-6]. Experimental models have consistently demonstrated elevated tau phosphorylation in the cortex and hippocampus of diabetic animals [7-8]. Notably, various models exhibit distinct phosphorylation profiles; for example, Otsuka Long Evans Tokushima Fatty rats show increased Ser199/202 and Ser396 phosphorylation [9], while tau transgenic mice treated with a high-fat diet exhibit hyperphosphorylation at Ser396/404 [10], Streptozotocin (STZ)-induced diabetic mice exhibit hyperphosphorylation at multiple sites, including Thr181, Ser199, Ser202, and Ser396/404 [11]. These findings underscore the interplay between metabolic dysfunction and tau aggregation, reinforcing the view that AD represents both a neurodegenerative and metabolic disorder [12].

One of the main regulators of tau hyperphosphorylation is glycogen synthase kinase-3 (GSK-3), a serine/threonine kinase that phosphorylates tau at multiple sites. GSK-3 is hyperactive in AD brains and is implicated in both tau aggregation and amyloid-ß pathology [13-14]. Given its central role, modulation of the Ak strain transforming (AKT)/GSK-3 pathway has been widely investigated as a therapeutic strategy for AD. Activation of Akt leads to the inhibitory phosphorylation of GSK-3, reducing tau phosphorylation and neuronal damage [15]. However, direct pharmacological inhibition of GSK-3 has been challenging due to toxicity and lack of specificity. This has prompted exploration of natural compounds, particularly omega-3-rich dietary interventions, as safer alternatives to modulate tauopathy.

Fish oil (FO), rich in long-chain omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has demonstrated neuroprotective effects through anti-inflammatory, antioxidant, and anti-apoptotic mechanisms [16-17]. Animal studies have shown that FO supplementation can reduce tau hyperphosphorylation and preserve neuronal function. In 5xFAD transgenic mice, short-term FO supplementation decreased hyperphosphorylated tau in the parietal cortex by 36% compared to untreated controls [18]. Similarly, FO combined with curcumin attenuated tau phosphorylation and improved insulin signaling by inhibiting c-Jun N-terminal kinase (JNK) pathways [19]. Human studies also indicate that DHA-rich FO can reduce GSK-3 activity and improve insulin sensitivity in overweight adults, suggesting potential cross-talk between metabolic and neurodegenerative pathways [20]. These findings highlight the potential of FO as a dietary intervention for AD prevention and management.

Menhaden fish oil (MFO) is regarded as a sustainable and environmentally friendly marine resource that provides omega-3 fatty acids (EPA and DHA) and is known for its health benefits, including cardiovascular protection and anti-inflammatory effects [21]. In contrast to plant-based sources like flaxseed or chia, which only provide alpha-linolenic acid (ALA) that must be converted into EPA and DHA through inefficient enzymatic processes in humans [22]. MFO, like other forms of FO that contain pre-formed DHA and EPA, can directly increase active fatty acids within cells and tissues effectively [23]. In comparison to other marine oils, such as cod oil or tuna oil, MFO provides a superior concentration of EPA and DHA, often containing between 1200mg and 2000mg of EPA and DHA per 100g, allowing for lower therapeutic dosages [24]. This unique composition strengthens its anti-inflammatory potential and its ability to modulate key signaling pathways such as AKT/GSK-3 and caspase-3 (CASP-3), which are critical for mitigating tau hyperphosphorylation and neuronal apoptosis. These biochemical advantages make MFO a promising candidate for research on complex neurodegenerative processes, particularly those associated with DM-related AD.

Although FO has been extensively studied, the molecular mechanisms by which MFO modulates tau pathology in models combining metabolic dysfunction and neuroinflammation remain underexplored. Previous studies have often focused on amyloid pathology rather than tauopathy and rarely examined dose-dependent effects or early phosphorylation sites such as Ser202, which are strongly associated with tau misfolding [25-26]. Furthermore, the direct influence of MFO on the expression of genes related to the AKT/GSK-3/CASP-3 pathway is still unclear. To address these gaps, the present study investigates the neuroprotective effects of MFO in

an STZ-LPS rat model that integrates metabolic dysfunction (STZ-induced diabetes) and neuroinflammation (LPS exposure), thus better replicating the multifactorial nature of DM-associated AD [27]. It is hypothesized that MFO supplementation will reduce total tau levels, decrease Ser202 phosphorylation, and regulate the expression of AKT-1, GSK-3, and CASP-3. Through ELISA, immunofluorescence, and RT-qPCR analyses, this study aims to provide novel insights into how MFO, with its unique omega-3 composition, can act as a multi-target neuroprotective agent.

2.1 General materials

Menhaden fish oil (Cat no: F8020) and lipopolysaccharide (LPS) (Cat no: L2630) were purchased from Sigma-Aldrich company (USA). Streptozotocin (Cat no: SC-200719) was purchased from Santa Cruz Biotechnology Company (USA). The Pro-prep protein extraction solution (Cat no: 17081) was purchased from Intron Biotechnology Company (South Korea). The total tau protein (Cat no: E1191Ra) was provided by Bioassay Technology Laboratory Company (China). The primary antibody against phospho-tau (Ser 202, Thr205) (Cat no:MN1020) from Thermo Fisher Scientific Company (USA). Meanwhile, the secondary antibody against Goat Anti-Rabbit IgG H&L (TRITC) (Cat no: ab6718) was purchased from Ab Cam Company (UK). Meanwhile, for the real-time-qualitative polymerase chain reaction (RT-qPCR) method, the Total RNA mini kit (Cat no: RT100) was purchased from Geneaid Biotechnology Company (Taiwan), ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Cat no: FSQ-301) from Toyobo Company (Japan), and SensiFAST SYBR No-ROX Kit (Cat no: BIO-98005) from Bioline Company (UK). Other chemicals used throughout this study were analytical grade.

2.2 Experimental animals

Male Wistar rats weighing 250-280 g were purchased from Anilab, Indonesia. The animals were acclimated for seven days in the Laboratory for Experimental Animal Development of the Faculty of Medicine (Universitas Brawijaya, Indonesia) before the experiment. Under conditions of well-ventilated conditions with 12-hour light/dark cycles, rats were housed in cages, with three rats per cage. They were also given unrestricted access to water and standard rat chow (Rat bio, Citra Ina Feedmill, Indonesia) on an *ad libitum* basis. The animal bedding was made of sawdust (Chipsi, Germany, JRS Germany GmbH & Co.) and was changed every morning during the study. The sample size (n=6 per group) was selected based on the resource-equation method [28] to account for attrition and maintain adequate statistical power (DF = 25). The Institutional Animal Care and Use Committee (IACUC) at Universiti Putra Malaysia, Selangor, Malaysia, approved all experimental care and procedures for the animal investigations (Approval NO: UPM/IACUC/AUP-R017/2022).

2.3 Experimental design and procedures

A total of thirty rats were randomly selected (n=6 for each group) into five groups: 1) Animal received Normal saline (NS) induction+NS oral (Control group), 2) Animal received NS induction+MFO with dosage of 3 g/kg (MFO control group), 3) Animal received STZ-LPS induction+NS oral (STZ-LPS group), 4) Animal received STZ-LPS induction+MFO with dosage of 1 g/kg (MFO 1g/kg group), 5) Animal received STZ-LPS induction+MFO with dosage of 3 g/kg (MFO 3g/kg group). Animal welfare was closely monitored throughout the STZ-LPS induction period. Body weight, food and water intake, and clinical signs of morbidity (e.g., lethargy, abnormal grooming, posture) were assessed daily. Humane endpoints were applied, and any rat exhibiting >20% body weight loss or severe distress was euthanized according to institutional ethical guidelines.

At the end of the experiment, all rats were euthanised using ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg). Following euthanasia, a necropsy was conducted. The brain samples were carefully isolated, washed, and separated by sagittal sections into two parts: left and right hemispheres. The left hemispheres were submerged in a 10% neutral buffered formalin (NBF) fixative solution at room temperature for 24 hours for immunofluorescence histological analysis. Meanwhile, the right hemispheres were kept frozen at -80°C until the ELISA and RT-PCR assays were conducted. Figure 1 illustrates the experimental design.

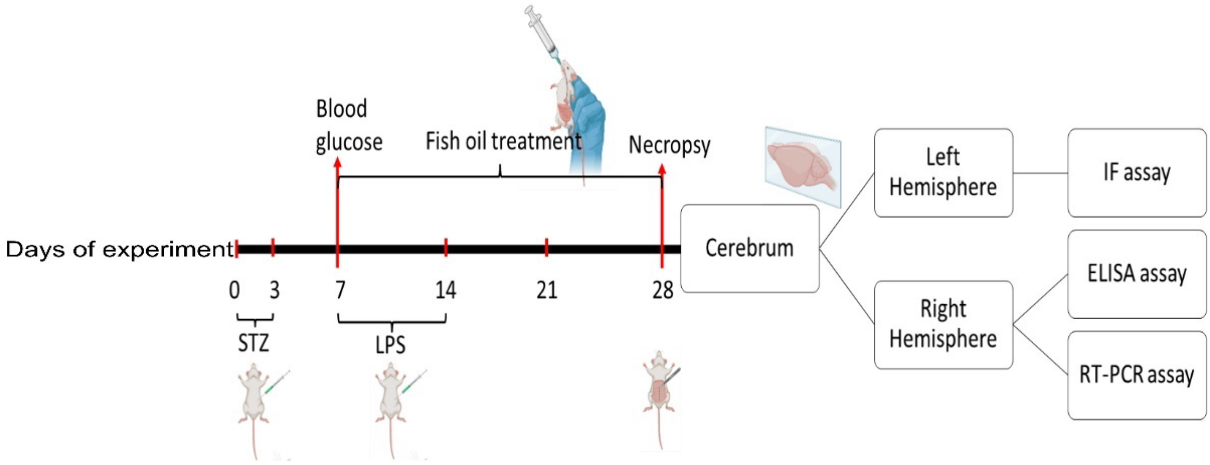


Figure 1. A simplified illustration of the experimental design timeframe. STZ was administered from days 1 to 3, followed by LPS injections from days 7 to 13. Fish oil treatment was initiated on day 7 and continued until day 27, with the experiment concluding on day 28. Abbreviations: STZ: streptozotocin; LPS: lipopolysaccharide; IF: immunofluorescence; ELISA: enzyme-linked immunosorbent test; RT-PCR: real-time polymerase chain reaction.

2.3.1 Induction of Diabetes Mellitus

Multiple intraperitoneal (i.p.) injections of freshly prepared STZ at a dose of 45 mg/kg were administered to overnight fasted rats for three consecutive days to induce diabetes mellitus [29]. STZ was dissolved in 0.1 mol/L sodium citrate buffer solution (pH 4.5). Following one week of STZ administration, blood was obtained from the tail vein to assess fasting blood glucose levels in rats that had been fasted for eight hours, utilizing a digital blood glucose meter (GlucoDr, Korea). Only rats with fasting blood glucose levels of 250 mg/dL or above were categorized as diabetic and included in the experiment.

2.3.2 Induction of Alzheimer's Disease

The onset of Alzheimer's disease (AD) was induced by daily administration of 250 µg/kg of LPS over a period of seven consecutive days [30]. LPS was administered intraperitoneally after being diluted in a physiological saline solution (0.9% NaCl). Throughout the administration of LPS, the body temperature of the animal was carefully monitored each morning. The success of AD induction was confirmed through behavioral assessment using the Y-maze test conducted after LPS injection. STZ-LPS-induced rats showed a significantly higher number of entries into the familiar arm compared to the start and novel arms, with the longest duration spent in the start arm and the shortest in the novel arm, indicating spatial memory impairment characteristic of AD-like cognitive deficits.

2.4 Brain protein extraction preparation

The extraction of brain proteins was conducted utilizing a Pro-prep protein extraction solution, involving the collection of 10-20 mg of cortical and hippocampus tissues. The tissues underwent homogenization in 600 µL of Pro-prep solution, subsequently transferred to a microtube and centrifuged at 13,000 rpm for 10 minutes. The samples underwent incubation for a duration of 30 minutes, followed by centrifugation at 13,000 rpm for a period of five minutes. The supernatant was subsequently transferred to a new 1.5 mL microtube and prepared for ELISA analysis. The procedure adhered to the specifications provided by the manufacturer.

2.5 Total tau protein analysis

All reagents, standard solutions, and brain samples were meticulously prepared in accordance with the kit instructions and allowed to acclimate to room temperature naturally. Initially, 50 µL of the standard solution was added to the designated well for standards. Subsequently, 40 µL of the brain sample was added to the sample wells, followed by the addition of 10 µL of rat tau protein antibody. Fifty microliters of Streptavidin-HRP

were added to the standard and sample wells and subsequently homogenized. The plate was carefully sealed and incubated for 60 minutes at a controlled temperature of 37℃. The plate underwent a rigorous cleaning process, being washed five times with a wash buffer. Each well was soaked with 300 μL for each wash for 30 seconds. Subsequently, the plate should be tapped onto paper towels before the sequential addition of 50 μL of substrate solution A and solution B to each well. Subsequently, the plate must be should be incubated with the new sealer in a dark environment for 10 minutes at a temperature of 37℃. Ultimately, it is essential to introduce 50 μL of stop solution into each well to halt the reaction before measurement. The absorbance at a wavelength of 450 nm ought to be assessed utilizing a Bio-Rad microplate reader (USA). The final cytokine concentrations were expressed as ng/g of wet tissue.

2.6 Phosphorylated tau protein (Ser 202) immunofluorescence analysis

The unstained brain tissues were heated for an hour at 60°C. The samples were then twice submerged for ten minutes each in xylol. The samples were then twice immersed in xylol for ten minutes each. After that, the samples must be rehydrated with ethyl alcohol in descending graded series, beginning at a concentration of 100% (2 changes), then 90%, 80%, and 70% every five minutes. The samples were rinsed three times using phosphate-buffered saline (PBS). The slides were subsequently covered with a solution of PBS Triton-X 100 at a concentration of 0.1%, which was allowed to remain for five minutes. The slides were subsequently covered with a 1% BSA solution and allowed to incubate for 30 minutes at room temperature. The slides are subsequently incubated with primary antibodies targeting tau phosphorylation at Ser202 (pSer202) at a dilution of 1:1000 for an extended overnight duration at 4°C. Following three washes with PBS, the slides underwent a 30-minute incubation at room temperature with secondary antibodies of Goat Anti-Rabbit IgG H&L at a dilution of 1:1000. For a duration of five minutes, DAPI was employed at a dilution of 1:1000 as a counterstain on the sections. Subsequently, the slides underwent a triadic washing process with PBS, mounted, and subsequently covered with glass. A fluorescence microscope (Olympus FV1000, Olympus, Tokyo, Japan) was employed to capture images of rat brain sections. In order to assess the immunoreactivity associated with tau phosphorylation at Ser202, the number of cells in each brain section was counted as the number of positive cells through the utilization of ImageJ software. Quantification of αß42 immunoreactivity was performed using ImageJ software. A blinded observer manually defined the region of interest (ROI) corresponding to the cortical area on each immunofluorescence image at 200× magnification (image resolution: 800 × 600 pixels). The red fluorescence channel, representing pSer202 signal, was analyzed using the 'Measure' function to obtain the integrated density values. These values were used to calculate the percentage of the ROI area exhibiting positive pSer202 staining. For each animal, five non-overlapping sections were analyzed, and the resulting percentages were averaged to yield a single representative value per subject.

2.7 Real time-qualitative polymerase chain reaction (RT-qPCR) procedures

The designated primer pairs for three genes associated with the tau phosphorylation pathway, along with one reference gene, were generated utilizing the Integrated DNA Technologies program (see Table 1). Gene sequences were derived from data sourced from the Gene Bank™ database pertaining to *Rattus norvegicus*. The gene specificity of the primer sequences was verified through BLAST searches, which revealed a lack of multi-locus matching at each individual primer site. The beta-actin gene (ACTB) served as an internal control or housekeeping gene to assess the quantity and quality of cDNA, which was then utilized as a standard for estimating the expression of candidate genes. Melt curve analysis was performed to ensure the specificity of amplification, with single, sharp peaks indicating the absence of primer-dimers or nonspecific products. Amplification efficiency for each primer pair was determined using standard curves, yielding efficiency values within the acceptable range of 90–110%. The ACTB gene was selected as the housekeeping gene due to its stable expression across all experimental groups, as confirmed by preliminary Ct analyses and supported by prior studies using STZ-LPS models.

Table 1. The parameters that were determined from the analysis of RT-qPCR data, along with the primers designated for the APP target genes and a reference gene.

Gene symbol	Gene name	Primer Forward (F)/ Reverse ®	Genbank	Amplicon size (bp)
GSK-3	Glycogen synthase kinase-3 beta	ACCTGCCCTCTTCAACTTTAC CACGGTCTCCAGCATTAGTATC	NM_032080.1	149
AKT-1	AKT serine/threonine kinase 1	GCTGGAGGACAACGACTATG CTTCTCATGGTCCTGGTTGTAG	NM_033230.3	109
CASP-3	Caspase-3	CCACGGAATTTGAGTCCTTCT CCACTCCCAGTCATTCCTTTAG	NM_012922.2	122
ACTB	Beta-actin	CCTAAGGCCAACCGTGAAA CAGAGGCATACAGGGACC	KJ696744.1	103

RT-qPCR was conducted with the SensiFAST SYBR No-ROX Kit on a Bio-Rad thermocycler (Bio-Rad, USA). The precise primer concentrations and PCR conditions were established during the initial optimization. Subsequent to optimization trials, tests were conducted using a total reaction volume of 10 µL, comprising equal concentrations of RNA, 5 µL of SYBR Green, 0.5 µL of each forward and reverse primer, 2 µL of ddH2O, and 2 µL of cDNA template at a concentration of 100 ng/µL. The reactions were conducted in a MicroAmp® fast 8-tube strip, covered with MicroAmp® optical 8-cap strips, under the following PCR conditions: initial denaturation at 45°C for 45 minutes, followed by 40 cycles consisting of denaturation at 95°C for five minutes, annealing at 95°C for 30 seconds, at 59°C for one minute, and extension at 72°C for 30 seconds. The concluding extension phase concluded at 72°C for a duration of ten minutes. In order to confirm the specificity of the products produced, a melting curve analysis was implemented promptly subsequent to the PCR. In order to ensure uniform amplification conditions, all samples were amplified on a single plate for each primer combination. Negative controls were implemented by substituting water for RNA templates.

The results were subsequently recorded as cycle threshold (Ct) values, and each sample was analyzed in duplicate. The comparative ΔCt method was employed to ascertain the relative expression levels of the genes in relation to the housekeeping genes for each sample. The Ct values were averaged. The ΔCt value was calculated as Ct (target gene) – Ct (ACTB), and the fold change (ΔΔCt) was calculated to quantify gene expression using the following equation: ΔΔCt = ΔCt (sample) – ΔCt (reference sample).

2.8 Statistical analysis

One-way ANOVA along with the Tukey test for post-hoc comparisons was employed to ascertain the distinctions between the control and experimental groups. The results are expressed as mean±standard error mean, with differences between groups deemed significant at $p<0.05$. Statistical analyses were conducted utilizing SPSS (version 20.0; SPSS Inc., Chicago, IL).

3. Results

3.1 Quantification of total tau protein

An ELISA kit was employed to quantify total tau, with absorbance readings acquired through a multiplate reader. The signals were subsequently transformed into ng/mL in accordance with the concentrations defined by the standard curve. Total tau protein levels in the cortex and hippocampus were measured using an ELISA assay, and the results are presented in Table 2. The STZ-LPS group exhibited a significant

Table 2. Measurement of overall tau concentrations in the cortical and hippocampal regions of the rat brain utilizing the ELISA approach.

Groups	Tau protein (ng/mL)	
	Cortex	**Hippocampus**
Control	32.48±5.76$^{\#\#\#}$	37.01±6.15$^{\#}$
MFO control	34.71±8.23$^{\#\#\#}$	38.79±12.68$^{\#}$
STZ-LPS	113.59±12.87**	77.29±3.97*
MFO 1g/kg	38.20±8.10$^{\#\#\#}$	41.78±9.23$^{\#}$
MFO 3g/kg	35.90±8.39$^{\#\#\#}$	39.03±7.64$^{\#}$

Data are presented as mean±SEM (n=6); *$p < 0.05$, **$p < 0.01$ vs. Control; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. STZ-LPS.

elevation in total tau protein levels (cortex: 113.59±12.87; hippocampus: 77.29±3.97) compared to the control (cortex: 32.48±5.76, $p<0.001$; hippocampus: 37.01±6.15, $p=0.019$) and MFO control (cortex: 34.71±8.23, $p<0.001$; hippocampus: 38.79±12.68, $p=0.027$) groups in both brain regions. Specifically, total tau levels increased by approximately 250% in the cortex and 110% in the hippocampus compared to control animals. In contrast, MFO treatment at both 1 g/kg and 3 g/kg doses significantly reduced total tau levels compared to the STZ-LPS group in the cortex (38.20±8.10, $p<0.001$; 35.90±8.39, $p<0.001$; respectively) and hippocampus (41.78±9.23, $p=0.047$; 39.03±7.64, $p=0.028$; respectively). In addition, the difference between the 1g/kg and 3g/kg MFO groups was not statistically significant ($p=0.999$), indicating that both doses effectively mitigated tau accumulation induced by STZ-LPS. Overall, the results demonstrate that MFO supplementation normalized tau levels in the cortex and hippocampus, with concentrations comparable to those observed in the control groups.

3.2 Phospo-tau-ser202 fluorescence intensity

Quantitative analysis of phospho-tau-Ser202 fluorescence intensity in the cerebral cortex of each experimental group is presented in Figure 2. The

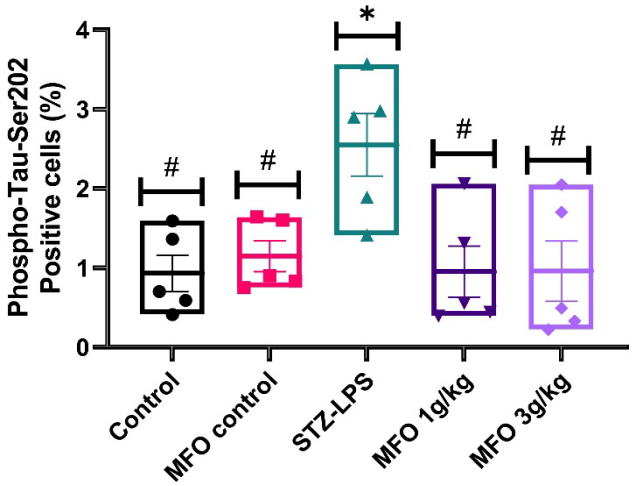


Figure 2. Quantification of phospho-tau-Ser202–positive area in the rat cortex. The bar graph shows the percentage of phospho-tau-Ser202–positive area relative to total ROI (800 × 600 pixels) across treatment groups. Data are presented as mean ± SEM (n=6), with individual data points overlaid. STZ-LPS group showed a significant increase in phospho-tau-Ser202 levels compared to controls, while MFO 1 g/kg and 3 g/kg treatments reduced this elevation. Statistical significance was determined by one-way ANOVA followed by a post hoc test. *$p < 0.05$ vs. Control and $^{\#}p < 0.05$ vs. STZ-LPS.

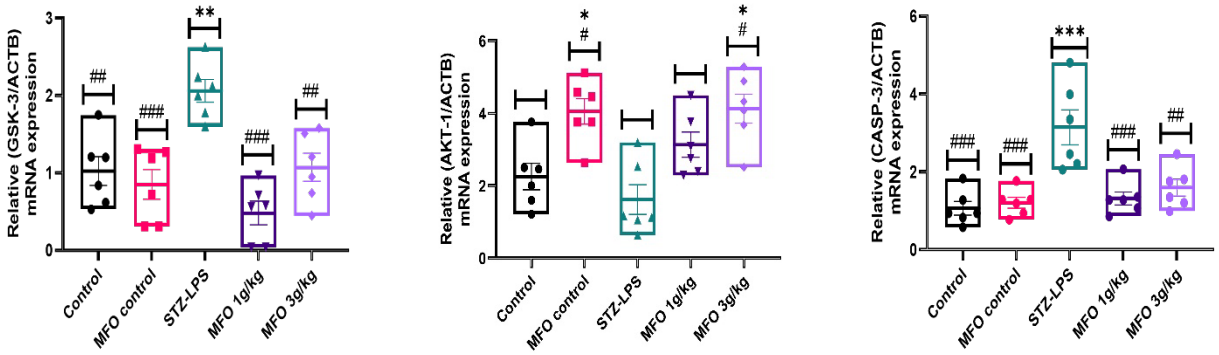
STZ-LPS group displayed a marked increase in the percentage of phospho-tau-Ser202-positive cells (2.55±0.39; p=0.013) compared to the control group (0.93±0.23), indicating enhanced tau hyperphosphorylation. Both MFO treatment groups (1 g/kg and 3 g/kg) significantly reduced phospho-tau-Ser202 levels relative (0.95±0.32, p=0.014; 0.96±0.38, p=0.014; respectively) to STZ-LPS group. There was no statistically significant difference (p=1) between the 1 g/kg and 3 g/kg MFO groups, although the 3 g/kg dose showed a slightly lower mean value.

3.3 Analysis of the relative expression of genes associated with tau phosphorylation

Figure 3 illustrates the levels of mRNA quantified through the quantitative PCR approach. The mRNA expression of genes linked to the AD pathway in the STZ-LPS group demonstrated a notable increase in GSK-3 (2.06±0.15; p=0.002) (Figure 3.A) and CASP-3 (3.15±0.45; p<0.001) (Figure 3.C) levels when contrasted with the control group (1.02±0.18 and 1.07±0.18, respectively). While the levels of AKT-1 (1.62±0.41; p=0.001) (Figure 3.B) demonstrated a reduction in comparison to the MFO control group (4.05±0.35). Conversely, the administration of fish oil 3g/kg in STZ-LPS-induced rats significantly diminishes the mRNA levels of GSK-3 (1.07±0.18; p=0.004) and CASP-3 (1.60±0.21; p=0.002), while concurrently elevating the levels of AKT-1 (4.13±0.4; p=0.001) when compared to the STZ-LPS group

4. Discussion

The STZ-LPS-induced rats in this study were able to develop tauopathy, which is considered important in AD pathogenesis. The combination of STZ-LPS induction could result in diabetic animals (range from 280-450 mg/dL), which may then promote AD development as evidenced by an increase in total tau and tau phosphorylation at serine202. In this current study also discovered that tau phosphorylation occurs via the AKT/GSK-3 pathway, as evidenced by the downregulation of the AKT-1 gene and the upregulation of the GSK-3 and CASP-3 genes. Furthermore, Wegmann and colleagues declared that phosphorylated tau proteins have been related to NFTs and tau accumulation in the brain [31]. It can be applied to differentiate AD from other forms of dementia as well [32]. Studies showed that different phosphorylation sites of the tau protein could differentiate between types of dementia. For example, phospo-tau-serine-396 distinguishes AD from chronic traumatic encephalopathy[33], phospo-tau-serine-231 distinguishes AD from vascular dementia [34], and phospo-tau-serine-181 distinguishes AD from dementia with Lewy bodies [35]. Meanwhile, total tau protein appears to be a general indicator of cortical axon damage or



A B C

Figure 3. The impact of fish oil supplementation on the relative fold change in mRNA expression across all genes among the various groups. (A) STZ-LPS significantly elevated GSK-3 expression compared to controls. MFO treatment reduced GSK-3 levels. (B) AKT-1 expression was lower in the STZ-LPS group and restored by MFO 3 g/kg supplementation. (C) STZ-LPS significantly increased CASP-3 expression, while MFO treatment (1 g/kg and 3 g/kg) attenuated this effect. Data are presented as mean±SEM; *p < 0.05, **p < 0.01, ***p < 0.001 vs. Control; $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 vs. STZ-LPS.

neurodegeneration, as studies have revealed that total tau is increased in AD [36].

Total and phosphorylated tau protein as a biomarker for neurodegenerative diseases such as AD is mostly evaluated in clinical studies on cerebrospinal fluid (CSF) [37-38], plasma [39-40] or even tear fluid [41]. In fact, previous studies reported that total tau protein increased in AD patients evaluated from CSF [42] or plasma [43]. In the meantime, study showed that total tau and phosphorylated tau levels in CSF reflect neuropathological changes in the brain, including AD progression [44]. Meanwhile, the results of the current study are consistent with those from a previous study on AD experimental animals [45-46], which discovered changes in total tau protein and phosphorylated tau protein in the brain sample. Similar findings were also observed in an STZ-induced diabetic animal study that linked the progression of AD due to DM [9-47]. Indeed, another study reported that DM causes brain insulin impairment, which can result in abnormal hyperphosphorylation of the tau protein [48]. In addition, this abnormal hyperphosphorylation has been widely established as a result of AKT inactivation, which causes GSK-3 overactivation [49-50].

On top of everything, the result of this study demonstrated the protective effect of FO supplementation against brain tauopathy associated with DM-AD animal model. The current findings support the role of MFO in normalizing tau protein levels in both the cortex and hippocampus of STZ-LPS rats. Both 1 g/kg and 3 g/kg MFO treatments significantly reduced total tau and phospho-tau-Ser202 to levels comparable to control groups, suggesting that MFO exerts neuroprotective effects regardless of dose within this range. While a slightly greater reduction was observed with 3 g/kg MFO, the difference between the two doses was not statistically significant. It is well known that tau hyperphosphorylation at Ser202 is recognized as an early marker of neurofibrillary tangle formation [25]. While we focused on Ser202 as a representative phosphorylation site, we acknowledge that evaluating additional sites such as Ser396, Tyr18, or Thr231 would provide a more comprehensive understanding of tau phosphorylation patterns [51]. Furthermore, animal studies have mentioned that the increases in phospho-tau-serine202 occur in the diabetic brain [52] or in AD brain [53]. Moreover, research confirmed that a higher total tau level increases the risk of AD by 25% [54]. Meanwhile, tau hyperphosphorylation is undeniably described as the cause of the NFTs that leads to AD [55-56]. In line with this, research has proven a relationship between DM and tau hyperphosphorylation and the elevated risk of AD [57]. Therefore, this strengthens the argument that tau-based therapeutic strategies for AD related to DM should also consider the ability of a substance to prevent the production of tau protein as well as tau hyperphosphorylation, such as MFO administration, as demonstrated in this current study. As numerous studies have also proven the positive effect of FO on tau development and also tau hyperphosphorylation [18-58].

In this current study, the tau-related genes showed that MFO could enhance the AKT/GSK signaling pathway in STZ-LPS-induced rats as evidenced by upregulating AKT-1 genes. This activation of AKT phosphorylates GSK-3, causing it to become inactive since GSK-3 is active in resting cells and phosphorylation inhibits its activity [59], In this study, the inactive GSK-3 due to MFO was represented by a lower number of GSK-3 relative gene expression compared to the STZ-LPS group. GSK-3 inhibition will delay tau protein hyperphosphorylation and NFT aggregation, ultimately preventing cognitive dysfunction [14]. Another study reported that an insulin dysfunction, such as in DM, will block the AKT pathway and activate GSK-3, which then promotes tau hyperphosphorylation and neurofibrillary tangle [60]. On the other hand, this current study and previous study [61-62], have demonstrated that FO administration has an impact on the AKT/GSK pathway, which prevents tau hyperphosphorylation and eventually neuron apoptosis. Additionally, MFO administration in this study also demonstrated the neuroprotective ability by lowering the activity of the CASP-3 gene in rats that had been exposed to in STZ-LPS. According to Wójcik and colleagues, CASP-3 has been linked to neuronal death, learning impairment, and memory loss [63]. Although the present study could not pinpoint the specific compound within MFO responsible for these effects, previous studies have shown that omega-3 fatty acids—a key component of fish oil—can facilitate AKT translocation, leading to efficient phosphorylation and activation of AKT, inhibition of GSK-3 activity, and suppression of CASP-3 activation and subsequent cell death [64,65].

Given that MFO contains a higher concentration of EPA and DHA compared to many other omega-3 sources, it is plausible that its unique composition enhances these neuroprotective pathways, warranting further investigation.

Several limitations of the current study should be noted. First, behavioral assays, such as the Morris Water Maze or Y-maze, were not included to assess cognitive function outcomes, which limits the ability to directly correlate molecular changes with functional improvements. This limitation will be addressed in future studies, as behavioral tests are critical for confirming the translational relevance of these findings. Second, only one tau phosphorylation site (Ser202) was analyzed via immunofluorescence, and Western blot confirmation of protein-level changes for AKT, GSK-3, and CASP-3 was not performed. Additionally, normalization of RT-qPCR data was performed using ACTB without validation of reference gene stability across all treatment groups, which we recognize as another limitation. Finally, MFO was the sole omega-3 source tested, thus limiting our ability to determine which fish oil is most effective in avoiding the onset of AD-related DM. This limitation is acknowledged, and additional study is required to directly compare MFO with other omega-3 sources to clarify its advantages.

In summary, this preclinical study demonstrates that MFO supplementation mitigates tauopathy in STZ-LPS-induced rats through modulation of tau phosphorylation and regulation of the AKT/GSK-3 signaling pathway. Both 1 g/kg and 3 g/kg MFO treatments significantly attenuated tau pathology to levels comparable to controls, supporting MFO's potential as a neuroprotective dietary intervention. Future studies incorporating behavioral assessments, additional tau phosphorylation sites, and protein-level validation are warranted to further elucidate MFO's therapeutic efficacy and mechanism of action.

5. Conclusions

In conclusion, this present study demonstrates that MFO supplementation effectively mitigates tauopathy in STZ-LPS-induced rats by reducing total tau levels and tau phosphorylation at Ser202, as well as modulating the AKT/GSK-3 signaling pathway and downregulating apoptotic markers such as CASP-3. Both 1 g/kg and 3 g/kg MFO doses achieved comparable neuroprotective effects, suggesting that MFO's therapeutic impact is robust within this dosage range. These findings indicate that MFO plays a significant role in preventing neurodegeneration through the inhibition of tau hyperphosphorylation and the prevention of neuronal apoptosis. By providing molecular evidence that links omega-3 fatty acids supplementation to the regulation of tau phosphorylation and neuronal survival, this study adds to the growing body of knowledge supporting nutritional interventions in neurodegenerative disorders. Future studies should incorporate behavioral assessments, additional tau phosphorylation markers, and protein-level validations (e.g., Western blotting) to further strengthen the mechanistic understanding and translational potential of MFO as a neuroprotective agent.

Availability of Data and Materials

All data are available in this study

Author Contributions

Conceptualization, N.T., and H.A.; Methodology, N.T., and H.A.; Investigation, N.T., and A.F.; Writing – Original Draft, N.T.; Writing – Review & Editing, N.T., A.F., and H.A.; Funding Acquisition, N.T., and H.A.; Supervision, I.S.A.R., and N.S.

Ethics Approval and Consent to Participate

The Institutional for Animal Care and Use Committee (IACUC) at Universiti Putra Malaysia, Selangor, Malaysia, approved all experimental care and procedures for the animal research (Approval NO: UPM/IACUC/AUP-R017/2022).

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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(Original Research)

Ethanolic Blueberry Extract Inhibits Tubular Injury, Inflammation, and Oxidative Stress in a Mouse Model of Kidney Fibrosis

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Abstract

Background: Kidney fibrosis is a progressive condition characterized by tubular injury, inflammatory cell infiltration, and oxidative stress resulting from increased reactive oxygen species. The blueberry exhibits strong antioxidant and anti-inflammatory properties, rendering it a promising natural therapy for inhibiting kidney damage. This study aimed to evaluate the therapeutic effects of this extract on tubular injury scores, the number of inflammatory cells, interleukin-1 beta (IL-1ß) expression, and malondialdehyde (MDA) levels in a Swiss Webster mouse model of kidney fibrosis. **Methods:** An experimental design was conducted using 25 male Swiss Webster mice, divided into five groups: a control group and four groups with unilateral ureteral obstruction (UUO), with or without blueberry extract therapy. The crude 70% ethanolic blueberry (*Vaccinium corymbosum*) extract was administered orally (1500 mg/kg body weight) via gavage for 7 or 14 days**,** followed by histological and biochemical analysis of the harvested kidneys. **Results:** UUO significantly increased tubular injury, inflammatory cell infiltration, IL-1ß expression, and MDA levels compared to the control group. Mice treated with blueberry extract showed a 22–13% reduction in tubular injury scores, a 25–21% decrease in inflammatory cell counts, a 39–34% reduction in IL-1ß expression, and a 7–5% decline in MDA levels at 7 and 14 days, respectively. These therapeutic effects were attributed to the extract's ability to suppress inflammation and inhibit lipid peroxidation triggered by oxidative stress. **Conclusions:** The crude ethanolic extract of *Vaccinium corymbosum* demonstrates significant potential as a natural therapeutic agent in reducing kidney damage, inflammation, and oxidative stress in kidney fibrosis.

Keywords

Vaccinium corymbosum, Kidney fibrosis, Tubular injury, Oxidative stress, IL-1ß marker

1. Introduction

Kidney fibrosis is a progressive pathological condition that contributes significantly to chronic kidney disease and eventually leads to end-stage renal disease. It is characterized by the excessive accumulation of extracellular matrix (ECM) proteins in the renal interstitium, which results in structural damage, impaired kidney function, and irreversible loss of renal tissue [1]. Since fibrosis is a common final pathway in nearly all forms of chronic kidney disease (CKD), it poses a major global health concern due to its rising prevalence and the lack of effective long-term therapies [2]. To better understand and develop

treatments for this condition, researchers often use the unilateral ureteral obstruction (UUO) model in animal studies. This model involves surgically blocking one ureter to mimic the obstructive damage that leads to kidney fibrosis [3]. The resulting pathological changes, such as tubular epithelial injury, immune cell infiltration, and ECM deposition [4], closely resemble those seen in human renal fibrosis, making UUO a reliable and relevant method for evaluating potential therapeutic interventions.

A key process driving the progression of kidney fibrosis is chronic inflammation. Injury to renal tissue activates pro-inflammatory signaling pathways and stimulates the release of cytokines like interleukin-1β (IL-1β), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) [5]. These cytokines are produced by resident renal cells and infiltrating immune cells, including macrophages and lymphocytes, in response to injury. The accumulation of these inflammatory mediators promotes the recruitment of additional immune cells, leading to a sustained inflammatory response [6]. Inflammation is linked to oxidative stress, where reactive oxygen species (ROS) are produced in excess, leading to lipid peroxidation, protein degradation, and DNA damage [7]. In the UUO model, ischemia and cellular injury cause elevated ROS generation [8], which enhances inflammatory pathways, stimulates fibroblast activation, and ECM deposition [9]. One of the by-products of lipid peroxidation is malondialdehyde (MDA), a reactive aldehyde commonly used as a biomarker for oxidative stress [10]. Elevated MDA levels in renal tissue indicate ongoing peroxidative damage to cell membranes and are associated with disease severity in kidney fibrosis [11]. Evaluating MDA concentration provides insight into the oxidative stress in the renal environment and the effectiveness of antioxidant therapies.

Given the key roles of inflammation and oxidative stress in kidney fibrosis, therapeutic strategies that target these mechanisms hold considerable promise [12]. Synthetic drugs have shown some effectiveness, but their long-term use is limited by adverse effects. Natural products with antioxidant and anti-inflammatory properties, such as blueberries (*Vaccinium spp.*), have gained interest due to their rich anthocyanin content, strong bioactive flavonoids [13,14]. This study investigates whether a crude ethanolic extract of blueberry can protect against UUO-induced kidney damage in mice by reducing tubular injury, suppressing IL-1β expression, lowering MDA levels, and ultimately attenuating fibrosis. The findings may support the use of natural dietary compounds in managing chronic renal diseases and improving therapeutic outcomes.

2. Materials and Methods

2.1 Blueberry extract preparation

Blueberry extract used in this study was prepared by maceration, a method known for effectively isolating bioactive compounds from plant material. A total of 500 grams of air-dried blueberries (*V. corymbosum)*, purchased from PT. Mustika Karya Anugrah, a certified supplier in Tangerang, Indonesia) were immersed in 2500 mL of 70% ethanol (Merck®, analytical grade, 96% ethanol diluted with distilled water). The maceration process lasted 72 hours at room temperature, allowing anthocyanins and other phenolic compounds to dissolve into the solvent. Following this, the solution underwent evaporation using a rotary evaporator (Buchi R-300, Switzerland) at 40°C to remove the ethanol and concentrate the extract. The final extract was re-dissolved in 0.5% carboxymethylcellulose (CMC) as a vehicle before oral administration. This procedure followed the optimized extraction method described by Ćujić *et al.* (2016), ensuring maximum retention of antioxidant constituents [15].

2.2 Experimental animals and housing conditions

The experimental subjects consisted of 25 male Swiss Webster mice, aged between six to eight weeks, with body weights ranging from 20 to 30 grams. Male mice were selected to minimize hormonal fluctuations present in females, which can influence physiological responses, thereby ensuring uniformity of data. The mice were obtained from a certified laboratory animal supplier and acclimatized for seven days before the experiment began. During the acclimatization period, animals were housed in standard laboratory cages under controlled environmental conditions: a temperature of 22–25°C, relative humidity of 50–60%, and a 12-hour light-dark cycle. Mice were provided with commercial rodent chow (702P, Gold Coin Feedmills Sdn Bhd, Malaysia) and water *ad libitum*. All animal procedures conformed to institutional ethical standards for laboratory animal research.

2.3 Experimental design and procedures

The mice were randomly divided into five groups, each consisting of five animals: Group A: Sham-operated control group (no UUO, no treatment); Group B: UUO-induced kidney fibrosis for seven days (UUO, no treatment); Group C: UUO-induced fibrosis with blueberry extract treatment for seven days (UUO, blueberry extract for seven days); Group D: UUO-induced kidney fibrosis for 14 days (UUO, no treatment); Group E: UUO-induced fibrosis with blueberry extract treatment for 14 days (UUO, blueberry extract for 14 days). This grouping allowed for comparisons between untreated and treated fibrosis, both at early and later stages of disease development.

2.4 Induction of kidney fibrosis: unilateral ureteral obstruction (UUO)

Kidney fibrosis was induced using the unilateral ureteral obstruction (UUO) technique, a widely validated model that mimics progressive renal interstitial fibrosis. Mice were anesthetized with a combination of ketamine (70 mg/kg body weight, bw) and xylazine (15 mg/kg bw), administered intraperitoneally. After confirming adequate anesthesia, the mice were positioned in lateral recumbency, and the right flank area was shaved and disinfected with povidone-iodine.

A 1.5 cm incision was made to expose the right kidney and ureter. The ureter was carefully dissected and ligated at both the proximal and distal ends using 3/0 silk sutures to prevent urine flow. The kidney was then returned to the peritoneal cavity, and the incision was sutured in layers using 2/0 silk thread. Post-operative care included oral administration of ibuprofen as analgesics (30 mg/kg bw) once daily for three days and daily monitoring for signs of distress. This method followed the procedure outlined by Hesketh *et al.* in 2014 [16].

2.5 Blueberry extract administration

Blueberry extract treatment was initiated 24 hours after the UUO procedure. Mice in groups C and E received blueberry extract orally via gavage once daily at a dose of 1500 mg/kg bw, as adapted from the study by Fauzi *et al*. (2020) [17]. Group C received the blueberry extract for seven consecutive days, while group E was treated for 14 days. The dose and duration were chosen based on prior evidence indicating the biological activity of blueberry extract and the development of kidney fibrosis. Groups A, B, and D, which served as the control group, received 10 mL/kg of 0.5% CMC by oral gavage.

2.6 Kidney Harvesting and Tissue Processing

At the end of the respective treatment durations (7 or 14 days), the mice were euthanized by cervical dislocation under anaesthesia. The abdominal cavity was dissected to expose the kidneys. The right kidney from each mouse, which had been subjected to UUO, was harvested and processed for histological and oxidative stress analyses.

For histological evaluation, kidney tissues were rinsed in phosphate-buffered saline (PBS) and fixed in 10% buffered formalin. Fixed tissues were embedded in paraffin and sectioned for staining. A portion of the kidney tissue was also snap-frozen in aluminium foil, stored in labelled tubes, and preserved on ice for MDA level assessment.

2.7 Assessment of tubular injury

Hematoxylin and eosin-stained histological scoring of tubular injury was performed by two independent observers who were blinded to treatment groups to ensure unbiased evaluation. Ten randomly selected non-overlapping fields from each slide were examined under 200x magnification, focusing on the renal cortex. The tubular injury parameters included dilatation, epithelial desquamation, and brush border loss. A semi-quantitative scoring system (Table 1) by Kim *et al.* (2009) was used to grade the severity of injury on a scale from 0 (normal) to 4 (extensive damage, >75% of tubules affected) [18].

2.8 Inflammatory cell quantification

To quantify inflammatory cell infiltration, kidney tissue sections were stained with hematoxylin and eosin and observed under 400x magnification. Mononuclear inflammatory cells were counted in five fields per sample using the

Table 1. Tubular injury scoring scale.

Tubular Injury Score	Description
0	Normal
1.0	Minor, tubular injury < 25%
2.0	Moderate, tubular injury 25–50%
3.0	Severe, tubular injury 50–75%
4.0	Extensive, tubular injury > 75%

Fiji ImageJ software, as described by Permata and Febrianto in 2019 [19]. This provided a reliable estimation of leukocyte infiltration in the renal interstitial area.

2.9 Immunohistochemical Analysis of IL-1β Expression

Interleukin-1 expression in kidney tissues was analyzed using immunohistochemistry. Sections were incubated with IL-1ß Polyclonal Antibody (bs-6319R Rabbit polyclonal, validated for mouse tissue, 1:250 dilution; Bioss®, USA) as the primary antibody. The staining protocol was conducted using the N-Histofine® Simple Stain™ MAX PO (MULTI) kit (Nichirei Biosciences Inc., Japan), which contains polymer-conjugated secondary antibodies and peroxidase. Diaminobenzidine (DAB, 1:40) served as the chromogen substrate, and Mayer's Hematoxylin (1:3) was used for counterstaining. Slides were mounted with Entellan mounting and examined under 400x magnification. Digital images were analyzed using Immunoratio software, which quantified IL-1ß expression as the percentage of DAB-stained nuclear area relative to total nuclear area.

2.10 Measurement of malondialdehyde (MDA) levels

Lipid peroxidation was assessed by determining MDA concentration using the thiobarbituric acid reactive substances (TBARS) method. Approximately 0.15 grams of kidney tissue were homogenized in a 0.9% NaCl solution. The homogenate was mixed with distilled water and 1% sodium thiobarbiturate, followed by centrifugation at 1000 rpm for 10 minutes. The supernatant was collected, and absorbance was measured at 532 nm using a spectrophotometer. MDA concentration was determined based on a standard curve and expressed in ng/mL, following the methodology outlined by Fauziah in 2018 [20].

2.11 Statistical analysis

Quantitative data, including MDA levels, IL-1ß expression, and inflammatory cell counts, were analyzed using One-way Analysis of Variance (ANOVA), followed by Tukey's post hoc test to determine statistical differences between groups. Prior to conducting the ANOVA, the data were tested for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test), which confirmed that the assumptions were met. Tubular injury scores, being ordinal in nature, were analyzed using the non-parametric Kruskal-Wallis test. Pairwise comparisons were conducted using Dunn's multiple comparison test where appropriate. A significance level of $\alpha = 0.05$ was used for all tests. Data analysis was performed using SPSS software. Statistical analyses were performed using IBM SPSS Statistics for Windows, version 26.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1 Macroscopic Kidney Changes

Kidneys of the sham group (Group A), which underwent surgery without ureteral obstruction, appeared normal in size, shape, and color. The kidneys were symmetrical, soft, and displayed a distinctive dark red coloration (Fig. 1), indicative of healthy renal morphology in mice. Meanwhile, untreated kidneys subjected to UUO (Groups B and D) demonstrated clear pathological changes. Specifically, these kidneys appeared enlarged with hydronephrosis, pale, and tense upon gross examination (Fig. 1). Prolonged obstruction for 14 days in Group D resulted in more pronounced discoloration and tissue rigidity. Nevertheless, kidneys treated with blueberry for seven days (Group C) and 14 days (Group E) showed improvement, as evidenced by a decrease in renal volume and the resolution of hydronephrosis features (Fig. 1).

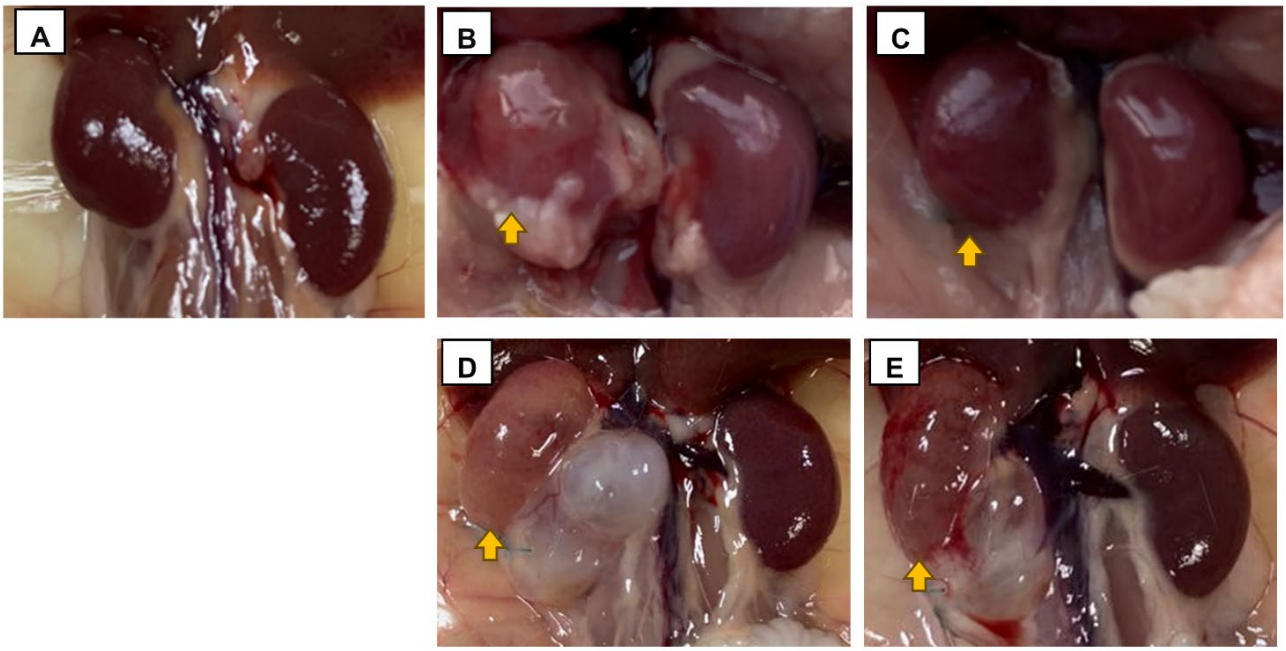

Figure 1. Macroscopic examination of kidneys from UUO-induced mice revealed distinct morphological differences among experimental groups. A) The sham-operated control group exhibited kidneys of normal volume and coloration. B) In contrast, kidneys from UUO-induced mice on day 7 appeared pale and enlarged. C) However, UUO-induced mice treated with blueberry extract for 7 days maintain kidney volume and color similar to the sham-operated control. D) By day 14, UUO-induced mice showed further deterioration, characterized by a pale appearance, approximately double the normal kidney volume, and evident hydronephrosis. E) Interestingly, blueberry treatment for 14 days partially mitigated these effects, resulting in kidneys with reduced hydronephrosis and less pallor than untreated UUO kidneys. Note: Yellow arrows indicate that the right kidneys are subjected to unilateral ureteral obstruction.

3.2 Tubular Injury Scores

The sham group (A) exhibited normal kidney histology, characterized by intact brush borders and no signs of desquamation or dilation (Fig. 2A). Statistical analysis using the Kruskal-Wallis test followed by the Mann-Whitney U test confirmed significant differences among the groups ($p < 0.05$) (Table 2). The UUO-7 group (B) exhibited moderate tubular damage, with an average injury score of 1.78 ± 0.13, which was significantly higher than that of the sham group (A). The severity increased further in the UUO-14 group (D), with a mean score of 3.85 ± 0.13, indicating extensive tubular injury. Groups treated with blueberry extract exhibited reduced injury scores. Group C (UUO + blueberry 7 days) had a mean score of 1.38 ± 0.10, representing a 22.47% decrease compared to Group B. Similarly, Group E (UUO + blueberry 14 days) recorded a score of 3.35 ± 0.06, a 12.99% reduction from Group D.

3.3 Inflammatory Cell Infiltration

The number of inflammatory cells in renal tissue (Table 3) varied significantly (p <0.05) among the experimental groups following UUO induction. Group A (sham-operated control) showed the lowest inflammatory cell count (5.5 ± 2.38), reflecting normal kidney histology. Group B (UUO for seven days) exhibited an increase in inflammatory cells (68.25 ± 11.33), while Group D (UUO for 14 days) had the highest level of infiltration (78.75 ± 6.99). Administration of blueberry extract notably reduced inflammatory cell infiltration. Group C (UUO for seven days + blueberry extract) had an inflammatory cell count of 50.75 ± 7.41, which was significantly lower than Group B. Group E (UUO for 14 days + blueberry extract) had a count of 62.25 ± 6.34, which was substantially lower than Group D.

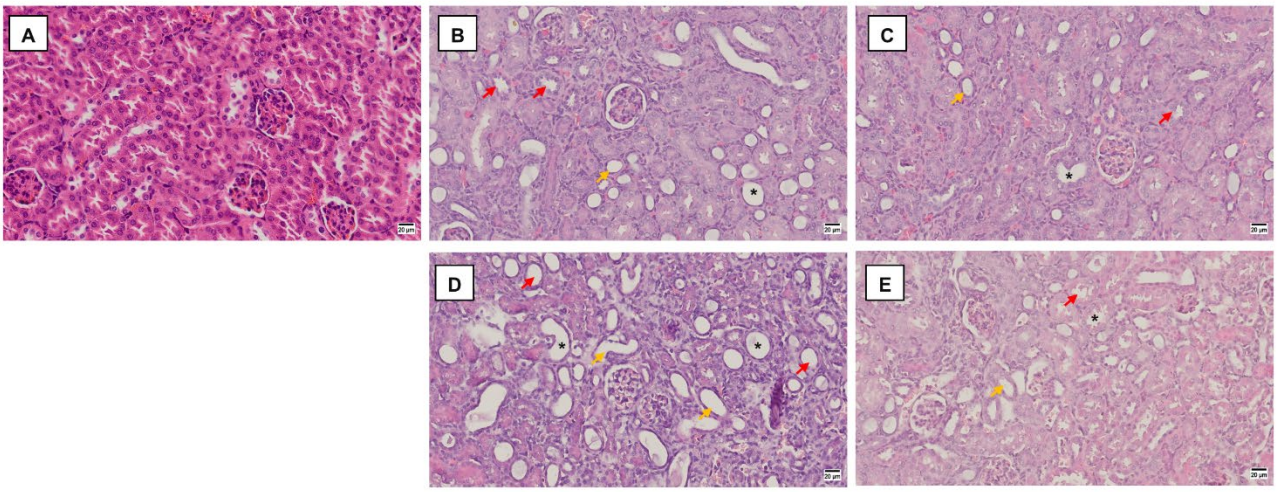

Figure 2. Microscopic evaluation of kidney tissues from UUO-induced mice treated with blueberry extract revealed progressive histological changes. The sham-operated control (A) exhibited normal renal architecture characterized by intact tubules and glomeruli. In contrast, the UUO-induced mice at day 7 (B) showed mildly dilated tubules (*), accompanied by epithelial desquamation (yellow arrow) and noticeable loss of brush borders (red arrow). However, UUO mice treated with blueberry extract for seven days (C) demonstrated improvements, with less dilation of tubules, reduced epithelial desquamation, and preservation of brush borders. By day 14, untreated UUO mice (D) displayed severe renal injury, including tubular necrosis and extensive epithelial loss. Notably, kidneys from mice treated with blueberry extract for 14 days (E) exhibited normal glomerular structures, despite persistent tubular necrosis and epithelial loss. Annotations in the images include dilated tubules (*), epithelial desquamation (yellow arrows), loss of brush borders (red arrows) (H&E staining; 200x magnification; 20 μm scale bar).

Table 2. Tubular injury score in the mouse kidney following the UUO procedure.

Experimental Groups	Tubular Injury Score Mean ± SD
Group A (Sham-operated control group)	0.00 ± 0.00^{a}
Group B (UUO-7 days)	1.78 ± 0.13^{abc}
Group C (UUO-7 days + blueberry extract)	1.38 ± 0.10^{ab}
Group D (UUO-14 days)	3.85 ± 0.13^{c}
Group E (UUO-14 days + blueberry extract)	3.35 ± 0.06^{bc}

Note: Different notations indicate significant differences between treatment groups (p <0.05)

Table 3. Inflammatory cell counts in the mouse kidney following the UUO procedure.

Group	Mean number of inflammatory cells
Group A (Sham-operated control group)	5.5 ± 2.38^{a}
Group B (UUO-7 days)	68.25 ± 11.33^{cd}
Group C (UUO-7 days + blueberry extract)	50.75 ± 7.41^{b}
Group D (UUO-14 days)	78.75 ± 6.99^{d}
Group E (UUO-14 days + blueberry extract)	62.25 ± 6.34^{bc}

Note: Different notations indicate significant differences between treatment groups (p <0.05)

3.4 Interleukin-1β Expression

IL-1β expression in kidney tissue sections increased in UUO-induced mice, and blueberry treatment reduced this expression (Fig. 3). Statistical analysis using the ANOVA, followed by Tukey's post hoc test, confirmed significant differences among the groups (Table 4). The sham-operated control group (Group A) showed the lowest IL-1β expression (9.77 ± 1.85%) among all groups. Group B (UUO for seven days) demonstrated a higher expression (25.03 ± 4.02%) compared to Group A. Further, Group D (UUO for 14 days) showed the highest expression among all groups (36.18 ± 1.52%). Meanwhile, Group C (UUO for seven days + blueberry extract) showed a significantly lower IL-1β expression (15.20 ± 2.34%) compared to Group B. Similarly, Group E (UUO for 14 days + blueberry extract) exhibited markedly reduced IL-1β expression (23.81 ± 1.69%) relative to Group D.

3.5 Malondialdehyde (MDA) Levels

Table 5 presents the MDA levels measured in mouse kidney tissue across all experimental groups. The sham-operated control group (Group A) exhibited the lowest MDA concentration, with a mean value of 364.94 ± 10.20 ng/mL, indicating basal oxidative status under normal conditions. A marked increase in MDA levels was observed in Group B (UUO for seven days), with a mean value of 414.11 ± 3.69 ng/mL, while a further increase was recorded in Group D (UUO for 14 days), which showed the highest MDA level among all groups (448.00 ± 8.56 ng/mL). Administration of blueberry extract was associated with lower MDA levels in both the 7-day and 14-day UUO groups. In Group C (UUO for seven days + blueberry extract), the MDA level was 385.50 ± 1.90 ng/mL, which was markedly reduced compared to Group B. Similarly, Group E (UUO for 14 days + blueberry extract) had an MDA level of 424.94 ± 2.63 ng/mL, which was significantly lower than that of Group D.

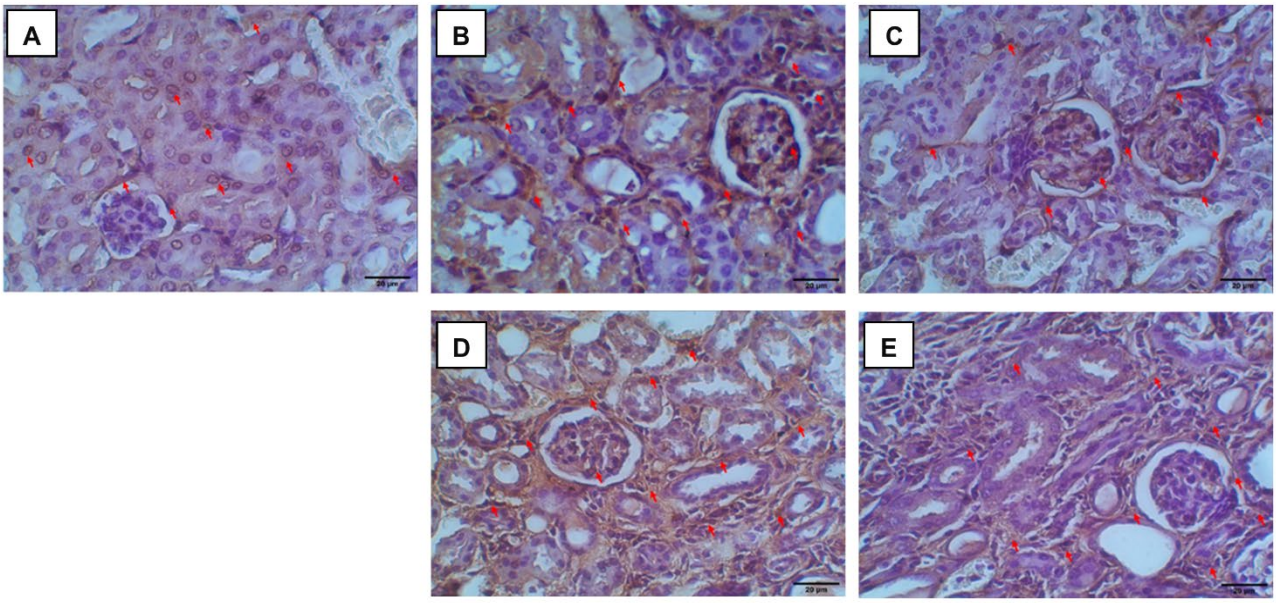

Figure 3. Micrographs illustrating IL-1β expression in the kidneys of UUO-induced mice treated with blueberry extract revealed distinct patterns across experimental groups. The sham-operated control group (A) exhibited minimal IL-1β expression. By day 7, untreated UUO-induced mice (B) demonstrated a mild increase in IL-1β immunoreactivity within glomeruli, renal tubules, and the interstitial areas. Conversely, UUO-induced mice treated with blueberry extract for seven days (C) showed comparatively lower IL-1β expression in these renal compartments. At day 14, untreated UUO-induced mice (D) exhibited severe IL-1β expression, particularly pronounced within interstitial areas, tubules, and glomeruli. However, the group treated with blueberry extract for 14 days (E) displayed substantially reduced IL-1β expression in the interstitial spaces, tubules, and glomeruli. Red arrows in the images specifically highlight IL-1β expression localized in the glomerulus and interstitial tubules. (400x magnification; 20 μm scale bar).

Table 4. Percentage area of IL-1ß expression in the mouse kidney following the UUO procedure.

Group	Mean IL-1β Expression (% area)
Group A (Sham-operated control group)	9.77 ± 1.85^{a}
Group B (UUO-7 days)	25.03 ± 4.02^{c}
Group C (UUO-7 days + blueberry extract)	15.20 ± 2.34^{b}
Group D (UUO-14 days)	36.18 ± 1.52^{d}
Group E (UUO-14 days + blueberry extract)	23.81 ± 1.69^{c}

Note: Different notations indicate significant differences between treatment groups ($p < 0.05$)

Table 5. MDA expression in the mouse kidney following the UUO procedure.

Group	Mean MDA Levels (ng/mL)
Group A (Sham-operated control group)	364.94 ± 10.20^{a}
Group B (UUO-7 days)	414.11 ± 3.69^{c}
Group C (UUO-7 days + blueberry extract)	385.50 ± 1.90^{b}
Group D (UUO-14 days)	448.00 ± 8.56^{d}
Group E (UUO-14 days + blueberry extract)	424.94 ± 2.63^{c}

Note: Different notations indicate significant differences between treatment groups ($p < 0.05$)

4. Discussion

This study investigated the therapeutic potential of an ethanolic extract of blueberry (*V. corymbosum*) in mitigating kidney damage induced by unilateral ureteral obstruction (UUO), a well-established animal model of renal fibrosis. Previous studies have established that UUO-induced animals develop kidney inflammation within three days, followed by histological alterations in one week, and develop into kidney fibrosis within two weeks [8]. In addition, a study reported that UUO induces tubular damage, macrophage infiltration, and interstitial fibrosis through upregulation of pro-inflammatory cytokines and oxidative pathways [21,22]. In this current study, findings demonstrated that blueberry extract significantly reduced tubular injury, inflammatory cell infiltration, IL-1ß expression, and MDA levels, suggesting its effectiveness in attenuating both inflammatory and oxidative pathways involved in the pathogenesis of kidney fibrosis.

Kidney fibrosis is a complex process characterized by excessive accumulation of extracellular matrix, tubular epithelial injury, and persistent inflammation [23]. UUO-induced kidney fibrosis mimics these conditions by mechanically obstructing urinary flow, resulting in ischemia, tubular damage, and inflammatory activation [24]. The observed macroscopic changes in the UUO groups, including enlarged and pale kidneys, confirm successful model induction. These morphological alterations reflect hydronephrosis and progressive fibrotic remodeling, which are consistent with earlier studies reporting progressive hydronephrosis and renal parenchymal loss in UUO models [25,26].

Histological analysis further supported these observations. In untreated UUO groups, particularly after 14 days, severe tubular injury was evident, including epithelial desquamation, tubular dilatation, and loss of the brush border. These are hallmarks of early and progressive fibrotic damage and are consistent with the ischemic injury described by Bonventre and Yang in 2011[27]. In contrast, mice treated with blueberry extract displayed notably reduced injury scores, especially in the 7-day treatment group. This suggests that the bioactive compounds in the crude extract may help preserve tubular integrity by counteracting oxidative and inflammatory insults at an early stage of fibrosis progression.

A major component in the pathogenesis of kidney fibrosis is the infiltration of mononuclear inflammatory cells, particularly macrophages and lymphocytes. These cells perpetuate tissue damage by releasing pro-inflammatory and pro-fibrotic cytokines. The significant increase in inflammatory cells observed in UUO kidneys aligns with previous reports that chronic inflammation plays a pivotal role in fibrosis [3,28]. Notably, administration of blueberry extract resulted in a substantial reduction in inflammatory cell counts, indicating that the extract effectively inhibited immune cell recruitment. This anti-inflammatory effect is consistent with prior reports on anthocyanin-rich blueberry extracts reducing inflammation and leukocyte infiltration [29].

The role of IL-1β as a pro-inflammatory cytokine was further emphasized in this study. IL-1β expression was significantly elevated in UUO groups and closely correlated with the extent of injury and inflammation. As a central mediator of inflammation, IL-1β is known to induce leukocyte recruitment, fibroblast activation, and extracellular matrix deposition [31,32]. The observed suppression of IL-1β expression in the blueberry-treated groups reinforces the conclusion that anthocyanins not only reduce leukocyte infiltration but also modulate the inflammatory cascade at a molecular level, as stated by several studies [33,34]. The mechanisms likely involve inhibition of ROS-induced inflammasome activation and reduced maturation of IL-1β from its precursor forms [35,36]. These findings are of particular relevance given that persistent IL-1β expression has been associated with chronic and progressive fibrosis [36].

Oxidative stress is another key contributor to renal fibrosis. UUO impairs renal perfusion, leading to hypoxia and excessive production of ROS. These molecules initiate lipid peroxidation, mitochondrial dysfunction, and cell apoptosis, thereby accelerating fibrotic changes [37]. The elevated MDA levels in UUO groups in this study reflect this ongoing oxidative damage. MDA, as a stable by-product of lipid peroxidation, serves as a reliable indicator of oxidative stress in renal tissues [38]. Treatment with blueberry extract significantly reduced MDA concentrations, indicating that the extract limited ROS production and protected lipid membranes from oxidative degradation. This antioxidant effect is consistent with the chemical properties of anthocyanins, which act as free radical scavengers and metal chelators [40,41].

The active compounds in blueberries, particularly delphinidin and malvidin, are known for their high radical-scavenging capacity [42,43]. In addition to directly neutralizing ROS, anthocyanins may also enhance the activity of endogenous antioxidant enzymes such as superoxide dismutase and catalase, further contributing to cellular defense [43]. The reduced MDA levels in the treatment groups, particularly in the 7-day after UUO surgery, highlight the potential of blueberry extract to attenuate oxidative damage in early-stage fibrosis. However, in the 14-day treatment group, while improvements were still significant, the effect size was smaller, possibly due to more extensive and irreversible damage at later stages of fibrosis. Indeed, during the later stages of fibrosis, this process becomes increasingly dysregulated and self-perpetuating, resulting in extensive and irreversible damage to the renal parenchyma [44].

Interestingly, across all parameters—tubular injury, inflammation, IL-1β expression, and MDA levels—both 7-day and 14-day treatments are effective, with slightly higher improvement observed on day seven. This finding suggests that earlier intervention yields greater benefits, likely because fibrosis is more responsive to antioxidant and anti-inflammatory therapies during the initial phase, before irreversible ECM deposition and tissue remodeling have occurred. Once fibrosis becomes entrenched, therapeutic agents may have limited efficacy in reversing structural changes, even if they continue to modulate inflammation or oxidative stress. A study indicated that the early phase of fibrosis is more responsive to therapy due to tissue repair dynamics, while the advanced phase of fibrosis creates a "mis-instructive" environment that activates fibrogenic cells and strengthens the pathological loop through tissue stiffness and TGF-β1 activation, making therapy difficult and often irreversible [45]. These findings underscore the importance of timely intervention in fibrotic kidney diseases and suggest that crude blueberry extract may be most effective when administered in the early stages of disease progression.

5. Conclusions

In summary, the results of this study support the hypothesis that crude ethanolic blueberry (*V. corymbosum*) extract possesses renoprotective properties in the context of obstructive kidney injury. The bioactive compounds within the extract may act through multiple mechanisms, including suppression of inflammation, modulation of cytokine expression, and inhibition of oxidative damage. These effects collectively contribute to reduced tissue injury and fibrosis. Since the extract used was crude and its phytochemical components were not quantified, further studies are needed to isolate and identify the bioactive compounds. Future research should expand upon these findings by investigating the molecular pathways modulated by its phytochemical compounds in greater detail, exploring dose-response relationships, and evaluating long-term outcomes. In addition, comparative studies involving other natural antioxidants could help contextualize the efficacy of blueberry extract within the broader spectrum of phytotherapeutic interventions. Given the increasing global burden of chronic kidney disease and the limitations of current pharmacological treatments, such natural compounds offer promising, low-toxicity alternatives or adjuncts to existing therapies.

Availability of Data and Materials

All data are available in this study

Author Contributions

Conceptualization, A.F., and N.T.; Methodology, A.F., N.T., and D.A.P.; Investigation, A.F., H.S., and V.K.; Data curation, H.S., and V.K.; Writing – Original Draft, A.F.; Writing – Review and Editing, N.T., and D.A.P.; Funding Acquisition, A.F.; Supervision, N.T., and D.A.P.

Ethics Approval and Consent to Participate

The Institutional Animal Care and Use Committee (IACUC) at Universitas Brawijaya, under number 045.KEP-UB.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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(Original Research)

Effects of Saba Banana [Musa 'Saba' (*Musa acuminata x Musa balbisiana)*] Peel Pectin Supplementation on Feeding, Fecal Weight and Adiposity Parameters of High-Fat Diet-Induced Obese Male ICR Mice

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Abstract

Background: Saba banana peels, often considered waste, are a rich source of pectin that can be used as a food ingredient and nutritional supplement. This study explored the potential of saba banana peel pectin in managing obesity, focusing on its impact on food intake and body fat. **Methods:** *In vivo* experiments were conducted using high-fat diet (HFD)-induced obese male mice. Mice were divided into groups receiving either HFD supplemented with saba banana peel (SP) pectin or commercial citrus pectin (CP) for nine weeks. Control groups included HFD-fed mice and a normal diet (ND) group. **Results:** Results showed no significant difference in daily feed intake among HFD and pectin-supplemented groups, though water intake increased with pectin supplementation. Notably, the total adiposity index (TAI) in the HFD+SP group was significantly lower than the HFD group and comparable to the HFD+CP group. Fecal weight increased in pectin-supplemented groups, suggesting the binding of unabsorbed fats, which likely contributed to weight reduction. **Conclusions:** Supplementation with 10% saba banana peel pectin significantly improved obesity-related parameters in high-fat diet (HFD)-induced obese male ICR mice, including a 13.39% reduction in body weight, 3.66% decrease in abdominal circumference, and 76.72% lower total adiposity index, along with increased water and fecal output. With a human equivalent dose of 4.87g per day for a 60-kg adult, saba banana peel pectin shows strong potential as a natural anti-obesity agent, warranting further investigation through clinical studies.

Keywords

Adiposity, Body fats, Commercial citrus pectin, Pectin, Saba banana peels pectin

1. Introduction

The World Health Organization (WHO) declared in 2016 that over 650 million people are obese and more than 1.9 billion adults are

overweight. This phenomenon has reached epidemic proportions globally and its prevalence tripled between 1975 and 2016 [1]. Rapid growth in obesity was experienced in Asia and the Pacific, with magnitude varying among countries. The increasing prevalence worldwide has a huge societal effect on the economy like health care costs, national productivity such as the cost of days of work, higher employer insurance premiums and lower wages, physiological and psychological consequences including discrimination, depression, anxiety, and self-esteem, and lower quality of life [2,3,4]. Additionally, obesity is considered a major risk factor for non-communicable diseases (NCDs) which includes diabetes mellitus (DM), cardiovascular diseases (CVDs), musculoskeletal disorders, several cancers, and metabolic syndrome [1,5,6]. In 2013, however, the American Medical Association already classified obesity as a NCD [5]. Numerous factors contribute to the development of obesity. These are genetics, low physical activity and exercise, poor diet, and other unhealthy behaviors. Except for genetics, all are modifiable factors. Hence, obesity is a preventable and curable disease [1,8,9], and lifestyle modification and a balanced diet are recognized as recommended preventive strategies [10,11,12].

Among dietary constituents, dietary fiber has been the subject of increasing attention for its effects on multiple mechanisms such as regulating food intake and body weight, greater feelings of satiety, and changes in blood glucose and insulin [12]. Dietary fiber is classified either as soluble or insoluble, with pectin as one of the widely available sources of soluble fiber. Pectin is commonly found in fruits, vegetables, and seed extracts. Commercially, it is commonly extracted from citrus peels and apple pomace and developed into a variety of products, including food supplements, gelling and thickening agents, and as an all-around stabilizer used by the food industry [12,13].

Studies have shown that pectin, a soluble dietary fiber found in both natural and artificial sources, might help manage and prevent obesity by reducing food consumption and adiposity, which is one of the many ways that dietary fiber is known to affect obesity [12,14]. One of the recently identified novel sources of pectin is the saba banana peel [Musa 'Saba' (*Musa acuminata x Musa balbisiana)*]. Saba is abundantly grown in the Philippines, but its peels currently have little to no use and, thus, are considered a waste product that can cause environmental problems. According to the study of Castillo-Israel *et. al.* [15], pectin from saba banana peels is comparable to commercial citrus pectin in terms of moisture content, gelling ability, and sensory qualities. Notably, it was found that ash content was much higher for the saba banana peels than that of commercial citrus pectin. These characteristics suggested the possible commercial use of extracted pectin from saba banana peels in food processing. However, studies are warranted to support its use as a nutraceutical product for obesity prevention and management.

Thus, the study investigated the anti-obesity potentials of pectin from saba banana peel via its effects on regulating food intake and adiposity *in vivo*. The results of this research hoped to provide bases to support initiatives promoting and utilizing saba banana peel pectin both as a functional food ingredient and an anti-obesity nutritional supplement. More importantly, this study is expected to contribute to the scant body of knowledge on the nutritional and health benefits of pectin from saba banana peels, specifically its role in obesity prevention and management.

2. Materials and Methods

Extraction of Saba Banana Peel Pectin

The 'saba' banana peel samples were obtained from banana processing industries in Lipa City, Batangas. The extraction was done at the Institute of Food Science and Technology (IFST) of the College of Agriculture and Food Science (CAFS), University of the Philippines Los Baños (UPLB) following the established protocol of Castillo-Israel *et. al.* [15] with some modifications. Briefly, the saba banana peels were washed with running water to remove adhering dirt and were dried in an oven (Memmert, Germany) at 55°C for 24 h. The dried peels were then processed into flour using a grinding mill (Oster, USA) and stored in polyethylene bags. Extraction of pectin was then facilitated by adding 20 g of ground banana peel powder to 400 mL 0.45 N citric acid (Chemline Scientific, Philippines) solution at pH 1.5. The resulting mixture was then heated at 85°C for 5h with continuous agitation. After the extraction process, the mixture was cooled and filtered

through an ordinary wire screen with a 1-mm mesh size and a two-layer cheesecloth. The filtrate was collected and added with twice its volume of 95% ethanol (Chemline Scientific, Philippines) to facilitate pectin precipitation. The precipitate was then recovered using a linen cloth lined on a Buchner funnel and was dried in an oven (Memmert, Germany) for 5h at 55°C. The dried pectin was then ground into powder and kept until used [16].

In Vivo Efficacy Test

All procedures performed in ICR mice for the efficacy study were approved by the UPLB Animal Care and Use Committee with approval no. CHE-2019-001.

Animals

A total of sixty (60) 6-week-old ICR male mice with initial weights between 22.0 ± 2 g obtained from the Research Institute for Tropical Medicine (RITM), Alabang, Muntinlupa City, were used in the study. Male ICR mice are an acceptable model for obesity and hyperglycemia studies [17], and being male prevents the interplay of sex with the variables of interest. Mice were housed individually in properly labeled polycarbonate cages with stainless steel tops (Techniplast, Italy) and maintained at 26°C, 50–60% humidity, and 12h: 12h light/dark cycle lights on at 7:00 AM and lights off at 7:00 PM at the laboratory animal experimental room, DBVS, CVM, UPLB. Commercial maintenance mice pellets (Altromin, Germany) and distilled drinking water were also provided *ad libitum* during the one-week acclimation period.

Components and Preparation of Diets

A regular diet (AIN-93G Purified Rodent Diet, Dyets, USA) and a custom high-fat (45% FDC) purified rodent diet were used for the normal and negative control groups (ND and HFD), respectively. The Dyets, Inc. (Pennsylvania, USA) feed formulation was used to develop the HFD, which contains 45 percent calories from fat. Pork lard used in the HFD was obtained by rendering fresh leaf fat surrounding the kidneys of swine—sourced from a certified local supplier—which was cleaned, chopped, and slowly heated using the dry heat method at 90–95 °C until golden cracklings formed, then filtered and stored at 4 °C for use in diet formulation. The agar solution was microwaved for 3 min to prepare the HFD. Once it reached 40°C, pork lard was added and heated for another one min at the same temperature. The mixture was transferred to an electric mixer (KitchenAid Stand Mixer ,USA) and blended thoroughly. The agar-lard solution was added to the dry ingredients and mixed until fully combined. The agar-lard solution was then added to all the dry ingredients (i.e.,Casein, L-Cystine, Sucrose, Cornstarch, Dyetrose, t-Butyl hydroquinone, Cellulose, Mineral Mix #210025, Vitamin Mix #310025, Choline Bitartrate, Salt Mix #210088, Dicalcium Phosphate, Calcium Carbonate, Potassium Citrate H20, Vitamin Mix #3000050) , and everything was combined well. In the preparation of HFD pectin mixes, the same process was followed, except that the powdered pectin was added together with all the dry ingredients. The mice's food was then transferred into a clean container, covered, labeled, and kept in a freezer until it was needed. Table 1 shows the composition and caloric content of the formulated diets.

Commercial Citrus Pectin (CP) (L# 11612929, Alysons' Chemical Enterprises, Inc., Quezon City, Philippines) and Saba Banana Peels Pectin (SP) were added to the HFD at 10% w/w with a few modifications to achieve isonitrogenous and isocaloric diets with 4.60 kcal per gram and carbohydrate-protein-fat compositions of 36-18-46 percent, respectively. The 10% dosage was based on the *in vivo* studies of Adam *et. al.* [12,14] wherein supplementation of 10% pectin (w/w) was found to significantly reduce body weight and adiposity index when using the HFD control.

Induction Period

The obesity induction was done for three (3) week, wherein mice were fed with HFD to induce obesity. Mice with more than a 20% increase from their pre-induction weight were considered obese [18] and were included in the supplementation period.

Supplementation Period

After the induction phase, the mice were randomly allocated to four (4) groups (n=7 per group), namely: 1) the normal diet group (ND), given a normal diet and served as a normal control;

Table 1. Composition and caloric content of the different diets.

Ingredients	kcal per gram	ND (g)	HFD (g)	HFD+CP (g)	HFD+SP (g)
Casein	3.58	100.0	116.54	115.77	115.55
L-Cystine	4.00	1.50	1.75	1.75	1.75
Sucrose	4.00	50.00	100.69	100.69	100.69
Cornstarch	3.60	198.7	42.42	0.00	0.00
Dyetrose	3.80	66.00	58.27	57.00	57.50
Soybean Oil	9.00	35.00	0.00	0.00	0.00
t-Butyl hydroquinone	0.00	0.007	0.003	0.003	0.003
Cellulose	0.00	25.0	29.13	29.13	29.13
Mineral Mix #210025	0.88	17.5	0.00	0.00	0.00
Vitamin Mix #310025	3.87	5.00	0.00	0.00	0.00
Choline Bitartrate	0.00	1.25	1.17	1.17	1.17
Salt Mix #210088	1.60	0.00	5.83	5.83	5.83
Dicalcium Phosphate	0.00	0.00	7.57	7.57	7.57
Calcium Carbonate	0.00	0.00	3.20	3.20	3.20
Potassium Citrate H20	0.00	0.00	9.61	9.61	9.61
Vitamin Mix #3000050	3.92	0.00	5.83	5.83	5.83
Pork Lard	9.00	0.00	118.00	117.72	117.35
Commercial citrus pectin	3.61	0.00	0.00	50.00	0.00
Saba banana peel pectin	3.59	0.00	0.00	0.00	50.00
Calorie		**500.00**	**500.00**	**505.30**	**505.20**
Total calories		**1880.02**	**2295.23**	**2312.77**	**2309.54**
Kcal per gram		**3.76**	**4.59**	**4.58**	**4.57**

ND – normal diet; HFD– high fat diet; HFD+CP– HFD w/10% commercial citrus pectin; HFD+SP– HFD w/10% Saba banana peels pectin

2) the HFD group, given with HFD, and served as a negative control; 3) the HFD + commercial citrus pectin group (HFD+CP), given with HFD + 10% (w/w) citrus pectin; and 4) the HFD + saba banana peel pectin group (HFD+SP), given with HFD + 10% (w/w) saba banana peel pectin. The ND group had mice with normal body weights, which served as a normal control, whereas the other three groups had obese mice. Animals received their respective diets *ad libitum* for nine (9) weeks of supplementation. Treatment-related effects were determined by measuring the feed and water intake, and adiposity parameters such as body weight, abdominal circumference, weight of body fats, total adiposity index (TAI), fecal weight, and adipocyte surface area.

Feed Intake Measurement

Each mouse was given 7-8g of feed (pre-weighed feeds) per day starting at 7 a.m. After 24h, the leftover feeds were measured daily for nine (9) weeks using a digital top-loading balance (Shimadzu, Japan). The leftover feeds were subtracted from the pre-weighed feeds (7-8g) to get the daily feed intake. Data were recorded to the nearest 0.01 gram. The caloric intake was computed by multiplying the daily feed intake by its equivalent calories per gram.

Water Intake Measurement

Each mouse was given 10 mL of distilled water daily, measured using a 60-mL syringe, and then placed in clean water bottles. The leftover water was measured using a 3 mL syringe cylinder starting at 7:00 a.m. daily for nine (9) weeks. The volume of leftover distilled water was deducted from the known volume of water given per mouse to get the daily water intake. Data was recorded to the nearest 1 mL.

Body Weight Measurement

Body weights of mice were measured weekly for nine (9) weeks using a digital top-loading

balance starting at 8:00 a.m. The measurement was done by placing the mouse in a container with a known weight and then placing the container with the mouse on the digital top-loading balance. The weight of the container was subtracted from the weight of the container + mouse to determine the weight of the mouse. The weight was recorded to the nearest 0.001 gram.

Abdominal Circumference Measurement

For abdominal circumference, mice were restrained by applying a scruff hold to the loose skin between ears using the thumb and forefinger with one hand while maintaining a grip on the tail with the other hand. The abdominal circumference was measured around the anterior abdomen of the mice [19], using a plastic non-flexible measuring tape with an accuracy of 0.1 cm.

Fecal Weight Measurement

Fecal samples were collected every week throughout the 9-week supplementation period. The collected feces were weighed using an analytical balance (Shimadzu, Japan) with the results recorded to the nearest 0.001g.

Euthanasia, Necropsy, and Tissue Collection, Processing, and Examination

Mice were euthanized via intraperitoneal injection with pentobarbital sodium using a 100 mg/kg dose. Necropsy was performed by licensed veterinarians, and any gross abnormalities in the pancreas and abdominal fat tissues were recorded. The weight of the pancreas and abdominal fats was also measured using an analytical balance. Pancreas and adipose tissue samples were obtained, fixed in 10% formalin for at least 72h, processed with routine paraffin technique, sectioned at 5 um thick, stained with H&E, and examined under the microscope. Histopathological changes in adipose tissues and the pancreas were assessed by a veterinary pathologist.

Total Adiposity Index (TAI)

The TAI, which accounts for the total fat tissues in the body of mice, served as a parameter of adiposity [20]. Different pad tissues were dissected and weighed. Total body fat was calculated as the sum of the following individual fat pad weights, and the TAI was computed following the formula indicated below [21].

Total body fat: epididymal fat + retroperitoneal fat + visceral fat.

Adiposity Index = (total body fat / final body weight) × 100.

Mean Adipocyte Surface Area

The mean diameters of abdominal white adipocytes were calculated as an indicator of adipocyte hypertrophy in restricted view fields on a computer monitor using an automated image analysis system (Image J). The mean diameter was expressed in μm, and a minimum of three white adipocytes per fat pad was measured.

Animal Dose to Human Dose Conversion

The human equivalent dose (HED) was calculated using the km value. HED was calculated following the formula indicated in the study of Shin *et. al.* [22]:

$$\text{HED (mg/kg)} = \text{animal dose (mg/kg)} \frac{\text{Animal Km}}{\text{Human Km}}$$

Statistical Analysis

The data was analyzed using SPSS Statistics Version 26 [23]. All tests were evaluated with a significance level of 0.05. A one-way ANOVA followed by Tukey-Kramer HSD as a post-hoc test was used to determine whether there were any significant differences in the variables across the various groups. A paired t-test and a Wilcoxon signed rank test were employed to determine the significant difference between time points in each group, while the Pearson R correlation test was used to measure the strength and significance of the relationship between the two variables.

3. Results

Effect of SP on Feed Intake

Feed Intake

The mean daily feed intake of the mice groups from the start of the obesity induction period up to

the supplementation period shows that the ND mice group consistently consumed a significantly lesser amount of food than the HFD, HFD+CP, and HFD SP groups throughout the feeding duration (Fig. 1). The HFD groups showed a consistently similar daily feed intake from baseline to endline supplementation. The mean daily intake of HFD+CP was 5.22 ± 0.185 g and HFD+SP was 5.31 ± 0.119 g, which were lower compared to the HFD group but higher than the ND group. Notably, the HFD group had the highest mean daily food intake (5.38 g±0.196 g), followed by HFD+SP and HFD+CP, but there was no significant difference between these groups.

Water Intake

Figure 3 shows the mean daily water intake of mice from the baseline of induction to the endline supplementation period. During the induction period, a significantly lower water intake was noted in the HFD group compared with the ND group. During the supplementation period, mice from the HFD group consistently consumed the least amount of water. The addition of pectin HFD, however, led to increased water intake, as evidenced by the significantly higher water intakes in the HFD+CP vs. HFD and HFD+SP vs. HFD groups ($p<0.000$ and $p<0.005$, respectively).

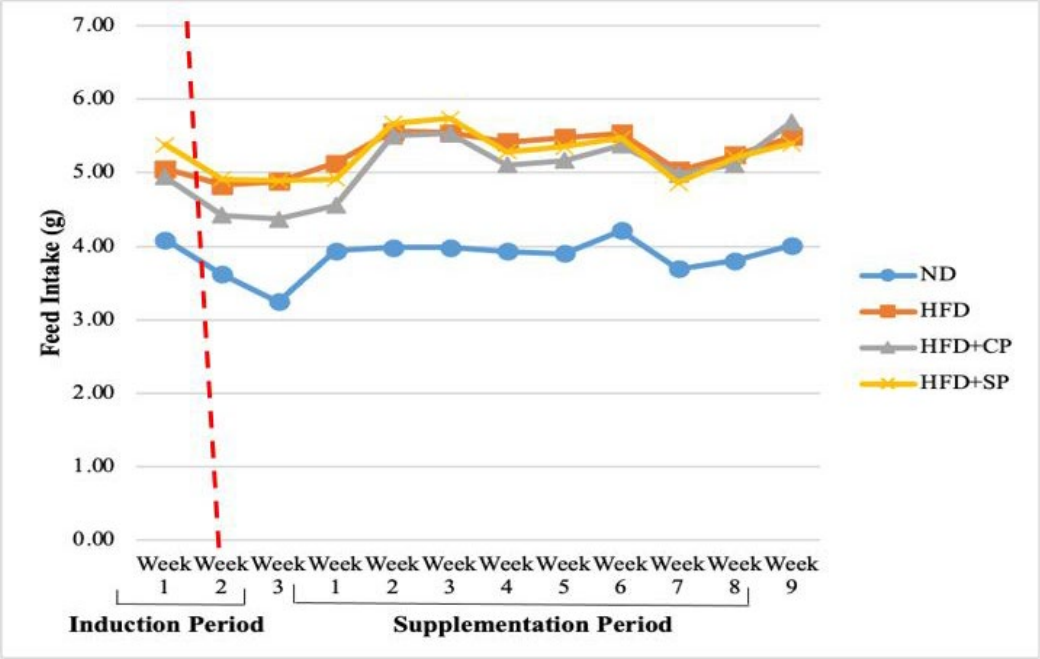

Figure 1. Mean daily feed intake of male ICR mice in the different treatment groups from induction to supplementation periods.

Caloric Intake

Figure 2 shows the mean daily caloric intake of all mice groups from the baseline of induction to the endline of the supplementation period. The same observation was made in the mean daily feed intake between groups in terms of caloric intake. Throughout the induction and supplementation periods, the ND group consumed significantly fewer calories than the HFD, HFD+CP, and HFD+SP groups ($p<0.000$, $p<0.000$, and $p<0.000$, respectively). There was, however, no significant difference in the caloric intake among mice in the HFD, HFD+CP, and HFD+SP groups.

Effect of Pectin from Saba Banana Peels on Adiposity Parameters

This section shows the mean body weights, abdominal circumference, body fats, TAI, and adipocyte area of ICR mice during the supplementation period. Data have revealed that the body weights of HFD+CP and HFD+SP groups were comparable and significantly different to HFD groups at endline supplementation ($p<0.003$, and $p<0.023$, respectively). Notably, the HFD+CP and HFD+SP groups' abdominal circumference was lower than the HFD group, although no significant difference was noted. The % TAI of the

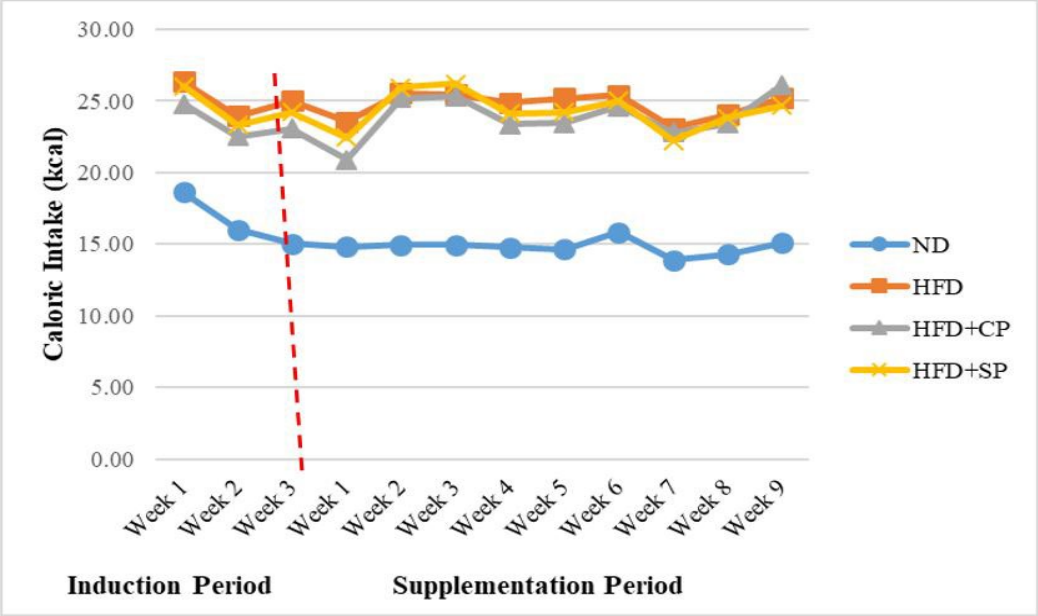

Figure 2. Mean daily caloric intake of male ICR mice in the different treatment groups from induction to supplementation periods.

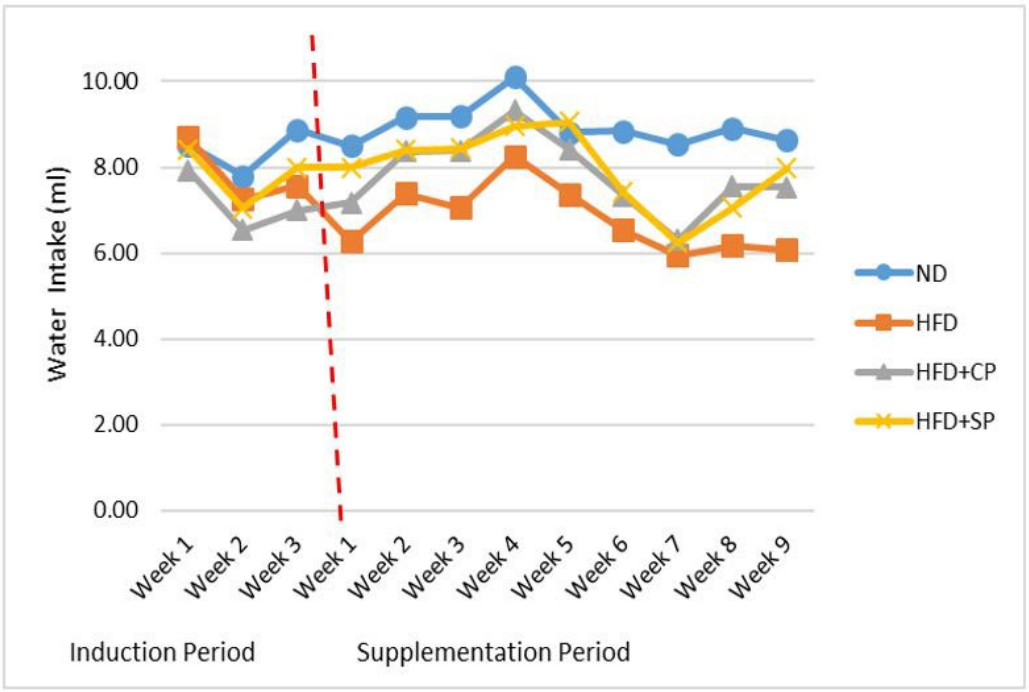

Figure 3. Mean daily water intake of male ICR mice in the different treatment groups from induction to supplementation periods.

HFD+CP and HFP+SP groups were comparable and significantly different from the HFD group ($p<0.001$ and $p<0.010$, respectively). The abdominal fat (A. fat), mesenteric fat (M. fat), and heart fat (H. fat) were lower in the HFD+SP group compared to the HFD+CP group and HFD group. In comparison, subcutaneous (S. fat) and epididymal fat (E. fat) in HFD+CP were significantly lower than in HFD+SP and HFD groups ($p<0.004$ and $p<0.006$, respectively). Lastly, the mean adipocyte surface area of HFD was higher compared to the HFD+SP and HFD+CP groups.

Bodyweight

The body weight of all mice groups increased throughout the supplementation period, as shown in Table 2. This indicates the normal growth of mice as they age. Also, the HFD+CP and HFD+SP groups had a steady weight gain, demonstrating no obvious treatment-related harm from pectin supplementation. As observed in the HFD+SP and HFD+CP groups, supplementation with pectin had almost the same effects on body weight except for the remaining four (4) weeks of supplementation. During this period, there was a modest increase in body weight among mice in the HFD+SP group. It was also noted that the HFD+SP and HFD+CP groups showed a clear steady body weight gain from week 1 to week 5, where mice were 10-14 weeks old. This was despite continued access to a high-fat diet as compared with the HFD group, which had a sharp increase in body weight.

Table 2. Mean body weight of male ICR mice in the different treatment groups during the supplementation period.

Animal Groups	Baseline Supplementation (g)	Endline Supplementation (g)
ND	$29.42 \pm 0.580^{a,*}$	$32.31 \pm 1.115^{a,+}$
HFD	$31.40 \pm 0.566^{b,*}$	$39.19 \pm 0.977^{b,+}$
HFD+CP	$31.40 \pm 0.566^{b,*}$	$33.19 \pm 0.889^{a,+}$
HFD+SP	$31.40 \pm 0.566^{b,*}$	$34.56 \pm 1.202^{a,+}$

ND – Normal Diet; HFD – HFD; HFD+CP – HFD w/ commercial citrus pectin; HFD+SP – HFD w/ Saba Banana peels pectin
Values represent the mean ±S.E. (ND n = 7; HFD n= 7; HFD+CP n=7; HFD+SP n=7)
Means in the same column followed by a different letter are significantly different at $p<0.05$.
Means in the same row followed by a different symbol are significantly different at $p<0.05$.

At the start of the supplementation period, the HFD group and pectin-supplemented (HFD+CP and HFD+SP) groups had comparable mean body weights and were significantly higher than the ND group ($p<0.005$). But at the end of the supplementation, the body weights of the HFD+CP and HFD+SP groups were significantly lower than the HFD group ($p<0.003$, and $p<0.023$, respectively) and statistically comparable with the ND group despite continued access to unlimited high-fat food. In terms of weight gain, the HFD+CP group had the lowest body weight gain and percent weight change (1.79 g and 5.69%, respectively), which were even lower than the ND group (2.89 g and 9.84%, respectively). The HFD+SP group's mean body weight gain and percent weight change (3.16 g and 10.05%, respectively) were a little higher than both the ND group and the HFD+CP group. Nonetheless, the weight gains of both pectin-supplemented groups showed no significant difference with the ND group while statistically lower than the HFD group ($p<0.005$). These findings may suggest the anti-obesity potential of both saba pectin and commercially available citrus pectin.

Abdominal Circumference

At baseline, the mean abdominal circumference of all mice groups was statistically comparable (Table 3). At the endline, the HFD group had the highest abdominal circumference and ND had the lowest value, but no significant difference was found between the control (ND and HFD groups) and pectin-supplemented (HFD+CP and HFD+SP groups). Interestingly, HFD+SP had a significantly greater reduction in mean difference and percent change in abdominal circumference, followed by the ND group and the HFD+CP group. Given that continued and prolonged access to a high-fat diet significantly increased the abdominal circumference in the HFD group ($p<0.000$), pectin supplementation therefore has positive effects on abdominal fats, thereby reducing the risk of abdominal obesity related to high-fat diet intake. These are interesting findings that can be explored further given the lack of published studies yet on the effects of pectin supplementation on abdominal obesity *in vivo*.

Body fats

Table 3 shows that the ND had the lowest A. Fat, M. Fat, and H. Fat, whereas the HFD+CP had the lowest S. Fat and E. Fat. Among HFD groups, HFD+SP had the lowest A. Fat, M. Fat, and H. Fat. Meanwhile, the HFD group consistently had the highest levels of fat in all body parts analyzed. The HFD group had the highest mean TAI, followed by

Table 3. Mean abdominal circumference during the supplementation period, mean body fats, and TAI% of male ICR mice in the different treatment groups at endline supplementation.

Animal Groups	Abdominal Circumference (cm)		Body Fats (g)					TAI (%)
	Baseline Supplementation	Endline Supplementation	A. Fat	S. Fat	E. Fat	M. Fat	H. Fat	
ND	7.31± 0.25 ^{a,*}	7.26± 0.15 ^{a,*}	0.29± 0.09 ^{a}	0.90± 0.19 ^{a}	0.93± 0.11^{a}	0.40± 0.06^{a}	0.15± 0.01 ^{a}	2.65± 0.30 ^{b}
HFD	7.11± 0.10 ^{a,*}	7.64± 0.11 $^{a,+}$	1.00± 0.80 ^{a}	1.87± 0.38 ^{b}	1.99± 0.23^{b}	0.87± 0.12 ^{a}	0.19± 0.06 ^{a}	5.92± 0.50 ^{a}
HFD+CP	7.27± 0.11 ^{a,*}	7.37± 0.18 ^{a,*}	0.56± 0.24 ^{a}	0.59± 0.17 ^{a}	0.98± 0.23^{a}	0.63± 0.16 ^{a}	0.13± 0.01 ^{a}	2.84± 0.57 ^{b}
HFD+SP	7.61± 0.22 ^{a,*}	7.37± 0.13 ^{a,*}	0.49± 0.18 ^{a}	0.87± 0.17 ^{ab}	1.26± 0.18^{ab}	0.62± 0.18 ^{a}	0.11± 0.03 ^{a}	3.35± 0.58^{b}

ND – Normal Diet; HFD – High Fat Diet; HFD+CP – HFD w/ commercial citrus pectin; HFD+SP – HFD w/ Saba Banana peel pectin
A.Fat. –Abdominal Fat; S.Fat.- Subcutaneous Fat; M.Fat.-Mesenteric Fat; H.Fat.-Heart Fat; TAI- Total Adiposity Index
Values represent the mean ±S.E. (ND n = 7; HFD n= 7; HFD+CP n=7; HFD+SP n=7)
Means in the same column followed by a different letter(s) are significantly different at $p<0.05$.

HFD+SP, HFD+CP, and the lowest in ND group. There were no significant differences in adiposity indexes between the ND group and the HFD+CP and HFD+SP groups. However, the TAI in HFD+CP was relatively lower than in HFD+SP. There were no significant differences in adiposity indexes between the ND group and the HFD+CP and HFD+SP groups. However, the TAI in HFD+CP was relatively lower than in HFD+SP.

Fecal Weight

Table 4 shows that at baseline, fecal weight was significantly higher in the HFD group and pectin-supplemented groups than in the ND group ($p<0.002$). At the end of supplementation, HFD+CP had the highest fecal weight, followed by HFD+SP, HFD, and the lowest in the ND group. The fecal weight of the HFD+CP group was significantly higher than that of ND, HFD**,** and HFP+SP. Among the HFD groups, HFD+CP had the greatest increase in fecal weight from baseline to endline (75.47%; 2.43g), followed by HFD+SP (26.65%; 0.89g), with the lowest increase observed in the HFD group (10.34%; 0.36g). A significant difference was observed between HFD and HFD+CP, as well as between HFD+CP and HFD+SP.

Table 4. Mean fecal weight of male ICR mice in the different treatment groups during the supplementation period.

Animal Groups	Mean Fecal Weight (g) Baseline Supplementation (Week 0)	Mean Fecal Weight (g) Endline Supplementation (Week 9)
ND	2.56 ± 0.16 ^{a,*}	3.05 ± 0.20 $^{a,+}$
HFD	3.48 ± 0.19 ^{b,*}	3.84 ± 0.13 ^{a,*}
HFD+CP	3.22 ± 0.14 ^{b,*}	5.65 ± 0.53 $^{b,+}$
HFD+SP	3.34 ± 0.12 ^{b,*}	4.23 ± 0.21 $^{a,+}$

ND – Normal Diet; HFD – High Fat Diet; HFD+CP – HFD w/ commercial citrus pectin; HFD+SP – HFD w/ Saba Banana peel pectin
Values represent the mean ±S.E. (ND n = 7; HFD n= 7; HFD+CP n=7; HFD+SP n=7)
Means in the same column followed by a different letter(s) are significantly different at $p<0.05$.
Means in the same row followed by a different symbol are significantly different at $p<0.05$.

Histopathology of Pancreas and Fats

Pancreas

Figure 4 shows the results of histological analysis of the pancreas from the ND, HFD, HFD+CP, and HFD+SP groups. Histological analysis from all the groups showed a normal architecture of the pancreas characterized by oval or elongated pancreatic islets surrounded by a thin connective sheath. No inflammatory or fibrotic changes were noted in any of the islets examined. The ND group did not show any adipocyte deposits and appeared normal. Several sections from the HFD group (Figure 4b) showed mild deposition of adipocytes in the parenchyma, while HFD+CP (Figure 4c) and HFP+SP (Figure 4d) showed occasional fat deposition.

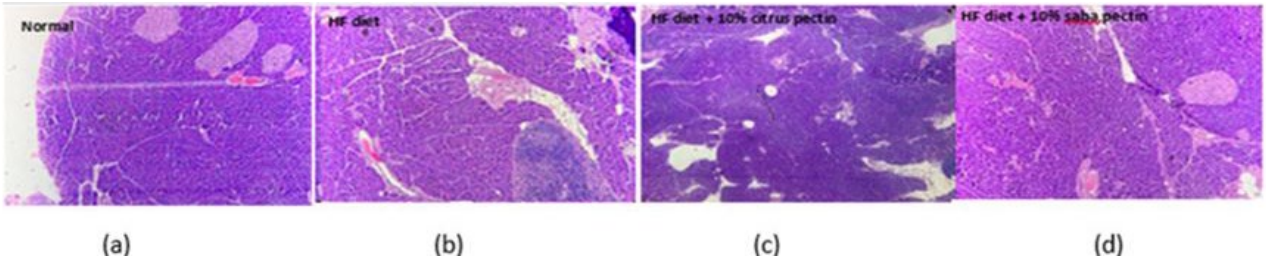

Figure 4. H&E image of a pancreas tissue: (a) normal diet (ND) group; (b) high-fat diet (HFD) group; (c) high-fat diet plus commercial pectin (HFD+CP) group and (d) high-fat diet plus saba banana peel pectin (HFD+SP) group.

Abdominal Fat

In Table 5, representative histological sections of the abdominal fat from HFD showed an increase in the diameter of the adipocytes (31.78µm). Similar to HFD, HFD+SP also showed an increase in the diameter of adipocytes (29.24µm) but to a lesser extent. Meanwhile, the HFD+CP (21.75µm) and the ND group (17.05µm) showed normal architecture of the adipocytes (Fig. 5) and were significantly lower in adipocyte diameter. Essentially, the histology of adipose tissue revealed that commercial pectin in HFD+CP was able to effectively ameliorate adipose tissue hypertrophy in obese diet-induced mice compared to HFD+SP.

Animal Dose to Human Dose Equivalent

The study demonstrated that pectin supplementation at 10% (w/w) of the total diet is beneficial in the prevention of weight gain and reduction of body fats in HFD-induced obese mice. When compared to the HFD group, SP supplementation at 10% (w/w) of the diet resulted

Table 5. Mean adipocyte surface area of different treatment mice after the supplementation period.

Animal Groups	After Supplementation Period (µm)
ND	17.05 ± 3.07^{b}
HFD	31.78 ± 13.04^{a}
HFD + CP	21.75 ± 7.37^{b}
HFD + SP	29.24 ± 11.20^{a}

ND – Normal Diet; HFD – High Fat Diet; HFD+CP – HFD w/ commercial citrus pectin; HFD+SP – HFD w/ Saba Banana peel pectin
Values represent the mean (ND n = 7; HFD n= 7; HFD+CP n=7; HFD+SP n=7)

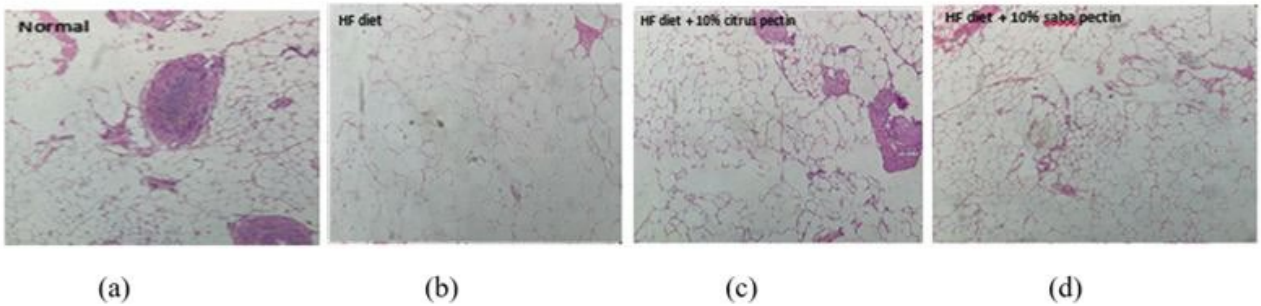

Figure 5. H&E image of Abdominal fat tissue: (a) Normal diet (ND) group (b) High-fat diet (HFD) group (c) High-fat diet plus commercial pectin (HFD+CP) group and (d) High fat diet plus saba banana peel pectin (HFD+SP) group.

in 14.61% higher water intake, 13.39% lower body weight, 3.66% lower abdominal circumference, and 76.72% lower TAI. The effective dose of HFD+SP at 10% (w/w) when converted to a human dose using the Reagan-Shaw formula [24] is equivalent to 4.87g per day for a 60-kg typical adult.

4. Discussion

The results of this study confirmed the ability of SP to reduce food intake and adiposity parameters, which play an important role in the prevention of fat accumulation and obesity. Specifically, this study demonstrated that SP added to a high-fat diet did not decrease the food but resulted in increased water intake and fecal weight, reduced body weight, and body fats in nine (9) weeks of supplementation. These findings are similar to the reports of several rat trials indicating that a high-fat diet supplemented with pectin from various sources has shown a lower body weight [14,33], and suppressed the development of adipose tissue [33]. The pectin added to the diet had resulted in increased water-holding capacity and therefore increased the bulk of the diet. Thus, it can be considered that weight and adiposity regulation in SP, like in CP can possibly be due to the increased water-holding capacity of pectin brought about by its gelling ability or increased viscosity, which both intakes and reduces the absorption of nutrients, including fats, thus resulting in lower body weight gain.

With body weight being an index of adiposity, the significant reductions observed in body weight of SP-supplemented groups may indicate the reduction of body fats in obese mice despite continued access to high-fat food. Similar to the findings of Adam *et. Al.* [14] dietary pectin supplementation in rats induced 23% body fat loss, leading to 12% lower final body weight and 44% lower total body fat mass than controls. Also, their study confirmed that pectin-supplemented rats showed that the decrease in body weight was associated with significant body fat loss. The highest reduction observed in HFD+SP in terms of abdominal circumference and lower weights of abdominal fats than HFD and HFD+CP suggest that SP may have positive effects on reducing the risk of abdominal obesity related to high-fat diet intake.

Furthermore, it was found in this study that SP, similar to CP, was also effective in increasing the fecal weight of mice. The significant increase in fecal weight seen in both pectin-supplemented groups may reflect the presence of pectin in the diet and may indirectly and partially indicate the amount of fat removed from the body, which may contribute to their anti-obesity effects. As previously noted in the study of Slavin [28], pectin binds to cholesterol and bile acids in the gut and promotes their excretion, as well as inhibiting fat deposition by removing them through bile production and stool [29]. The bacterial mass formed from fermentable substances such as pectin and the retained water is responsible for the increase in fecal bulk [30]. These effects are assumed to decrease fat accumulation, increase fecal weight, decrease body weight, increase water intake, and lower TAI, indicating that it may indirectly and partially increase the amount of fat removed from the body, which may contribute to the anti-obesity effects of pectin.

In terms of the association between feed intake and body weight, a positive correlation was noted in pectin-supplemented groups. However,

the effect was more pronounced in commercially available citrus pectin, where a significantly strong positive correlation was noted. Likewise, in the study of Adam *et. al.* [14], it was also determined that there is a strong positive correlation between food intake and weight gain in rats in a four-week pectin-based dietary intervention. It was also found that the body weight was lower in rats fed with pectin-containing diets compared with rats on high-fat diets. Based on these findings, it can be said that the pectin from saba peels when supplemented with high-fat diets, is effective in lowering body weight and adiposity similar to its commercially available counterpart, citrus pectin.

Comparing the two (2) pectin sources, the current study revealed no significant difference in the effect of SP and CP as pectin sources in the variables that were investigated, except in terms of subcutaneous fat, epididymal fat, and fecal weight. Both CP and SP supplementation resulted in higher water intake and a lower mean abdominal circumference compared to the HFD diet after the supplementation period. Also, the mean adipocyte surface area was lower in HFD+CP and HFD+SP compared to HFD at endline supplementation. However, the effect of CP supplementation tended to reduce daily feed and calorie intakes; lower body weight; lesser body weight gain; higher fecal weight; lesser TAI; subcutaneous fat and epididymal fat; and adipose tissues. On the other hand, SP supplementation resulted in higher water intake and lower fat accumulation in the pancreas, abdominal fat, mesenteric fat, and heart fat. These may suggest that saba peel pectin in HFD+SP could have a more pronounced effect on reducing the degree of body fat in some body parts, such as A. Fat, M. Fat, and H. Fat, whereas commercial pectin in HFD+CP has a more pronounced effect on S. Fat and E. Fat. Differences in their effects can be partly attributed to differences in the source of pectin, as backed up by several studies [31,32,33,34]. In certain mice studies, data have revealed that pectin lyase-modified red ginseng extract supplementation reduced the size of adipocytes from epididymal adipose tissue in a dose-dependent manner when compared to HFD-obese mice [34]. In arabinoxylan supplementation, the arabinoxylan treatment normalized the subcutaneous adipose tissues as compared to HFD, which increases the amount of subcutaneous adipose tissues [6]. In inulin intake, the HFD-induced increase in subcutaneous and epididymal white adipose tissue [25] was observed; in an arabinoxylan-supplemented diet, a lower fat mass development was observed through the weight of epididymal, subcutaneous, and visceral adipose tissues [32]. Among other sources of DF, (1-3), (1-4) Beta-D-glucan from oats was shown to have a lower adiposity index (visceral fat/final body weight) in high-glucan-fed mice than the low glucan-fed mice [30]. In the study of Schroeder et. al. [26], it was mentioned that the lowest epididymal fat pad weight was observed in high-viscosity fiber, whereas the cellulose group had the heaviest epididymal weight. In a gum arabic supplementation study, the adipose tissues of the supplemented group, such as mesenteric fat, perinephric fat, and periovarian fat, relative to body weight and mean adipocyte area, tended to be smaller compared to a normal group [32].

The chemical properties and purity of pectin from CP and SP may partially explain their closely similar effects on food intake and adiposity parameters. According to Castillo-Israel et. al. [15], pectin from banana peels is comparable to commercial citrus pectin in terms of gelling ability, sensory qualities, moisture content, and degree of esterification. However, the more pronounced effect of CP in ameliorating some of the adiposity parameters could be attributed to the significantly higher methoxyl content (9.09%) as compared to SP (5.25%) [15]. The higher methoxyl content indicates greater solubility in water [35], which may increase the gelation capacity of the pectin, thus promoting gastric distention once ingested in the body. The relatively low purity of pectin extracted from Saba peels, as indicated by its lower % Anhydrogalacturonic Acid (AUA) (39.68% vs. 74.26% in commercial pectin) and higher ash content exceeding the 10% maximum limit for good gel formation [37], may have diminished its effectiveness in regulating feed intake, body weight, and adiposity biomarkers [15]. These impurities suggest that residual substances in SP may interfere with efficient gelation, thereby potentially diminishing its effectiveness in regulating feed intake, body weight, and adiposity biomarkers [15], which are key mechanisms in anti-obesity activity attributed to pectin's gelling properties.

Taken collectively, it can be said that the pectin from saba peels, when supplemented in high-fat diets, is effective in lowering body weight

and adiposity to a level closely similar to its commercially available counterpart, citrus pectin. Pectin is considered viscous and capable of holding water and forming gels, resulting in higher viscosity, gastric distention, and inhibition of gastric emptying, thereby altering food digestion and fat absorption [29,38]. All these mechanisms could be responsible for the observed anti-obesity effects of SP supplementation.

5. Conclusion

In general, when compared to the HFD group, SP supplementation at 10% (w/w) of diet resulted in 14.61% higher water intake, 13.39% lower body weight, 3.66% lower abdominal circumference, 76.72% lower TAI and 9.22% higher fecal weight. The study demonstrated that pectin supplementation at 10% SP fraction of the total diet is beneficial in the prevention of weight gain and reduction of body fats in HFD-induced obese male ICR mice. The human dose equivalent of 10% (w/w) saba banana peel pectin is 4.87g per day for a typical adult weighing 60 kg.. Given these findings, saba banana peels, a considered waste material, are a cheap source of pectin that has the potential to regulate feed intake and adiposity parameters. These interesting findings merit further investigations through clinical studies to fully establish the anti-obesity potential of saba banana peel pectin supplementation.

Availability of Data and Materials

All data are available in this study.

Author Contributions

Conceptualization - L.M.A., M.A.C.E., and K.A.C.I.; Methodology - L.M.A., E.M.F.O., P.A.A.B., C.J.M., M.A.C.E., K.A.C.I., R.P.G., J.R.C., and P.J.V.G.; Validation - L.M.A., M.A.C.E., and K.A.C.I.; Formal analysis - L.M.A., M.A.C.E., and K.A.C.I.; Investigation - E.M.F.O., P.A.A.B.,.C.J.M., M.A.C.E., K.A.C.I., L.M.A., R.B.N., R.P.G., J.R.C., and P.J.V.G.; Resources - E.M.F.O., P.A.A.B., C.J.M., M.A.C.E., and K.A.C.I.; Data curation - L.M.A., M.A.C.E., and K.A.C.I.; Writing - Original Draft - L.M.A., E.M.F.O., and A.D.F.; Writing - Review and Editing - L.M.A., E.M.F.O., and P.A.A.B.; Visualization-L.M.A., M.A.C.E., and K.A.C.I.; Supervision - L.M.A., M.A.C.E., and K.A.C.I.; Project administration - L.M.A., M.A.C.E., and K.A.C.I.

Ethics Approval and Consent to Participate

All animal experiments conducted were authorized by the University of the Philippines Los Baños Animal Care and Use Committee (UPLB IACUC) under approval number CHE-2019-001.

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Conflict of Interest

The authors declare no conflict of interest.

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(Original Research)

Prevalence, Associated Risk Factors, and Transmission Risk Scoring of Classical Swine Fever in Smallhold Farms in the Philippines

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Abstract

Background: Classical swine fever (CSF) is a notifiable disease, and the limited epidemiological data in the Philippines underscores the need for effective disease surveillance. **Methods:** This study aimed to determine the prevalence of antibodies against CSF virus (CSFv) using enzyme-linked immunosorbent assay and CSFv RNA using real-time reverse transcription polymerase chain reaction in pigs from smallhold farms in 21 Philippine provinces. The association between seropositivity and factors from interviews of abattoir officers was analyzed using the least absolute shrinkage selection operator regression. A semi-quantitative method was also adapted to estimate the transmission risk. **Results:** Our study found an overall seroprevalence of 36.0% (153/425, 95% Confidence Interval: 31.5%-40.8%), while all 423 samples tested negative for CSFv. A positive association was found in water treatment, swill feeding, CSF vaccination, and keeping vaccination records, while CSF history, proximity to residential areas, and raising native pigs negatively impacted seropositivity. Nueva Ecija was considered high-risk for CSF transmission, while others fell within the moderate, low, and very low risk categories. **Conclusions:** Our findings highlight the CSF seroprevalence and factors to consider for improved prevention and control. Classifying the provinces according to transmission risk also provided insights on future targeted surveillance and efficient resource allocation.

Keywords

Philippines, Prevalence, Risk scoring, Transmission

1. Introduction

Classical swine fever (CSF), also known as hog cholera, is a notifiable animal disease affecting all pig species [1]. As a highly contagious disease with the potential to cause severe economic consequences, it is also recognized as a transboundary animal disease [2]. The causative agent, CSF virus (CSFv), is a single-

stranded, positive-sense RNA virus from the genus *Pestivirus* of the *Flaviviridae* family, and it is taxonomically related to the bovine viral diarrhea and border disease viruses [3]. While there is only one known CSFv serotype, this virus has three genotypes, with genotypes 2 and 3 causing recent outbreaks in Europe and Asia [4,5]. Despite these genetic differences, the CSFv does not produce a clinically distinct disease in all age groups of pigs [1]. Following infection, the acute disease starts with leukopenia and immunosuppression, predisposing pigs to microbial co-infections [1]. As the disease progresses, pigs may exhibit fever, inappetence, constipation, lethargy, and petechial hemorrhages on the ears, abdomen, and thighs, and mortalities often occur 1 to 4 weeks after acute infection [1]. Similarly, chronic and persistent infections, which are common in piglets infected *in utero,* also result in delayed mortalities [1]. Aside from vertical transmission, CSFv also spreads directly between infected and healthy pigs through saliva, urine, and feces [6] as well as indirectly through contaminated feeds, swills, water, clothing, and farm equipment [7].

In the 1990s, the Netherlands reported one of the largest CSF occurrences, where 10 million pigs across 429 farms were culled, resulting in USD 2.3 billion in economic losses [8]. Around USD 12 million was also lost in Belgium in 1997 from eight affected farms with low pig density [9]. Given these serious economic impacts, investigating risk factors linked to outbreaks can benefit CSF control and prevention. Among the identified factors in global occurrences were increased herd size, denser pig population, increased pig transportation, proximity of infected and susceptible herds, swill feeding, a longer interval between disease onset and reporting, the introduction of diseased pigs, and having no CSF vaccination [10–12]. The disease also reemerged in Japan in 2018, 26 years after its last reported case, and intervention strategies such as stamping out, movement control, disinfection protocols, and active surveillance in both domestic and wild pigs were implemented [13]. In the absence of effective treatment, the World Organization for Animal Health (WOAH) also recommends a robust reporting system, decontamination of swill or prohibition of its feeding, compartmentalization strategies in affected areas, vaccination, proper handling and treatment of pig products and by-products, and surveillance programs as CSF control and prevention [14].

Given the non-specific CSF symptoms, surveillance and monitoring are based on a combination of clinical observation, viral detection, and serology tests [15,16], such as enzyme-linked immunosorbent assay (ELISA) and real-time reverse transcription polymerase chain reaction (qRT-PCR). ELISA is a reliable diagnostic tool for detecting anti-CSFv antibodies, and discrimination between infected and vaccinated animals has been possible with modifications in the coated antigens and improvement in vaccine development [17,18]. It is commonly applied to large-scale epidemiology studies for seroconversion and post-vaccination surveillance [19]. On the other hand, qRT-PCR offers precise viral detection by targeting the highly conserved region within the 5′ UTR of the CSFv genome in experimentally infected pigs. With around two hours of turnaround time, it is considered a rapid tool for CSF diagnosis [20]. Integrating these two diagnostic methods into the CSF surveillance program is, therefore, crucial in undertaking and enhancing disease monitoring and control.

Currently, the Philippine swine industry faces significant production and economic losses due to the ongoing African swine fever (ASF) crisis [21]. Disease prevention and control remain challenging, especially in smallhold farms, due to biosecurity lapses and limited access to vaccines for economically important animal diseases [22]. With the focus on ASF, other swine diseases such as CSF appear to be neglected. In the 2000s, prevalence in 14 provinces ranged from 30 to 40%, based on the laboratory tests conducted by the Philippine Animal Health Center [23]. Bulacan, Pampanga, and provinces in Mindanao were also affected in the following years [24,25], highlighting the national challenge in CSF control. With the potential enzootic distribution of CSF in the Philippines [26], disease surveillance, along with examining risk factors and assessing transmission risk, will enable a better understanding of its local epidemiology to prevent future outbreaks and ensure food security. Hence, this study aimed to determine the distribution of CSF in the Philippines using ELISA and qRT-PCR, identify associated risk factors, and evaluate the transmission risk in selected provinces to aid policymakers in designing evidence-based prevention and control strategies.

2. Materials and Methods

2.1 Ethical Statement

The existing Institutional Animal Care and Use Committee of the University of the Philippines Los

Baños carefully reviewed and approved all animal procedures in this study under approval number UPLB-2022-001, in full compliance with the national guidelines and policies governing the ethical use of animals in scientific research.

2.2 Study Sites

A total of 21 provinces (Benguet, Ilocos Sur, Pangasinan, Nueva Ecija, Pampanga, Batangas, Cavite, Laguna, Occidental Mindoro, Palawan, Camarines Sur, Aklan, Negros Occidental, Bohol, Cebu, Samar, Zamboanga del Sur, Bukidnon, Davao del Sur, North Cotabato, and Surigao del Norte) were purposively chosen based on the following criteria: geographic representatives from different administrative regions of the Philippines, with high swine population size, and willingness of the local government units to participate. The term "municipality" used in this study refers to both municipalities and cities. A maximum of four municipalities were selected from each province based on these same criteria, and sample collection was conducted in slaughterhouses that primarily served smallhold pig farms within a municipality. Overall, samples were collected from 43 municipalities from January 2022 to November 2023.

2.3 Sample Size Determination

A sample size of 384 was computed using the formula [27] for estimating disease prevalence in an infinite population with 50% expected prevalence, 5% desired margin of error, and 95% confidence level. In this cross-sectional study, a total of 425 blood samples were collected, higher than the computed sample size to improve the precision of the sample estimate.

2.4 Sample Collection

Aseptic blood sampling (3–5 mL) was performed via the external jugular vein from a total of 13 to 30 domestic pigs, regardless of sex and breed, aged at least 5 months, in pig holding pens of selected slaughterhouses. The collected blood samples were transferred to a properly labeled VacugenTM tube (BioSpectra Marketing, Iloilo City, Philippines) and allowed to clot at room temperature for approximately 30 to 45 minutes. The samples were centrifuged at 2,000 x *g* for 10 min to collect the sera and transferred to a properly labeled Labopette cryovial tube (Labotech Trading, Las Piñas City, Philippines). All samples were transported to the laboratory in an insulated specimen transport box and kept at -80°C for further analysis.

2.5 Enzyme-linked Immunosorbent Assay (ELISA)

The serum samples were thawed under controlled cold temperature and tested in duplicate using the Classical Swine Fever Antibody Test kit (IDEXX, Montpellier, France) following the manufacturer's competitive ELISA protocol. Optical density (OD) was recorded at 450 nm with the MultiskanTM Go Microplate Spectrophotometer (Thermo Fisher Scientific Corporation, Vantaa, Finland). The assay was considered valid if the mean OD of the negative control exceeded 0.50 and the blocking percentage of the positive control was greater than 50%. The presence of anti-CSFv antibodies was determined by calculating the percentage of the test samples' absorbance relative to the negative control. The absorbance value of the sample was subtracted from that of the negative control, then divided by the absorbance of the negative control, and then multiplied by 100 to express it as a percentage. A sample was classified as positive if its blocking percentage was at least 40% and negative if it was at most 30%.

2.6 Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

All samples were handled in a Biosafety Level 2 (BSL-2) laboratory, adhering to standard BSL-2 procedures. The samples were thawed in a cold, controlled environment, and the total RNA was consequently extracted using the QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purity and quantity of extracted RNA were checked spectrophotometrically using a NanodropTM 2000/2000c (Thermo Fisher Scientific, DE, USA) prior to further testing.

The total RNA was reverse transcribed using random hexamer primers with the cDNA synthesis kit (HiScript® III RT SuperMix, Vazyme International LLC, Nanjing, China) following the manufacturer's instructions with minor modifications. Sixteen (16) µl of the gDNA wiper-treated total RNA and 4 µl of the mastermix were incubated at 37 °C for 15 minutes and 65 °C for 5 seconds. The qRT-PCR assay was conducted using the Toptical Gradient 96 (Analytic Jena, Göttingen,

Germany) with the Primerdesign™ Classical Swine Fever virus genesig Advanced kit in tandem with Oasig lyophilized 2x qPCR standard mastermix. For CSFv detection, a mixture was prepared using 10 µl of 2x qPCR Mastermix, 1 µl of CSFv specific primer/probe mix, 3 µl RNAse-free water, and 5 µl cDNA. The primers were designed to target the 5' UTR of the CSFv genome, based on the reference sequence with GenBank accession number HQ148062.1, producing an amplicon of 106 bp anchored at nucleotide position 158. Amplification was carried out under the following thermal conditions: enzyme activation at 95 °C for 2 minutes, followed by 50 cycles of denaturation at 95 °C for 10 seconds and annealing/extension at 60 °C for 60 seconds with signal acquisition. Results were interpreted based on the detected FAM-labeled channel amplification as cycle threshold (Ct) values, wherein samples with a Ct value of at most 35 were classified as positive.

2.7 Interview of Abattoir Officers

Using a structured questionnaire, an interview with meat inspectors and veterinarians in each slaughterhouse and local government unit, respectively, was conducted. Farm demographics, common farm practices, history of CSF vaccination and outbreak, and details of the CSF surveillance program were obtained for the association test with seropositivity. A written consent form was provided only to those who agreed to the interview, and demographic data were treated with the utmost confidentiality.

2.8 Data Analysis

All data were recorded and organized in Excel (Microsoft, WA, USA). To calculate the positivity rates, the number of positive samples was divided by the number of tested samples and multiplied by 100. Statistical analyses were conducted in R (Posit Software, MA, USA). Using the *imputeMCA* function of the *missMDA* package [28] with default settings, imputation of missing information under 14 factors from the survey data was undertaken, except for Benguet with one sampled municipality due to the absence of interview responses. The association between these factors and seropositivity was assessed using the least absolute shrinkage and selection operator (LASSO) regression analysis [29] with the *glmnet* package [30]. The open-access Quantum Geographic Information System version 3.40 was also used to generate the geographical distribution maps of the positivity rates and risk classifications.

2.9 Semi-quantitative Risk Scoring

The risk of CSF transmission was assessed semi-quantitatively, adapting the methods of the European Food Safety Authority in ASF risk assessment with minor modifications in assigning risk scores [31]. Specifically, the probability of transmission as measured by eight factors—four from this study and four from published databases—was estimated for each sampled province. A greater weight in the final risk score was allocated to factors that were directly linked to CSF occurrence.

Seropositivity was given a maximum risk score of "5.0". The range between the highest and lowest positivity rates was split into five equal intervals, and the risk score was inversely proportional to seropositivity (i.e., 0-20%=5.0, 21-40%=4.0, 41-60%=3.0, 61-80%=2.0, 81-100%=1.0). Swill feeding, vehicle disinfection, and water treatment from interviews were factors influencing seropositivity in this study, resulting in their inclusion in the risk scoring. Municipalities were scored based on the percentage of farms engaging in swill feeding (i.e., 0%=0.0, 1-25%=1.0, 26-50%=2.0, 51-75%=3.0, and 76-100%=4.0). Under vehicle disinfection, a municipality received a grade of "0" if it was practiced both before entry and after leaving the farm, "1.0" if it was done either before or after, and "2.0" if not done at all. Those employing water treatment methods in farms were given a "0" score; otherwise, a grade of "1.0" was assigned. For each of these three factors, the mean of the risk scores of all municipalities reflected the risk of their respective provinces.

For the succeeding four factors, data covering the sampling period in each province were obtained from online databases. A large herd size was associated with CSF occurrence [10], so the percentage of pigs in commercial farms was computed by dividing the number of pigs raised in commercial farms by the total number of pigs raised in a province, then multiplied by 100 [32]. The swine population density (heads/km^2), which reflected the closeness of pigs linked to CSF transmission [10], was also estimated by getting the ratio between the total number of pigs in a province [32] and the most recent provincial data in total land size [33]. The assignment of risk scores in these two factors followed the method employed in seropositivity, except for the direct relationship between factors and risk score and the assignment of

a "0" score for those who had "0" raw data. As the CSFv survives in and can be transmitted through pork and pork products [7], the regional data on total frozen pork inventory (metric tons) [34] was also used to estimate its contribution to transmission at the provincial level. Similar to swill feeding, a maximum score of "4.0" was assigned, and four equal intervals were derived from the range of the pork inventory. The role of increased human interactions in the potential mechanical transmission of CSFv [7] was considered minimally. As represented by the human population density (heads/km^2) in a province, the human population size [33] was divided by the total land size [34]. Provinces with a population density greater than the median received a score of "1.0"; otherwise, a "0" score was assigned.

The scores across these eight factors were summed for each province. The range between the maximum and minimum possible values of the overall risk scores was partitioned into six equal intervals, and each province was categorized into one of the following risk bands: extreme, very high, high, moderate, low, and very low [35].

3. Results

3.1 Serological and Molecular Detection of CSF

Out of 425 samples subjected to competitive ELISA, 153 (36.0%, 95% Confidence Interval: 31.5-40.8%) tested positive (Table 1). The highest

Table 1. Positivity rates in classical swine fever from smallhold farms in 21 Philippine provinces.

REGION	PROVINCE	ELISA		qRT-PCR	
		TESTED SAMPLES	POSITIVE SAMPLES (%)	TESTED SAMPLES	POSITIVE SAMPLES (%)
CAR	Benguet	17	17 (100.0)	17	0 (0.0)
I	Ilocos Sur	20	3 (15.0)	20	0 (0.0)
	Pangasinan	16	0 (0.0)	16	0 (0.0)
III	Nueva Ecija	15	6 (40.3)	15	0 (0.0)
	Pampanga	15	9 (60.0)	15	0 (0.0)
IV-A	Batangas	13	4 (30.8)	13	0 (0.0)
	Cavite	21	11 (52.4)	21	0 (0.0)
	Laguna	16	6 (37.5)	16	0 (0.0)
IV-B	Occidental Mindoro	16	0 (0.0)	16	0 (0.0)
	Palawan	16	0 (0.0)	14	0 (0.0)
V	Camarines Sur	15	5 (33.3)	15	0 (0.0)
VI	Aklan	27	0 (0.0)	27	0 (0.0)
	Negros Occidental	15	8 (53.3)	15	0 (0.0)
VII	Bohol	13	2 (15.4)	13	0 (0.0)
	Cebu	22	15 (68.2)	22	0 (0.0)
VIII	Samar	28	7 (25.0)	28	0 (0.0)
IX	Zamboanga del Sur	30	1 (3.33)	30	0 (0.0)
X	Bukidnon	20	12 (60.0)	20	0 (0.0)
XI	Davao del Sur	30	19 (63.3)	30	0 (0.0)
XII	North Cotabato	30	4 (13.3)	30	0 (0.0)
XIII	Surigao del Norte	30	24 (80.0)	30	0 (0.0)
	TOTAL	***425***	***153 (36.0)***	***423***	***0 (0.0)***

ELISA: enzyme-linked immunosorbent assay; qRT-PCR: real-time reverse transcription polymerase chain reaction

seropositivity was observed in Benguet (100.0%, 17/17), followed by Surigao del Norte (80.0%, 24/30), Cebu (68.2%, 15/22), and Davao del Sur (63.3%, 19/30) (Fig. 1). Conversely, all samples in Pangasinan (n=16), Occidental Mindoro (n=16), Palawan (n=16), and Aklan (n=27) were seronegative (Fig.1). In the qRT-PCR assay, all of the 423 tested samples were negative for CSFv RNA (Table 1).

3.2 Factors Associated with Seropositivity in CSF

Around 38.1% of the municipalities (16/42) had farms with 6-10 pigs, while a majority (59.5%, 25/42) reported that native pigs were raised in about 1-25% of farms (Table 2). Most municipalities (54.8%, 23/42) also indicated that 50% of swine farms were close to residential areas.

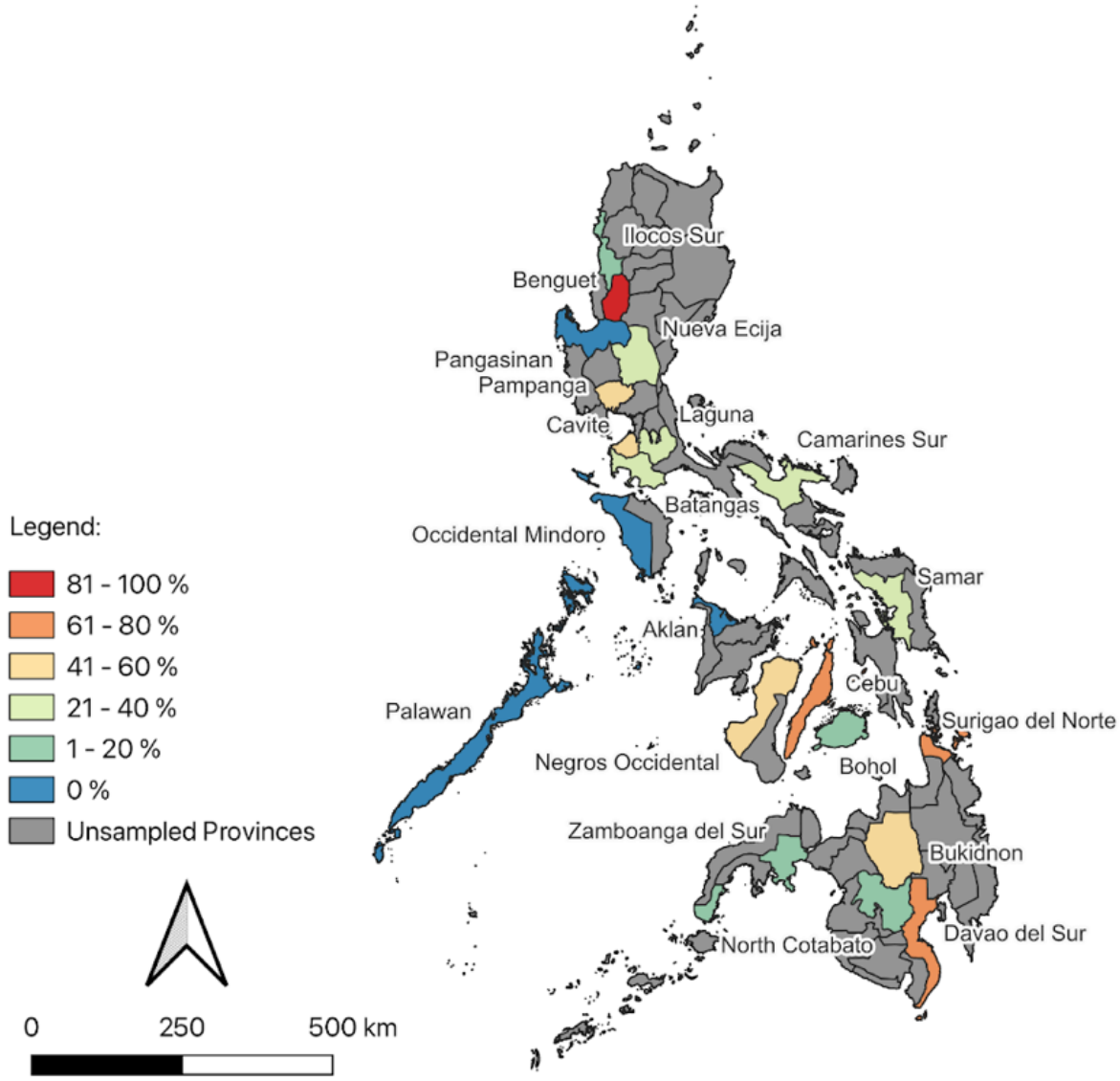

Figure 1. Geographic distribution of classical swine fever seropositive pigs in smallhold farms across 21 sampled provinces in the Philippines. The map was generated using Quantum Geographic Information System 3.40.

Table 2. Distribution of interview responses from 42 municipalities across 14 variables.

Variables	Number of municipalities (%)	Variables	Number of municipalities (%)
Average herd size		*Percentage of farms keeping vaccination records*	
1-5 pigs	14 (33.3)	0 %	14 (33.3)
6-10 pigs	16 (38.1)	1-25%	16 (38.1)
11-15 pigs	7 (16.7)	26-50%	1 (2.4)
16-20 pigs	5 (11.9)	51-75%	3 (7.1)
Percentage of farms raising native pigs		76-100%	8 (19.0)
0 %	9 (21.4)	*Housing system*	
1-25%	25 (59.5)	Pen-type	38 (90.5)
26-50%	7 (16.7)	Free range	1 (2.4)
51-75%	1 (2.4)	Mixed	3 (7.1)
76-100%	0 (0)	*Water treatment*	
Percentage of farms proximal to residential areas		No	23 (54.8)
0 %	4 (9.5)	Yes	19 (45.2)
1-25%	9 (21.4)	*Disinfection of transport vehicles in the farm*	
26-50%	10 (23.8)	No	12 (28.6)
51-75%	10 (23.8)	Before entry or after exit	7 (16.7)
76-100%	9 (21.4)	Before entry and after exit	23 (54.8)
Percentage of farms with biosecurity measures		*Disease reporting*	
0 %	0 (0)	No	4 (9.5)
1-25%	16 (38.1)	Yes	38 (90.5)
26-50%	12 (28.6)	*Surveillance program for classical swine fever*	
51-75%	6 (14.3)	No	24 (57.1)
76-100%	8 (19.0)	Yes	18 (42.9)
Percentage of farms engaging in swill feeding		*Vaccination against classical swine fever*	
0 %	15 (35.7)	No	31 (73.8)
1-25%	18 (42.9)	Yes	11 (26.2)
26-50%	5 (11.9)	*History of classical swine fever*	
51-75%	2 (4.8)	No	41 (97.6)
76-100%	2 (4.8)	Yes	1 (2.4)
Percentage of farms raising animals other than pigs			
0 %	1 (2.4)		
1-25%	21 (50.0)		
26-50%	11 (26.2)		
51-75%	3 (7.1)		
76-100%	6 (14.3)		

A majority of the municipalities (38.1%, 16/42) also had 1-25% of farms with biosecurity measures. Approximately 35.7% (15/42) did not engage in swill feeding. Half of the municipalities (21/42) responded that less than a quarter of their swine farms raised other animals aside from pigs. Fourteen (14) out of 42 municipalities (33.3%) had farms not keeping their vaccination records, while a majority (38.1%, 16/42) had an estimated 1-25% of farms that did. The most frequent housing type was pens (90.5%, 38/42), while water in farms in around 54.8% of the municipalities (23/42) was not treated. The majority of municipalities (54.8%, 23/42) noted that disinfection of transport vehicles was also practiced before entry and after leaving the farm. Most municipalities (90.5%, 38/42) also stated that farms reported swine disease occurrences, while around 57.1% (24/42) indicated the absence of CSF surveillance programs. Thirty-one (31) municipalities (73.8%) responded that farms were not vaccinating against CSF, and forty-one municipalities (97.6%) had farms without CSF history.

The LASSO regression revealed the association of seven (7) factors from interviews of abattoir officers with seropositivity (Table 3). The greatest positive impact was found in water

Table 3. Least absolute shrinkage selection operator coefficient of 14 variables from survey data against seropositivity in classical swine fever.

Variable	Beta Coefficient
Intercept	27.1
Average herd size	0.0
Percentage of farms raising native pigs	-1.7
Percentage of farms proximal to residential areas	-2.8
Percentage of farms with biosecurity measures	0.0
Percentage of farms engaging in swill feeding	6.6
Percentage of farms raising animals other than pigs	0.0
Percentage of farms keeping vaccination records	0.6
Housing system	0.0
Water treatment	10.7
Disinfection of transport vehicles in the farm	0.0
Disease reporting	0.0
Surveillance program for classical swine fever	0.0
Vaccination against classical swine fever	5.2
History of classical swine fever	-17.8

treatment (β=10.7), followed by swill feeding (β=6.6), CSF vaccination (β=5.2), and maintaining vaccination records (β=0.6). On the other hand, a history of CSF (β=-17.8), farms near residential areas (β=-2.8), and raising of native breeds (β=-1.7) were negatively associated with seropositivity.

3.3 Transmission Risk of CSF in 21 Provinces

A total of eight factors—four identified in our study and four obtained from online databases—were included in the risk scoring of CSF transmission in 21 sampled provinces. For the four factors from our study, an inverse relationship between seropositivity and risk score was employed, as our results suggest that the detected antibodies in ELISA and CSF vaccination were positively associated. The seropositivity rates across 21 provinces ranged from 0 to 100.0% (Table 4). Benguet had the highest seropositivity (100.0%) and was assigned a score of "1.0" (Table 5). On the other hand, eight provinces earned a "5.0" score due to very low seropositivity rates, falling within the 0 to 20% interval. In swill feeding, Nueva Ecija, Cavite, and Bukidnon had at least a score of "2.0", while farms in Ilocos Sur, Batangas, Zamboanga del Sur, Davao del Sur, North Cotabato, and Surigao del Norte were not engaging in swill feeding, leading to a "0" score. Vehicle disinfection was not practiced in farms from Bohol, Zamboanga del Sur, and Bukidnon, earning a score of "2.0", while Ilocos Sur, Pampanga, Batangas, Camarines Sur, Negros Occidental, Davao del Sur, and North Cotabato were given a "0" score for implementing vehicle disinfection before entry and after exit from farms. Under water treatment, all sampled municipalities in Ilocos Sur, Pangasinan, Nueva Ecija, Occidental Mindoro, Bohol, Zamboanga del Sur, and Bukidnon indicated that farms did not treat agricultural water, resulting in a score of "1.0", while Camarines Sur, Davao del Sur, North Cotabato, and Surigao del Norte received a "0" score for practicing water treatment.

From online databases, provinces were scored based on commercial farm proportion, swine population density, frozen pork inventory, and human population density. The proportion of commercial farms in sampled provinces ranged from 0 to 97.0%. Cavite had approximately 97.0% commercially raised pigs (Table 4), leading to a score of "5.0" (Table 5), while Samar and Surigao del Norte were given "0" scores due to the absence of commercial farms. For swine population density, the maximum value was observed in Batangas (250.4 heads/km^2), while Samar had the lowest density at 2.5 heads/km^2. After dividing the range into five equal intervals, Batangas had a score of "5.0" (200.9-250.4 heads/km^2), while Pampanga received a "4.0" score (151.3-200.8 heads/km^2). Eleven provinces also earned a score of "1.0" due to values falling within the 2.5 to 52.1 heads/km^2 interval. Under the frozen pork inventory, four equal intervals were derived between the range of 31,795.1 and 6.1 metric tons, resulting in a score of "4.0" in Nueva Ecija (23,848.0-31,795.1 metric tons) and "3.0" in Batangas (15,900.7-23,847.9 metric tons). Meanwhile, ten out of 21 provinces exceeded the median of the human population density (349.6 heads/km^2), receiving a score of "1.0".

The sum of the scores of each province across these eight factors was calculated to estimate the transmission risk of CSF (Table 5). No province fell within the extreme (26.8-32.0) or very high (21.4-26.7) risk bands, while Nueva Ecija was the lone high-risk province (16.1-21.3) (Fig. 2). The transmission risk of CSF was found to be moderate (10.8-16.0) and low (5.4-10.7) in nine provinces each, while two provinces had a very low risk level (0-5.3).

Table 4. Provincial data on eight factors for risk scoring of classical swin fever transmission.

Region	Province	Seropositivity Rate (%)	Swill Feeding	Vehicle Disinfection	Water Treatment	Pigs in Commercial Farms (%)	Swine Population Density (heads/km²)	Frozen Pork (metric tons)	Human Population Density (heads/km²)
CAR	Benguet	100.0	ND	ND	ND	12.9	15.6	ND	292.6
I	Ilocos Sur	15.0	0.0	0.0	1.0	4.2	24.3	272.9	272.0
	Pangasinan	0.0	0.5	1.5	1.0	55.2	25.7	310.5	580.3
III	Nueva Ecija	40.0	3.5	1.5	1.0	40.1	14.8	31,795.1	406.0
	Pampanga	60.0	1.7	0.0	0.3	73.7	151.8	8,809.1	1,404.9
IV-A	Batangas	30.8	0.0	0.0	0.5	7.7	250.4	19,733.1	933.7
	Cavite	52.4	2.7	1.0	0.3	97.0	51.3	6,835.7	2,846.7
	Laguna	37.5	0.5	0.5	0.8	70.6	40.8	7,587.1	1,754.0
IV-B	Occidental Mindoro	0.0	1.0	0.3	1.0	16.2	15.7	6.1	89.8
	Palawan	0.0	0.5	1.0	0.5	2.7	18.2	270.2	73.2
V	Camarines Sur	33.3	1.0	0.0	0.0	0.4	62.8	338.2	375.2
VI	Aklan	0.0	0.3	0.7	0.3	15.5	40.0	84.1	349.6
	Negros Occidental	53.3	1.5	0.0	0.5	10.6	61.0	176.4	400.7
VII	Bohol	15.4	1.0	2.0	1.0	34.9	53.9	4,376.0	292.2
	Cebu	68.2	1.0	1.7	0.3	67.6	107.1	8,052.0	964.3
VIII	Samar	25.0	1.0	1.0	0.5	0.0	2.5	142.5	131.1
IX	Zamboanga del Sur	3.3	0.0	2.0	1.0	10.5	61.2	76.7	343.8
X	Bukidnon	60.0	2.0	2.0	1.0	54.3	66.6	952.8	146.8
XI	Davao del Sur	63.3	0.0	0.0	0.0	59.9	78.6	1,005.8	533.3
XII	North Cotabato	13.3	0.0	0.0	0.0	15.2	24.1	374.4	136.9
XIII	Surigao del Norte	80.0	0.0	1.0	0.0	0.0	4.2	135.8	273.8

ND: No data

Table 5. Risk scores and classifications in classical swine fever transmission of 21 selected provinces across the Philippines.

Region	Province	Seropositivity Rate	Swill Feeding	Vehicle Disinfection	Water Treatment	Pigs in Commercial Farms	Swine Population Density	Frozen Pork	Human Population Density	Risk Score	Risk Level
CAR	Benguet	1.0	ND	ND	ND	1.0	1.0	ND	0.0	**3.0**	**Very Low**
I	Ilocos Sur	5.0	0.0	0.0	1.0	1.0	1.0	1.0	0.0	**9.0**	**Low**
	Pangasinan	5.0	0.5	1.5	1.0	3.0	1.0	1.0	1.0	**14.0**	**Moderate**
III	Nueva Ecija	4.0	3.5	1.5	1.0	3.0	1.0	4.0	1.0	**19.0**	**High**
	Pampanga	3.0	1.7	0.0	0.3	4.0	4.0	2.0	1.0	**16.0**	**Moderate**
IV-A	Batangas	4.0	0.0	0.0	0.5	1.0	5.0	3.0	1.0	**14.5**	**Moderate**
	Cavite	3.0	2.7	1.0	0.3	5.0	1.0	1.0	1.0	**15.0**	**Moderate**
	Laguna	4.0	0.5	0.5	0.8	4.0	1.0	1.0	1.0	**12.8**	**Moderate**
IV-B	Occidental Mindoro	5.0	1.0	0.3	1.0	1.0	1.0	1.0	0.0	**10.3**	**Low**
	Palawan	5.0	0.5	1.0	0.5	1.0	1.0	1.0	0.0	**10.0**	**Low**
V	Camarines Sur	4.0	1.0	0.0	0.0	1.0	2.0	1.0	1.0	**10.0**	**Low**
VI	Aklan	5.0	0.3	0.7	0.3	1.0	1.0	1.0	0.0	**9.3**	**Low**
	Negros Occidental	3.0	1.5	0.0	0.5	1.0	2.0	1.0	1.0	**10.0**	**Low**
VII	Bohol	5.0	1.0	2.0	1.0	2.0	2.0	1.0	0.0	**14.0**	**Moderate**
	Cebu	2.0	1.0	1.7	0.3	4.0	3.0	2.0	1.0	**15.0**	**Moderate**
VIII	Samar	4.0	1.0	1.0	0.5	0.0	1.0	1.0	0.0	**8.5**	**Low**
IX	Zamboanga del Sur	5.0	0.0	2.0	1.0	1.0	2.0	1.0	0.0	**12.0**	**Moderate**
X	Bukidnon	3.0	2.0	2.0	1.0	3.0	2.0	1.0	0.0	**14.0**	**Moderate**
XI	Davao del Sur	2.0	0.0	0.0	0.0	3.0	2.0	1.0	1.0	**9.0**	**Low**
XII	North Cotabato	5.0	0.0	0.0	0.0	1.0	1.0	1.0	0.0	**8.0**	**Low**
XIII	Surigao del Norte	2.0	0.0	1.0	0.0	0.0	1.0	1.0	0.0	**5.0**	**Very Low**

ND: No data

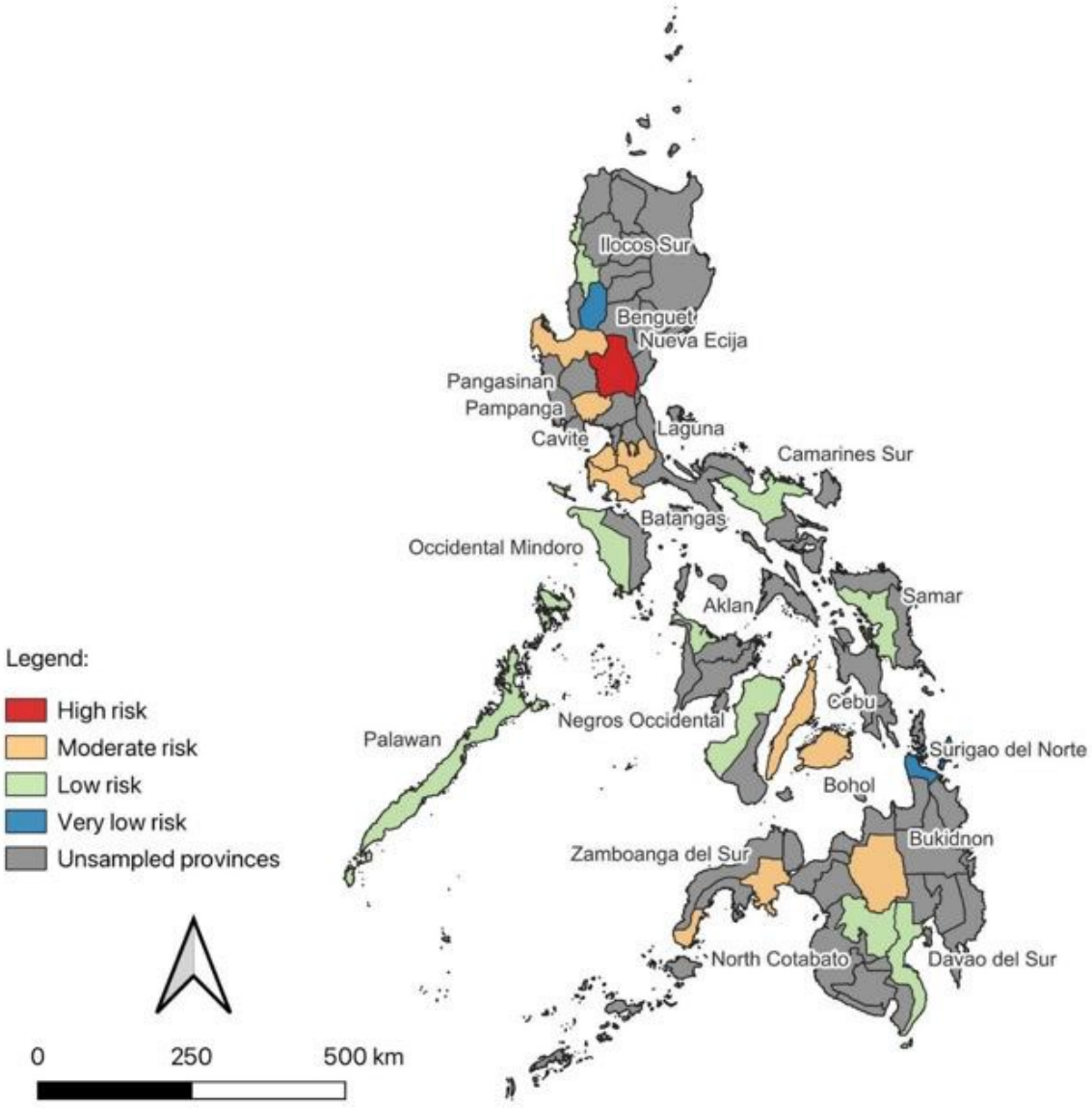

Figure 2. Geographic distribution of the risk classifications for classical swine fever transmission in 21 sampled provinces in the Philippines. The map was generated using Quantum Geographic Information System 3.40.

4. Discussion

CSF belongs to the WOAH list of notifiable diseases of domestic and wild pigs [7]. Due to its transboundary nature, it poses a serious risk to pig health, the swine industry, and both local and international trade, impacting both economic stability and food security worldwide [2]. The disease is thought to be endemic in the Philippines, with outbreaks between 2007 and 2009 affecting more than 4,000 pigs nationwide [23–25]. With the potentially severe CSF consequences and the scarce data on occurrences, surveillance using reliable and valid diagnostic methods such as ELISA and qRT-PCR can be useful in determining the CSF spread. Investigating risk factors linked to positivity and estimating transmission risk can also provide insights on how to prevent and control future occurrences while considering the local swine farming context.

Our results revealed the presence of seropositive pigs in smallhold farms across the 21 sampled provinces. This is consistent with

previous CSF research in Nueva Ecija and Pampanga, which reported seropositivity in a smaller sample size of both vaccinated and unvaccinated pigs [36]. In our study, the detected antibodies against CSFv may be attributed to passive immunity, the diagnostic kit's performance, and humoral immune response. Maternally derived antibodies (MDAs), transferred via colostrum, were found to persist for up to 10 weeks in domestic pigs [37], and these might have waned in the sampled five- to six-month-old pigs. Antibodies against other members of the genus *Pestivirus* may cross-react with those against CSFv using the kit in our study [17], potentially contributing to the seropositivity rate. Investigation of other pestiviruses, such as bovine viral diarrhea virus, causing reproductive losses in pigs and affecting sheep and goats [38,39], as well as border disease virus, which was not yet reported in the Philippines, will rule out this source of false positive samples. Additionally, examining samples from suspected CSF cases at the molecular level after a serological assay may also achieve increased specificity. For example, differentiation of CSFv from other pestiviruses was successful using qRT-PCR [20,40]. Another study developed a multiplex qRT-PCR assay for diagnosing CSF that was capable of detecting as few as 8 copies of the viral genome with exceptional specificity for CSFv, ensuring no cross-reactivity with other pestiviruses [41]. Post-infection with a wild type of CSFv also elicits antibody production, except for persistently infected pigs with immunosuppression [42]. Pigs surviving CSF were known to have prolonged and even lifelong immunity [15], and the WOAH recommends examining antibodies in probable CSF cases within a seroconversion period of 14 to 21 days post-infection [1,15]. Considering the negative association of CSF history on seropositivity from our regression analysis, it was highly likely that the detected antibodies were not derived from field infection. CSF vaccination is typically performed at five to nine weeks of age, either as a single dose or with a booster depending on vaccine type and existing CSF situation [43,44]. In one study, seroconversion takes around one to three weeks post-vaccination, and pigs remained seropositive at 45 days [45]. Other studies showed varying persistence of vaccine-induced antibodies [18,46,47], which may have lasted in the sampled pigs. Although our test kit cannot distinguish the various drivers of antibody production [17], the association of CSF vaccination with seropositivity in our study provides sufficient evidence on the increased likelihood of vaccine-derived antibodies. This is consistent with research findings in Timor-Leste and Indonesia wherein the odds of seropositivity were increased by two to three times with CSF vaccination[48,49]. For improved sensitivity and specificity, future surveillance programs may benefit from using Differentiating Infected from Vaccinated Animals (DIVA) serological tests or vaccines to reduce ambiguity in interpreting seropositivity [17,50]. On the other hand, the immunosuppressive effect of the CSFv, especially in persistently and chronically affected herds, may account for the seronegative samples, as shown previously [42,51]. Vaccination failure may also occur if the timing of administration and seroconversion coincide with the presence of MDAs [52,53]. Finally, seronegativity may also be interpreted as the complete absence of CSFv exposure.

Despite considerable seropositivity, no CSFv RNA was detected in any of the tested samples. Early viremia may lead to negative qRT-PCR outcomes due to very low and undetectable viral loads [41]. Moreover, chronic cases typically show prolonged viral shedding and would still be positive when subjected to molecular testing. These attributes demonstrate the absence of active CSF infection, whether acute or chronic, in the sampled animals. The inconsistency between our serologic and molecular findings is also similar to published studies. For instance, a study on the E2CD154 subunit vaccine showed that vaccinated pigs maintained protective immunity for at least nine months post-vaccination, with no evidence of adverse effects or prolonged viral presence [46]. The widely used live Chinese strain vaccine has also been reported to confer solid immunity within a few days after a single vaccination, with lifelong immunity and without prolonged viral shedding [18]. The Thiverval strain vaccine was also documented to offer complete protection five days after inoculation, effectively inhibiting CSFv replication post-challenge [47]. These findings underscore the efficacy of the CSF vaccines in eliciting sustained humoral immune responses without viral persistence and the significance of vaccination as part of a CSF intervention program. Also, the results of the two laboratory-based methods may also be used together in the future direction of CSF surveillance, particularly in declaring CSF-free areas.

Apart from CSF vaccination, three other factors from survey data positively impacted seropositivity. In Kenya, income level and educational attainment were substantially linked to knowledge of farm irrigators on integrated water resource management [54]. Similarly, we hypothesize that water treatment having the highest positive impact on seropositivity may be related to the smallhold farmer's capacity to afford and access vaccination, which can be confirmed by undertaking a knowledge, attitude, and practice study, along with economic analysis. Swill feeding has been implicated as a route of CSFv infection, and a study found the survival of infectious CSFv in pork sausage casing for 37 days [1,55]. This emphasizes the role of pork and pork products in CSF transmission and infection, which may result in antibody production. Furthermore, the practice of feeding untreated leftovers to pigs is common in smallhold farms as a cost-saving measure [56]. In our study, some municipalities reported both swill feeding and CSF vaccination, accounting for the association between these factors. Maintaining vaccination records was also weakly associated with seropositivity. This practice is essential in determining the proper timing of vaccination in growers, sows, and piglets farrowed from vaccinated sows to avoid vaccination failure and promote successful seroconversion [53,57].

Results of LASSO regression also showed three factors being negatively associated with seropositivity. The commonality of farrow-to-finish operations in smallhold farms in the Philippines was previously demonstrated [58]. The ongoing ASF issue may have also increased the farms employing this operation type to minimize the probability of introducing infected herds. Under this intensive system, CSF occurrence may result in a higher probability of vertical transmission, which commonly results in immunosuppression and persistent viremia in farrowed pigs [59]. This explains the inverse relationship between seropositivity and the history of CSF in smallhold farms. An increased proportion of farms near residential areas was also observed to result in lower seroprevalence. Compared to commercial farms, smallhold farms are typically found in residential backyards, have fewer biosecurity measures in place, and have less access to veterinary care [60]. These characteristics may have contributed to the lower seropositivity in the increased percentage of farms close to residential zones. Increasing native pig breed distribution also negatively impacted seropositivity, which may be attributed to the perceived resistance of these breeds to swine diseases, resulting in a lesser willingness to have these pigs vaccinated [61]. However, it is crucial to emphasize the susceptibility of all pigs to CSFv infection as shown in the comparative study between indigenous and commercial breeds in Lao's People Democratic Republic [62].

Among the sampled provinces, only Nueva Ecija was categorized as high-risk for CSF transmission due to consistently high scores across all factors, leading to an elevated overall risk. On the contrary, it is worth noting that the missing responses in Benguet influenced its very low risk classification, reinforcing the significance of complete working data for a more precise analysis. In our semi-quantitative scoring, seropositivity was inversely related to the risk score. Generally, sufficient antibody levels—whether from vaccination, field infection, or passive immunity—confer protection to pigs. For example, the Thiverval vaccine offered robust defense five days following vaccination, and protection was maintained even when vaccinated pigs were housed with CSFv-positive pigs [47]. The E2CD154 candidate vaccine also demonstrated capacity to prevent vertical transmission of CSFv [46], and MDAs were shown to be effectively transferred to piglets, providing early and short-lived immunity [37]. Antibodies in pigs surviving infection by the wild CSFv strain also persisted for long periods of time [15]. A cross-sectional study also found the lack of vaccination as a significant risk factor for CSF occurrence [12]. Collectively, these findings indicate that the lack of exposure to CSFv increases the susceptibility of pigs to contracting the disease and being a source of transmission.

Direct contact with infected pigs is a major route in CSF transmission [1]. To estimate the potential contact rate among a high volume of pigs, we considered the proportion of pigs in commercial farms and swine population density, which were among the factors found to be associated with increased odds of CSF occurrence. Commercial farms typically raise a large herd size of more than 50 pigs [63], and it was previously estimated that this farm type had a contact rate of 1.24 times a day, higher than in smallhold farms [64]. As the herd size increases, the number of susceptible pigs also rises, along with the chance of effective

contact [10]. While higher contact rates suggest greater odds of CSF occurrence, it is important to note that these farms have sufficient capacity to employ appropriate biosecurity measures and disease intervention strategies such as vaccination and disease monitoring, which can mitigate CSF transmission risk. The swine population density may also more effectively reflect the contact rate than population size. In Cuba, a denser swine population was associated with a 1.25-fold increased chance of infection [65]. Additionally, airborne transmission or "neighborhood effect" within a distance of one meter was considered during CSF outbreaks in areas with high farm density [66]. This evidence underscores the potential heightened risk of CSF transmission in areas with larger swine herd sizes and higher swine population density.

The association of farm practices with seropositivity in our study prompted an examination of these factors as contributors to CSF transmission risk. The CSFv has been shown to survive in water for 6 to 24 days at 20 °C [67]. Its infectiousness requires further assessment, and minimal studies were conducted to elucidate the role of drinking water in transmission. Given its persistence under certain conditions, disease prevention efforts may benefit from water treatment and ensuring a safe and clean water supply for pigs as a form of reducing CSFv risk. Vehicle disinfection is a critical element of farm biosecurity. In Denmark, truck disinfection at borders was mandatory due to varying persistence of CSFv and other important swine viruses in the outside environment, especially in the presence of protein and organic materials [67,68]. Moreover, trucks coming from another livestock farm were banned from entering new livestock premises for 48 hours after disinfection [68]. Model simulations found that these practices decreased the likelihood of CSFv occurrence [68] due to the susceptibility of the virus to common disinfectants such as sodium hypochlorite, quartz, and aldehydes [69]. Without vehicle disinfection practices, the risk of CSF occurrence and transmission can be higher.

The effect of frozen pork inventory and swill feeding was closely related in CSFv transmission. Studies have documented the survival of CSFv in frozen pork for years, in chilled pork for up to 85 days, and in cured or smoked pork for 17 to 188 days [67]. The volume of pork imported both legally and illegally was also estimated to pose a serious risk in Denmark because of the potential use of pork as swill [68]. Feeding kitchen leftovers to pigs was also shown to increase the chances of CSF occurrence by 8.53 times in Ecuador [12] and by 2.25 times in Bhutan [70]. These findings substantiate the inclusion of frozen pork volume and swill feeding in our risk scoring scheme. Several strategies were implemented and recommended to mitigate this risk. For instance, banning swill feeding was found to decrease the risk of CSFv introduction through frozen pork [68], though non-compliance remains an issue, particularly in smallhold farms, where it is commonly practiced to reduce production cost. It was also recommended to uniformly heat the meat for consumption to 70°C for at least 30 minutes to effectively inactivate CSFv [14]. For swills, inactivating the virus can be achieved by heating to at least 90 °C for at least 60 minutes with constant stirring or to 121 °C for at least 10 minutes [14]. The role of human population density, which reflects the increased human interaction, was also considered minimally in the risk scoring. Limited studies dealt with the role of increasing human movements in the spread of CSFv, but indirect or mechanical transmission via contaminated clothing of humans was possible [7], contributing low risk to CSFv transmission.

Considering all eight factors in assessing the risk of CSF transmission improves our understanding of its epidemiology and potential danger to the swine industry and pig health. Despite no active infection detected, the results of risk scoring provide useful information that can aid in designing future disease surveillance programs while considering efficient use of resources [66]. While a similar study including all provinces in the Philippines may be done, we recommend focusing on the identified moderate- to high-risk provinces in terms of conducting CSF vaccination programs, enforcing strict biosecurity measures, and undertaking other CSF intervention strategies to prevent disease occurrence and mitigate related impacts.

5. Conclusions

This study provides critical information on the epidemiological status of CSF in smallhold farms across 21 provinces in the Philippines. The seroprevalence implies prior CSFv exposure, which was likely due to vaccination. On the other hand, the absence of detected CSFv RNA suggests

no active infection in sampled pigs from the sampled areas. The combined results of the two laboratory methods may also be used in future disease surveillance frameworks in declaring CSF-free areas. Factors associated with seropositivity were related to on-farm practices, farm demographics, and human-driven practices. Furthermore, the 21 sampled provinces were classified into six risk levels, with no province having extreme and very high risk classifications. Overall, these findings highlight the need for strategic disease monitoring with a requirement for better vaccination strategies, strict biosecurity measures, and efficient resource allocation for CSF control and prevention in the Philippines.

Availability of Data and Materials

All data may be provided upon reasonable request to the corresponding author.

Author Contributions

Conceptualization, B.R.G.M. and M.S.E.G.L.; Methodology, B.R.G.M. and M.S.E.G.L.; Formal Analysis, G.M.V.P., J.M.G.B., and M.A.O.A.; Investigation, C.M.D.P., A.P.R.S., M.A.O.A., E.N.G.L., K.A.S.D.R., B.R.G.M., A.W.B.R., and M.S.E.G.L.; Data Curation, G.M.V.P., J.M.G.B., and M.A.O.A.; Writing – original draft, C.M.D.P., J.M.G.B., C.P.F.C., and K.A.S.D.R.; Writing – review and editing, C.M.D.P., A.P.R.S., M.A.O.A., E.N.G.L., K.A.S.D.R., B.R.G.M., A.W.B.R., J.M.G.B., G.M.V.P., C.P.F.C., and M.S.G.L.; Supervision, M.S.E.G.L., B.R.G.M., and C.P.F.C.; Project Administration, M.S.E.G.L. and B.R.G.M.; Funding Acquisition, M.S.E.G.L., B.R.G.M., and C.P.F.C.

Ethics Approval and Consent to Participate

Only abattoir workers who signed a written consent form were included in the interview.

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Conflict of Interest

The authors declare the absence of competing interests.

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(Original Research)

Evaluating Sugarcane Water, Coconut Water, and Honey as Diluents for Philippine Native Chicken Semen at Two Storage Temperatures

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Abstract

Background: Natural extenders present a cost-effective, biodegradable and less toxic alternative to chemical extenders in semen extension and storage. The experiment was designed to find a suitable semen extension medium for Banaba native chicken by comparing the effects of four different extenders: egg yolk-citrate (EY-C), sugarcane water (SW), coconut water (CW), and honey solution (HS) under two storage temperatures: low temperature (3–5°C) and room temperature (25–27°C). **Methods:** The experiment employed a 4 x 2 factorial design in a randomized complete block design (RCBD) with four types of diluents and two storage conditions, using the collection period as a blocking factor. The quality of pooled semen samples from 10 collection periods was assessed using Computer-Assisted Semen Analyzer (CASA). Only samples with ≥ 70% total motility were further analyzed. **Results:** The type of extender had significant effect on sperm total motility, progressive motility, and morphology of the Banaba native chicken. Except sperm progressive motility, temperature had no significant influence on semen parameters. **Conclusions:** All extenders were effective in protecting the spermatozoa of the roosters at room temperature compared to that of low temperature. The sugarcane water diluent was numerically superior to the other diluents and can be used for semen extension.

Keywords

Banaba native chicken, Coconut water, Sugarcane water, Honey, Semen extension

1. Introduction

Poultry ejaculate has a unique characteristic, high concentration of spermatozoa in a limited volume, [1] and is one of the constraints in artificial insemination (AI) [2]. This limited volume necessitates an extension of the ejaculate for AI. AI is essential in poultry genetic improvement, presenting an incomparable control over breeding programs and allowing rapid propagation of desirable traits. Also, AI allows the use of cryopreservation and gene banking, preserving valuable genetic resources for future use, and therefore contributing to biodiversity preservation and the prevention of genetic loss [3].

Materials used for extension are collectively called extenders and are commercially available for poultry. These extenders provide requirements for sperm survival during fresh and storage at low temperature. Therefore, adding energy sources that support cellular metabolism, control pH and osmolality, prevent bacterial growth, and maintain a favorable microenvironment is essential for preserving good sperm motility and viability [4]. Although commercial chemical extenders are readily available, they are costly, require careful preparation, are environmentally

unfriendly, and are reported to be toxic to stored or extended semen. In contrast, extenders from naturally occurring ingredients are less expensive, can be easily adopted by local farmers and breeders, biodegradable, and less toxic.

Selecting the correct extender is a vital precondition of handling semen for AI. Natural extenders are alternatives to chemical extenders in semen extension and cryopreservation of various farm animals [5,6,7]. These may contain natural ingredients obtained from coconut, sugarcane, and honey. Coconut water (CW) is a clear liquid inside coconut fruits that contains various nutrients necessary for cell preservation [8]. In 2019, Rochmi and Sofyan [9] reported that CW is a good candidate for preservation of spermatozoa motility and viability in roosters for up to seven days. It is an alternative semen extender that is non-toxic, low-cost, practical, and effective for semen extension and cryopreservation [6,8]. Sugarcane water (SW), as a natural product, contains appreciable amounts of total sugars, minerals, vitamins, antimicrobial and cytoprotective effects, and antioxidants [10]. These components contribute to the maintenance of sperm motility and viability. Honey, on the other hand, is rich in sugars, proteins, lipids, vitamins, minerals, amino acids, enzymes, volatile chemicals, antioxidants, phenolic acids, organic acids, and flavonoids, all of which are helpful in the sustenance of sperm cells, leading to improved survivability and sperm motility quality [7,11].

Native chicken in rural areas contributes to protein supply, poverty alleviation, and additional income for poor rural farmers [12]. The Banaba is one of the Philippines' native chicken breeds, predominantly found in Batangas province. It is commonly used as a source of good-quality meat and eggs, and it is also favored for leisure activities such as cockfighting by locals. The Banaba chicken produces a relatively high volume of sperm cells. This high semen volume can be exploited in our quest to maximize poultry production efficiency to meet the increasing demands for sustainable human food resources through propagation using extended semen. Thus, the purpose of the study was to formulate an extension medium suitable for Banaba native chicken semen extension that could maintain and sustain sperm viability when processed for a short-term storage.

2. Materials and Methods

The Institutional Animal Care and Use Committee (IACUC) of the University of the Philippines Los Baños (UPLB) approved the experimental procedures with allotted procedure number CAFS-2018-006. Semen collections were done at the University Animal Farm (UAF) in Brgy. Putho-Tuntungin, Los Baños, College, Laguna, Philippines with location coordinates 14°09'24.4"N, 121°15'06.6"E. The semen samples were immediately sent to the Animal Physiology Laboratory, Villegas Hall, Institute of Animal Science (IAS), University of the Philippines Los Baños (UPLB) for pre-processing quality assessment, processing, and evaluation using a computer assisted sperm analyzer (CASA).

2.1 Experimental Design and Storage Temperature

The experiment employed a 4 x 2 factorial design in a randomized complete block design (RCBD) with four types of diluents and two storage conditions, using the collection period as a blocking factor. The types of extenders were: EY-C (egg-yolk citrate), SW (40% sugarcane water + 30% distilled water + 30% EY-C), CW (20% coconut water + 30% distilled water + 50% EY-C), and HS (15% honey solution + 20% distilled water + 65% EY-C). The storage temperatures used were low temperature (3-5°C) and room temperature (25-27°C).

2.2 Experimental Animals Management and Care

Twelve (12) 29-month-old Banaba native roosters with an average weight of 2.24 kg were obtained at five (5) months old from the National Swine and Poultry Research and Development Center, Bureau of Animal Industry, Tiaong, Quezon, Philippines. The roosters were housed in an open-sided housing system with temperature conditions at the farm between 21–26°C in individual cages with a floor space of 2.0 ft^2/bird. Birds were offered commercial chicken breeder feeds with clean drinking water provided *ad libitum*. The birds' cages were cleaned at regular intervals with the manual scraping of feces, washing with water, and disinfection of the floor to ensure good sanitary conditions on the farm. The roosters were trained for semen collection using the abdominal massage method.

2.3 Semen Collection

A trained person at the University Animal Farm was responsible for the semen collection. The semen collection was done twice a week at 7:00 AM. The roosters were fasted for 15 h (from 16:00 to 07:00) to reduce the chances of fecal contamination during semen sample collection. Additionally, feathers were occasionally plucked at the peri-cloacal region to avoid dirt contamination during collection. During collection, care was taken to ensure collection of clean semen. The duration of semen collection was between 10-15 min per collection. During collection, each collected semen sample was independently assessed for color and consistency through visual appraisal, while volume was determined using Indoplas® sterile disposable 1 mL syringe with 0.01 mL calibration. The 1 mL syringes containing the semen sample were put inside a clean, well-disinfected foam-padded ice chest and sent to the laboratory for processing and evaluation. The transportation time was usually 10–15 min. During the experiment, Electrogen® D+, an anti-stress supplement, was offered to the experimental birds for revitalization of energy. Semen samples were collected ten times, totalling 120 ejaculates.

2.4 Semen Processing and Evaluation

Semen samples were pooled to remove individual variability effects among the semen donor roosters. Subsequently, using a conventional hemocytometer slide, sperm concentration was determined according to the procedures of [13]. The pooled ejaculate was gently mixed and divided into equivalent volume and was arbitrarily allotted to each experimental treatment with a dilution rate of 1:25 (semen: extender) for CASA (Ceros II, IMV Technologies, China) evaluation. Semen with preliminary microscopic parameters of ≥ 70% were used in this study. The diluted semen of the various treatments (EY-C, SW, CW, and HS) was then divided into two parts (Part A and Part B). Part A was stored at 3–5^{o}C in the refrigerator, while part B was stored at room temperature of 25–27°C. About 2 µL sample from EY-C per batch was used for preliminary spermatozoa motility (%) evaluation. The Gallus setup/module of CASA was used in this study with a frame capture speed of 60 Hz and camera exposure of 4 ms. Normal microscope slides were used in analyzing the semen samples. The slides were put in a MiniTherm Stage Warmer, maintaining the sample temperature at 37 °C. Five (5) frames were taken for every single analysis, with an average time of 60 seconds to complete. The % total motility, % progressive motility, and % normal morphology were analyzed at 2 h intervals up to 10 h post extension. Sperm viability was determined using eosin-nigrosin procedure [13].

2.5. Semen Extender Preparation

2.5.1 Preparation of Sodium Citrate Solution (13.6 mL)

The sodium citrate solution (CS) was prepared by dissolving 0.37 g of sodium citrate and 0.20 g of D-fructose in 13.6 mL of distilled water. The mixture was then thoroughly mixed using a vortex mixer resulting in 13.6 mL of solution. This served as the base solution.

2.5.2 Preparation of Standard Extender (10 mL)

The standard extender (EY-C) was prepared using 8.5 mL of the base solution and 1.5 mL of egg yolk. The mixture in a 50-mL conical tube was then gently mixed, resulting in 10 mL of the standard extender (EY-C).

2.5.3 Preparation of Coconut Water Extender (10 mL)

The fresh young coconut fruits were bought from the open market throughout the experiment, but from the same source. The fruits were washed to remove any contaminants and wiped dry using paper towels. The fruit's pericarp (epicarp) was then cut open through the mesocarp to reach the endocarp, exposing the water-containing meat. The water of the fruit was then withdrawn using disposable 1-mL syringes piercing through the flesh of the fruit. The coconut water (CW) extender was made up of 2 mL of CW, 3 mL of distilled water, and 5 mL of EY-C (20% CW+30% dH_2O+50 % EY-C) and mixed carefully. This formed the coconut water extender (CW).

2.5.4 Preparation of Sugarcane Water Extender (10 mL)

The 2004-1011 sugarcane variety obtained from the Institute of Plant Breeding (IPB) was used for the experiment. The freshly cut sugarcane pieces with a maximum of four internodes were washed clean under running water. The skin of the sugarcane of one-piece internode was peeled off and rinsed under running water. Subsequently, the piece was divided into four quarters and placed in a juice extractor, pressed, and extracted the liquid. The extract was then sieved using the sperm filtering paper in conjunction with a cheesecloth. The SW extender was made up of 4 mL of sugarcane water, 3 mL of distilled water, and 3 mL of EY-C (40% SW+30% dH_2O+ 30% EY-C), which was gently mixed in a 15 mL conical tube. This was designated as SW extender.

2.5.5 Preparation of Honey Extender (10 mL)

The honey used in the experiment was purchased from Institute of Biological Science (IBS). The honey extender was prepared by dissolving 1 mL of honey in 9 mL of distilled water in a 15 mL conical tube, resulting in 10% honey solution. Then, 1.5 mL of the honey solution was added to 2 mL of distilled water and 6.5 mL EY-C (15% (10% HS) +20% dH_2O + 65% EY-C) and mixed carefully in a conical tube. This formed the honey extender (HS).

The optimized composition of the above natural extenders used in the experiment were obtained through the "trial and error method" of different concentrations before arriving at various inclusion rates.

2.6 Statistical Analyses

All data gathered were first tested for normality and homoscedasticity using Shapiro-Wilk's test and Levene's test, respectively. All data satisfying both assumptions were analyzed using analysis of variance (ANOVA) while Tukey's LSD was used as a post hoc analysis tool to determine the level of significance among the means at 5%. All statistical analyses were done using STATA V 15.

3. Results and Discussion

In this experiment, macroscopic and microscopic characteristics of Banaba native chicken semen were determined from 120 ejaculates during a three-month long experimentation. The macroscopic characteristics were semen color, semen consistency, semen volume and semen pH while the microscopic characteristics were sperm concentration, sperm motility (total motility and progressive motility), sperm morphology and semen viability. All observed values on macroscopic semen characteristics and sperm concentration are summarized in Table 1.

Table 1. Macroscopic semen characteristics and sperm concentration of Banaba Philippine native chicken.

Parameter	Results
Semen volume, mL	0.15±0.02
Semen color	Creamy
Semen consistency	Thin
Semen pH	7.27±0.06
Semen concentration, x10^9 spz/mL	4.99±1.32

spz- spermatozoa

3.1 Semen Characteristics of Banaba Native Chicken

3.1.1 Semen Volume

The semen volume values ranged from 0.07±0.01 to 0.34±0.03 mL with a mean value of 0.15±0.02 mL. The mean semen volume obtained in this current

study is higher than that in earlier study by [14] which was 0.13 ± 0.01 mL of Kampung broiler chicken. Similarly, Telnoni *et al*., in 2017 [15] reported a similar semen volume of 0.15±0.02 mL in Sentul Kampung Kedu (SK Kedu) chicken in Indonesia. On the contrary, the results in this report are lower than those obtained by [16] who reported an average semen volume of 0.24 ± 0.06 mL in Kampung chicken. The differences in semen volume could be attributed to breed variations, as meat-type chickens tend to produce more semen than egg-type chickens [6]. Other contributing factors include management practices, the condition of reproductive glands, and the extent of exploitation of the breed's genetic potential [17].

3.1.2 Semen Color

Majority of the ejaculates (98) representing 81.67% were creamy while 22 ejaculates (18.33%) were watery/clear. These findings collaborate with the findings of [18] who reported semen color of white milky/creamy from four different breeds of local chicken. They associated creamy color with high sperm concentration hence good quality while bright white color/clear semen was associated with lower concentrations of sperm and poor quality. Esguerra *et al*., in 2020 [6] reported 53.21% of the ejaculates being watery in Paraoakan native chicken which differs from the findings of this current study. This may be attributed to breed difference and the season in which the experiments were conducted [19].

3.1.3 Semen Consistency

The consistency of the various ejaculates observed were 60, 21.67 and 18.33% for thin, thick, and watery, respectively. This partly agrees with [20] who reported a consistency of fresh rooster sperm as viscous/thick using dorso-abdominal massage method in collecting the semen. Ideally, the normal semen consistency in chicken ranges from thin to thick creamy. The semen in this experiment can best describe as creamy thin.

3.1.4 Semen pH

The semen pH values ranged from 7.17 ± 0.06 to 7.35 ± 0.08 with a mean value of 7.27 ± 0.06. The semen pH recorded in this study agrees with the reported pH value of 7.31 ± 0.06 by [14]. In contrast, the pH value obtained in this study is lower compared to [21] who reported 7.70 ± 0.90 for the Ovambo breed but higher than the previous reports of [17]. Hambu *et al*., in 2016 [20] stated that the stimulation of the accessory sex glands and method of semen collection could be factors contributing to variations in semen pH. However, the findings in this report are within the normal pH of chicken sperm of 6.0 to 8.0 in most studies [22].

3.1.5 Sperm Concentration

Sperm concentrations vary within breeds and between species and range from 2.0-10.0 x 10^9 spermatozoa/mL. It is an essential indicator of the viability of the spermatozoa and provides information on the extent of dilution necessary to obtain required sperm numbers per insemination dose [23]. The sperm concentration of the pooled semen from the Banaba native chicken in this study ranged between 4.73 x 10^9 to 5.25 x 10^9 spermatozoa/mL with an average of 4.99 x 10^9 spermatozoa/mL. The values in this study are within the findings of [24], who reported range values of 3.0 x 10^9 to 8.0 x 10^9 spermatozoa/mL in Anak 2000 broiler breeder cocks. However, the results in this report are higher than that of [14] (2.62± 51.1x10^9 spermatozoa/mL), [19] (4.16-4.39 x 10^9 spermatozoa/mL), [16] (2.81 ± 0.40 x 10^9) in indigenous breeds of chicken. On the other hand, the results in the current study are lower than the results reported by [25](6.60 x 10^9 spermatozoa/mL) in Ross broiler breeder roosters. The variation in sperm concentration is attributable to strain genetic makeup, environmental adaptability, season, ejaculate volume and age [24].

3.2 Effects of Storage Duration and Storage Temperature on Sperm Motility of Banaba Native Chicken

Percent (%) total sperm motility and % progressive sperm of extended semen from Banaba native chicken were analyzed using CASA after extension at a 2 h intervals for 10 h. Irrespective of the extender, there was a decreasing trend observed in percentage total motility (TM) and progressive motility (PM) over time among the four experimental extender treatments and maintained at two storage temperatures (Fig. 1, 2, 3, & 4).

In Figure 1, CW extender showed a wave-like trend while the other three extenders showed linear trends. However, EY-C extender showed superior quality in maintaining the % TM of the sperm cells at room temperature closely followed by SW extender.

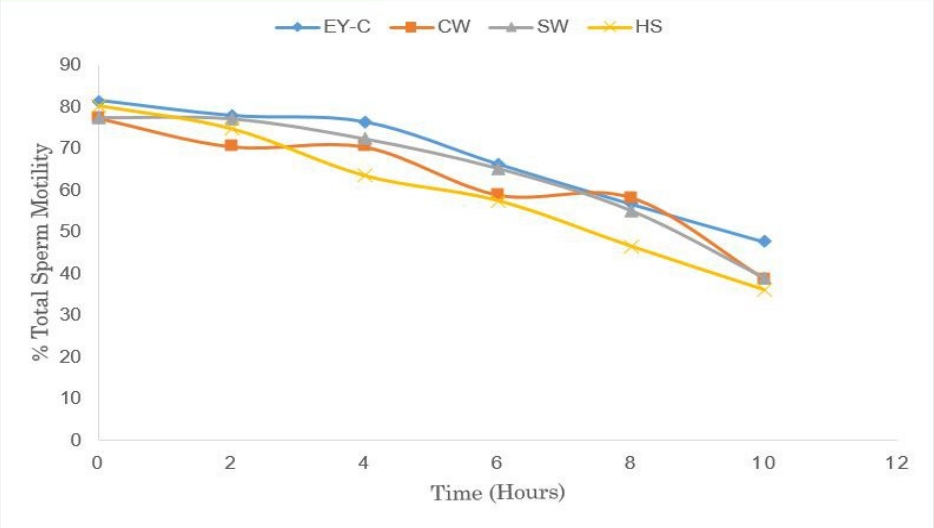

Figure 1. CASA-assessed percentage total sperm motility changes in Banaba native chicken semen diluted with four natural extenders and maintained at room temperature (n=10).

In Figure 2, EY-C, CW, and HS showed wave-like trends of declining progressive sperm motility while SW showed linear trend as in Fig. 1.

In Figures 3 and 4, SW showed superiority over the other extenders in the % TM and % PM in the extended ejaculate stored at low temperature. However, HS extender supported the survival of the sperm cells up to 8 h while the other extenders maintained the viability of sperms cells up to the 10 h designed experiment.

Similar results have been reported by

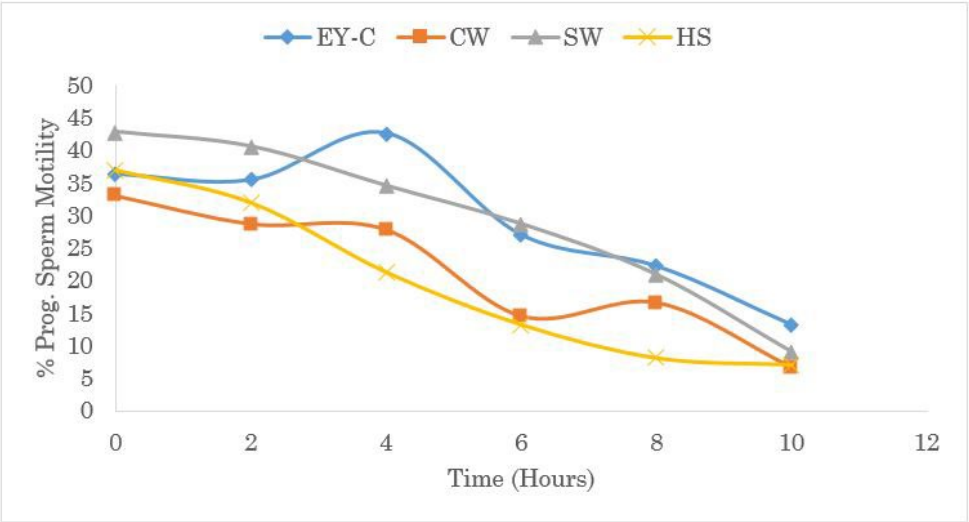

Figure 2. CASA-assessed percentage progressive sperm motility changes in Banaba native chicken semen diluted with four natural extenders and maintained at room temperature (n=10).

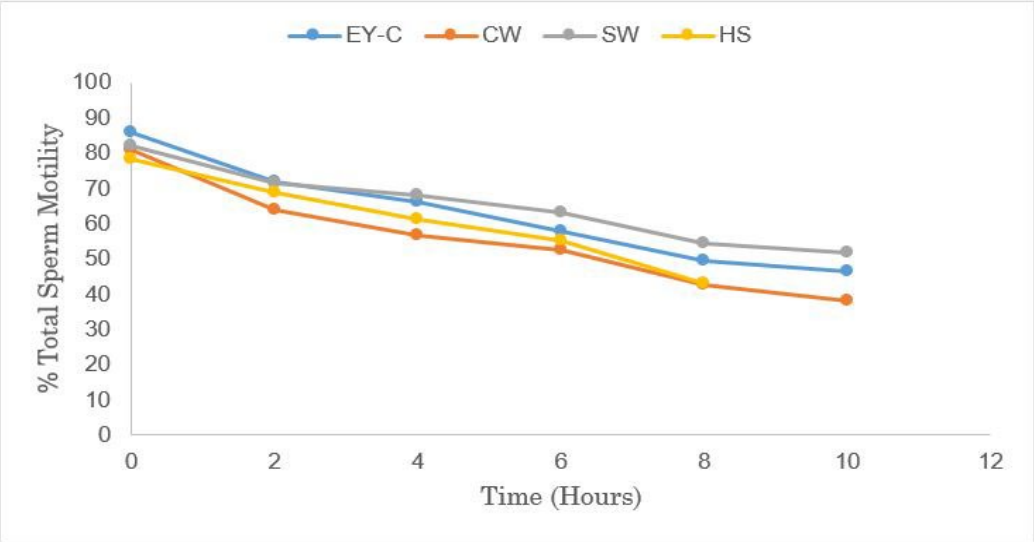

Figure 5. CASA-assessed percentage total sperm motility changes in Banaba native chicken semen diluted with four natural extenders and preserved at low temperature (n=10).

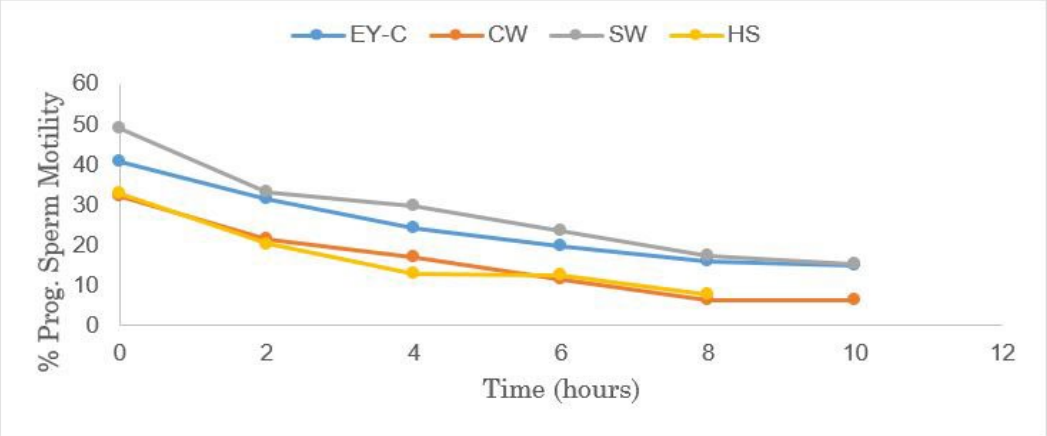

Figure 4. CASA-assessed percentage progressive sperm motility changes in Banaba native chicken semen diluted with four natural extenders and preserved at low temperature (n=10).

previous researchers. Masoudi *et al.*, in 2019 [26] observed significant reduction of all sperm parameters measured in Ross broiler semen stored at 5°C for 48 h. Rakha *et al.*, in 2016 [27] also recorded decreasing number of motile sperm cells in all extenders at any time measurements were taken during 48 h cold storage of semen from the Indian Red Jungle fowl. A decreasing sperm motility with increasing storage periods has been reported in all extenders for other birds [28,29,30]. Evaluation of progressive sperm motility of ejaculate indicates the reproductive efficiency of the cock.

3.3 Effects of Storage Duration on Extender pH Values

The initial pH values of the extenders were 7.39 for EY-C, 7.45 for HS, 6.79 for SW, and 6.99 for CW. After storing the semen samples in these extenders at 5°C for 10 h, the pH values in all extenders showed a slight increase. Specifically, the pH values increased by 0.54% for EY-C, 0.40% for HS, 1.91% for SW, and 1.72% for CW.

Low temperature storage of semen is a common technique used to decrease sperm metabolism and to maintain sperm viability over

an extended period [31]. Dilution and holding of semen in low temperatures reduce sperm metabolic activity as a result, conserving energy and prolonging the accumulation of sperm metabolites in storing media [6]. Also, the harmful effects of microbial contamination and competition are curtailed at low temperature, hence protecting sperm cells, and making nutrients available to them. The decrease in sperm metabolism and delayed accumulation of sperm metabolites may have contributed to the observed stable or slight increase in the pH.

Conversely, extenders with semen samples stored at 25-27°C exhibited a decrease in pH values. The percentage decreases were 2.30% for EY-C, 3.62% for HS, 5.30% for SW, and 2.15% for CW, with the most significant decrease observed in the SW extender. This decline in pH is often linked to increased sperm metabolic activity, which leads to the production of lactic acid and subsequently a reduction in sperm motility [7]. Additionally, factors such as changes in nutrient composition due to sperm metabolite release, alterations in medium osmolarity, increased chloride ion production in certain extenders, and potential physical damage to spermatozoa during the dilution process can also contribute significantly to reduced sperm motility [32]. Surprisingly, SW extender with the lowest pH value in both storage conditions performed comparable to that of EY-C at room temperature and better than all the other extenders at low temperature. The decrease in sperm motility during storage may be due to other factors rather than decreasing pH in this extender.

3.4 Effects of the Type of Extender and Storage Temperature on Sperm Characteristics of Banaba Native Chicken

3.4.1 Sperm Motility

The results on sperm motility (%) of the Banaba native chicken showed significant ($p=0.016$) difference among the type of extender (Table 2). However, the interaction effect between temperature and type of extender and the temperature main effect on sperm motility (%) were found to be insignificant.

Table 2. Mean (± SEM) motility (%) of Banaba native chicken semen diluted with four natural extenders and maintained at two different storage temperatures (n=10).

	Temperature		Mean for type
Treatment	Low (3-5°C)	Room (25-27°C)	of extender
EY-C	63.21 ± 2.22[a]	68.24 ± 1.99[a]	65.70 ± 1.51
SW	65.18 ± 1.95[a]	64.95 ± 2.20[ab]	65.06 ± 1.46
CW	56.43 ± 2.54[b]	62.69 ± 2.29[b]	59.59 ± 1.73
HS	60.27 ± 2.34[ab]	64.471 ± 1.17[ab]	60.98 ± 1.85
Mean for Temperature	61.46 ± 1.14	64.47 ± 1.17	
		p-values	
Type of extender		0.0160	
Temperature		0.0569	
Type of extender x tem.		0.4497	

[a, b], Means in a column with different superscript letters differ significantly ($P<0.05$). Each value is the mean of 10 independent observations. Tem- Temperature.

Under the low temperature, the EY-C, SW and HS extenders were statistically similar in maintaining motility and viability of the spermatozoa. Though, there was similarity among the three extenders, SW was numerically superior compared to the EY-C and HS extenders. There was no significant difference between CW and HS while CW was significantly ($p<0.05$) lower compared to EY-C and SW extenders in maintaining percent sperm motility.

In the room temperature condition, EY-C was numerically superior to the three natural extenders but only statistically differs from CW extender. The SW, HS and CW were similar in maintaining sperm motility and viability under this temperature condition.

Low temperature storage of extended semen is commonly employed to reduce sperm metabolic activity and enhance sperm longevity, thereby preserving sperm quality during storage [33]. Higher temperatures, in contrast, can lead to rapid depletion of energy from the extension medium, reducing sperm motility and increasing the risk of bacterial growth [30]. Slanina *et al*., in 2015 [30] found that spermatozoa stored in chilled media (5°C) exhibited higher motility compared to those incubated at room temperature (22°C). However, this study found that extended semen from Banaba roosters stored at room temperature showed comparable results to those stored at low temperatures in terms of sperm motility and morphology, with significantly higher progressive sperm motility ($p<0.05$) at room temperature. This finding aligns with previous research by [34,35]. Castro *et al*., in 2020 [7] attributed this result to the adaptability of native animals to warmer environments or inherent characteristics that influence sperm longevity and motility.

Given that all extenders proved most effective for Banaba native chicken semen when stored at room temperature, refrigeration and its associated costs can be avoided. This finding offers a more practical and cost-effective solution for small rural farmers, facilitating the adoption of artificial insemination and enhancing the local rearing of Banaba native chickens.

In respect to percentage sperm progressive motility under low temperature condition in Table 3, the type of extender and temperature interaction did not significantly affect the percent progressive motility. However, the type of extender and temperature had significant effect on the percent progressive motility of the Banaba native chicken.

Table 4. Mean (± SEM) progressive sperm (%) of Banaba native chicken semen diluted with four natural extenders and maintained at two different storage temperatures (n=10).

Treatment	Temperature		Mean for type of extender
	Low (3-5°C)	Room (25-27°C)	
EY-C	24.65 ± 1.74^{aB}	29.93 ± 1.95^{aA}	27.29 ± 1.32
SW	27.96 ± 1.85^{aB}	29.40 ± 1.88^{aA}	28.68 ± 1.32

Table 3. Mean (± SEM) progressive sperm (%) of Banaba native chicken semen diluted with four natural extenders and maintained at two different storage temperatures (n=10).

Temperature	
	p-values
Type of extender	0.000
Temperature	0.002
Type of extender x temperature	0.629

^{a,b}, Means in a column with different superscript letters differ significantly ($P<0.05$).
^{A,B}, Means within a row with different uppercase superscript letters differ significantly ($P<0.05$).
Each value is the mean of 10 independent observations.

The SW and EY-C showed superiority in terms of maintaining sperm forward progressing and were significantly higher than HS and CW extenders, but HS and CW were similar statistically at 5% confidence level. The same trend was exhibited under the room temperature condition with SW and EY-C showing better performance over the HS and CW extenders. The observed significant effect of temperature on the % progressive motility is in line with the findings of [30,34].

Sugarcane water (SW) is a natural product rich in total sugars (glucose, fructose, and sucrose), minerals (potassium, phosphorus, calcium, magnesium, and iron), and vitamins (A, B1-6, C, and E). It also possesses antimicrobial, anti-inflammatory, cytoprotective, and antioxidant properties [36]. The sugar content in SW helps rehydrate spermatozoa and provides the necessary energy to maintain their motility and viability. Additionally, the high mineral content, particularly potassium, plays a crucial role in sperm viability [6]. SW also enhances natural immunity by protecting host cells from microbial damage, including bacterial infections, potentially contributing to its effectiveness as an extender [10]. This combination of beneficial properties may explain the superior performance of SW as an extender.

The continuous survival of the spermatozoa in SW extender may be attributed to the antioxidant properties of the sugarcane water. The phenolic and flavonoid compounds in SW are responsible for the high antioxidant activity and cyto-protection [37]. These properties can prevent the free radicals induced oxidative DNA and cell membrane damage. It is of no doubt that, the cytoprotective compounds in SW extender protected spermatozoa from oxidative stress resulting from variety of sources during the semen processing. In addition to the mentioned properties, SW has slightly low acidity in nature together with its antimicrobial effect is adequate to inhibit the growth of many types of bacteria and can therefore be used as an alternative to antibiotics in the preparation of extenders [7]. SW as an extender has shown to be successful in preserving the semen of pigs [38] and fishes [39]. Akandi *et al*., in 2015 [38] reported that the viability of boar semen stored in sugarcane juice was comparable to the honey-supplemented extender but significantly higher than that of tomato- and pineapple-supplemented extenders. They concluded that spermatozoa can be stored in extenders containing natural products especially sugarcane juice and honey.

Coconut water (CW) is sterile and slightly acidic natural solution composed of sugars, proteins, vitamins, salts, neutral fats, antimicrobial and antioxidant properties [5,6] and commonly consumed by people around the globe because of its health benefits. Esguerra *et al*., in 2020 [6] stated that CW is locally available and widely used in the Philippines and the natural buffering effect of CW led to the testing of its efficacy in semen extension and cryopreservation. It has been tested for preservation of semen in chicken [5], cattle [40], goats [41] African catfish [42], and dogs [43].

Daramola *et al*., in 2015 [42] reported that the improvement in sperm parameters in their study was attributed to sugar content in CW which provides energy and increases osmotic potential of spermatozoa thereby protecting their membranes against chilling-induced injury. It was further stated that improvement in sperm viability is due to the potassium levels in the holding medium and hence its positive effect on the viability of extended spermatozoa. Similarly, essential amino acids especially arginine and lysine in CW improve the shelf-life of sperm cells and sperm motility [6].

Honey is a natural product rich in nutrients like sugars, proteins, lipids, vitamins, minerals, amino acids, enzymes, volatile chemicals, antioxidants, phenolic acids, organic acids, and flavonoids [11,44]. All of these are beneficial to improving sperm motility quality and viability. Honey also contains defensin-1, an antimicrobial peptide and hydrogen peroxide and appreciable amounts of methylglyoxal upon water dilution. These inherent qualities of honey are responsible for the antimicrobial effects on bacteria contributing to the motility maintenance of spermatozoa stored in honey-supplemented diluents during extended period. The antimicrobial effect of honey-supplemented media reduces the competition for available nutrients between the sperm and bacteria, as a consequence improves viability of spermatozoa in the medium. It has also been reported that, honey has an antimicrobial effect on several bacteria species that are immune to antibiotics and can serve as an alternative for antibiotics in extender preparation.

Though HS extender did poorly in maintaining sperm motility and viability compared to EY-C and SW in this current study, it has been successfully used in preserving semen in many studies: chicken/bulls [11], rams [45] and stallions [46].

Castro *et al.*, in 2020 [7] reported nonsignificant differences between honey-supplemented extender and commercial extender in % sperm motility and % progressive motility in semen of Duroc and Quezon boars. Similarly, [11] recorded no significant differences ($p>0.05$) in sperm motility of native chicken between honey

sperm motility of boar semen stored under room temperature condition.

3.4.2 Sperm Morphology

As shown in Figures 5 and 6, EY-C and SW extenders were superior in maintaining

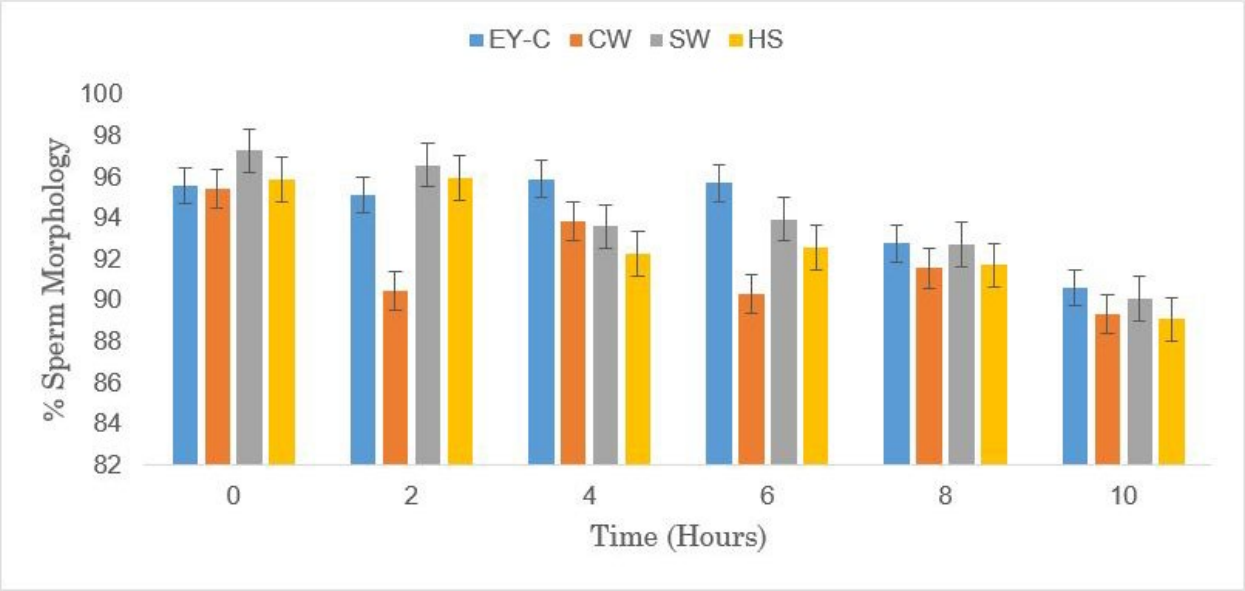

Figure 6. CASA-assessed percentage sperm morphology changes in Banaba native chicken semen diluted with four natural extenders and maintained at room temperature for 10 h (n=10).

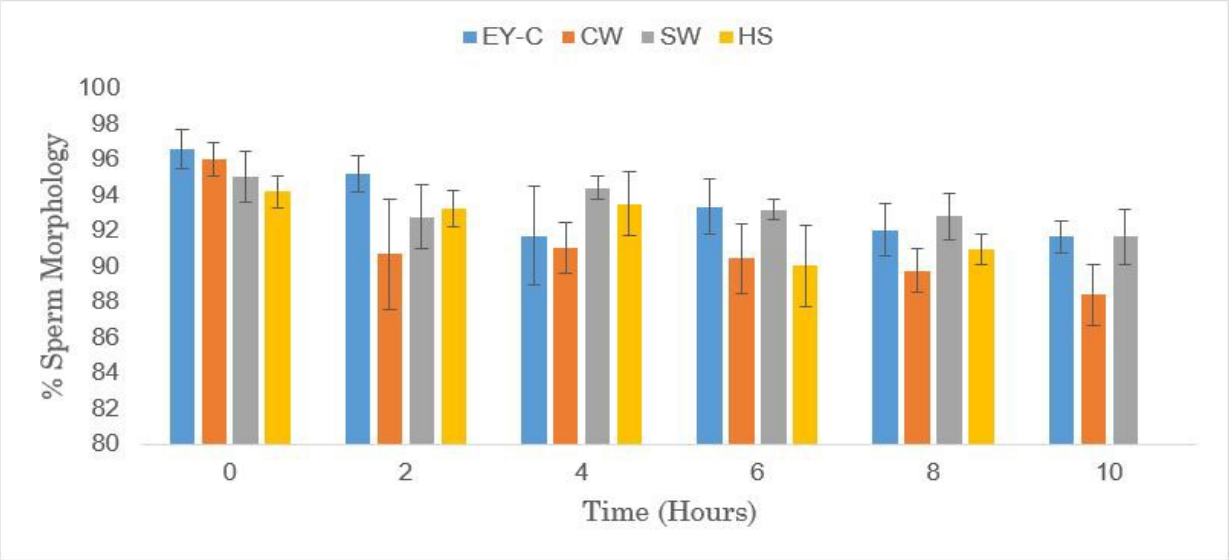

Figure 7. CASA-assessed percentage sperm morphology changes in Banaba native chicken semen diluted with four natural extenders and maintained at low temperature for 10 h (n=10).

extenders (4-6% inclusion rate) and the basic extender. Also, [38] reported honey extender superiority over other natural extenders in %

morphologically normal cells than CW and HS extenders. At hours 0 and 2 under the room temperature (Fig. 5), SW extender was superior in

maintaining morphologically normal sperm cells than the other three extenders. At hours 4-10, EY-C extender surpassed SW in keeping the sperm cell normal morphology while CW performed poorly throughout the 10 h period.

In Figure 6, EY-C extender out-performed SW, HS, and CW extenders in minimizing cell damage to sperm cells under the cold storage. However, SW performed better from the 4th h till the 10th h while HS could not maintain viability until the 10th h. It was generally observed that, morphologically abnormal spermatozoa increase with the passage of time.

There was no significant interaction observed between the type of extender and storage temperature on morphologically normal sperm (%) in Banaba native chicken semen. Also, storage temperature condition has no significant effect on normal sperm morphology. However, the type of extender significantly influenced percent sperm morphology (*p*-value = 0.001) in Banaba native chicken semen in both storage conditions (Table 4), showing that some extenders are better than others in maintaining sperm morphology during semen dilution. The EY-C and SW extenders were significantly higher than CW but was similar to the HS extender.

Sperm morphology gives a useful information about the quality of the semen collected, efficiency of collection and handling and the effectiveness of storage conditions of fresh and frozen semen [22]. Generally, it is accepted that, higher percentage of morphologically normal spermatozoa in semen corresponds with higher fertility rates [47].

Spermatozoa of chicken are filiform in shape [48]. A normal chicken sperm is made of the head (long and narrow) approximately 10.48 µm long and 1.39 µm wide, midpiece approximately 9.51 µm and tail 82 µm long [49]. The dilution processes, preservation, osmotic variance and storage time are known to cause structural damages to sperm morphology [6]. During storage, a decrease in live and an increase in morphologically abnormal dead spermatozoa with bent heads have been reported in earlier studies on avian [28]. Time-dependent decrease in viability and an increase in abnormal sperm morphology of chilled rooster semen are due to osmotic pressure, reactive oxygen species (ROS) and lactic acid produced during storage which cause damage to sperm plasma membrane hence reduces quality in chilled-stored semen.

Table 5. Mean (± SEM) morphologically normal sperm (%) of Banaba native chicken semen diluted with four natural extenders and maintained at two storage temperatures (n=10).

Treatment	Temperature		Mean for type of extender
	Low (3-5°C)	Room(25-27°C)	
EY-C	93.46 ± 0.68[a]	94.38 ± 0.54[a]	93.92 ± 0.43
SW	93.32 ± 0.53[a]	94.13 ± 0.64[a]	93.72 ± 0.41
CW	91.23 ± 0.76[b]	91.93 ± 0.65[b]	91.58 ± 0.49
HS	92.27 ± 0.68[ab]	93.19 ± 0.69[ab]	92.74 ± 0.48
Mean for Temperature	92.62 ± 0.33	93.44 ± 0.32	
		p-values	
Type of extender		0.001	
Temperature		0.067	
Type of extender x temperature		0.998	

[a, b], Means in a column with different superscript letters differ significantly (P<0.05). Each value is the mean of 10 independent observations.

3.4.3 Sperm Viability

As indicated in Figure 7, HS and SW showed superiority over EY-C and CW in maintaining the % viability in semen of the Banaba native chicken.

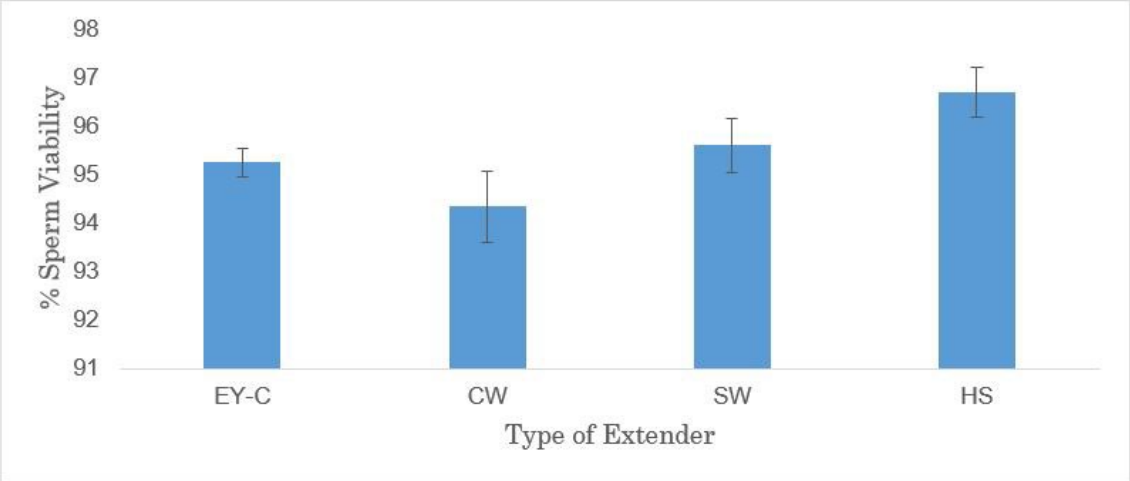

Figure 8. Percentage viability through staining of Banaba native chicken semen extended with four natural extenders and incubated for an hour (n=10).

The results showed sperm viability of 95.25, 94.34, 95.60, and 96.70 % for the EY-C, CW, SW, and HS extenders, respectively. This report agrees with [15,16]. Sperm viability is one of the most vital semen quality parameters used to determine the sperm quality and fertilizing potential of the semen [50]. From the practical viewpoint, [51] stated that, the most critical indicators of male fertilizing potency is the number of live, morphologically intact spermatozoa.

4. Conclusion

The study results indicate that the choice of extender significantly affects sperm total motility, progressive motility, and morphology. However, aside from progressive motility, the holding temperature did not significantly impact semen parameters. Storing semen at room temperature proved more effective in preserving Banaba native chicken spermatozoa. Natural extenders were successful in maintaining sperm motility, morphology, and viability. Among these, the sugarcane water (SW) extender demonstrated the highest suitability for diluting Banaba native chicken semen. Consequently, it can be concluded that SW extender is ideal for processing semen intended for short-term storage.

Availability of Data and Materials

All data are available in this study.

Author Contributions

Conceptualization, A.R.S.S., and P.P.S.; Methodology, A.R.S.S., P.P.S., and M.M.L.; Investigation, A.R.S.S., G.A.D., and M.M.L.; Writing – Original Draft, A.R.S.S.; Writing – Review & Editing, A.R.S.S., and P.P.S.

Ethics Approval and Consent to Participate

The current research has followed the accepted principles of ethical conduct by The Institutional Animal Care and Use Committee (IACUC) of the University of the Philippines Los Baños (UPLB), Philippines, protocol number CAFS-2018-006.

Acknowledgment

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interventions for the conservation of genetic diversity of Philippine native pigs, chickens, and ducks" implemented by the IAS, CAFS, UPLB.

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Conflict of Interest

The authors declare no conflict of interest.

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(Original Research)

Evaluation of β-1,3/1,6-glucan Supplementation on Growth Performance, Immune Parameters, and Gut Health of Broiler Chickens Vaccinated with Live Attenuated *Eimeria* spp. Vaccine

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Abstract

Background: Coccidiosis, caused by protozoa of the genus *Eimeria*, remains one of the most economically important poultry diseases. Beta-glucans**,** naturally occurring polysaccharides derived from yeast, possess potent immunomodulatory properties and have been shown to enhance innate and adaptive immune responses. The present study investigated the effects of ß-1,3/1,6-glucan (Polymune®), produced from a novel strain of *Aureobasidium pullulans*, on growth performance, immune function, and intestinal health in broiler chicks vaccinated against coccidiosis. **Methods:** A total of 108 day-old Cobb 500® broiler chicks were assigned to a 2 × 3 factorial design with ß-glucan supplementation at 0%, 0.1%, or 0.3% via drinking water from days 1–14, with vaccinated groups receiving an 18× dose of a live attenuated *Eimeria* vaccine (EVANT®) on day 7. Performance indicators included body weight gain (BWG), feed conversion ratio (FCR), gut morphometry, oocyst per gram (OPG) counts, qPCR of *Eimeria* spp. and heterophil-to-lymphocyte (H/L) ratio. Ileum and liver histological lesions were scored using the "I See Inside" (ISI) methodology. **Results:** On day 14, significant differences in BWG were observed due to vaccination ($P = 0.002$), with no interaction between vaccination and ß-glucan supplementation. Vaccination significantly increased relative liver weight ($P=0.010$), while ß-glucan supplementation had no effect ($P > 0.05$). OPG counts were significantly elevated in all vaccinated groups ($P < 0.0001$), peaking on day 12, while unvaccinated groups remained negative. ß-glucan dosage did not significantly influence OPG counts ($P=0.7771$); however, high-dose supplementation (0.3%) showed a numerical reduction in OPG among vaccinated birds. Birds supplemented with 0.3% ß-glucan without vaccination showed higher ISI scores in ileum (17.0 ± 3) and liver (16.0 ± 3) which is comparable to the vaccinated groups. **Conclusion:** Overall, ß-1,3/1,6-glucan showed mixed effects on immune and gut health parameters, with higher doses potentially contributing to enhanced immune response.

Keywords

Beta glucan, *Eimeria*, Live Coccidiosis Vaccine, Broilers

1. Introduction

Coccidiosis is a parasitic disease affecting poultry, causing significant detriment to the global poultry industry annually. It stands out as one of the most economically impactful diseases in chickens, resulting from parasites belonging to the genus *Eimeria* and *Isospora* within the phylum *Apicomplexa* [1]. Each species of these parasites predominantly targets and invades the avian intestinal tract, causing inflammation to epithelial cells and resulting in a range of clinical manifestations in infected chickens. This disruption allows opportunistic bacteria such as *Clostridium perfringens*, *E. coli*, and *Salmonella* to colonize and proliferate in the damaged gut. These effects encompass necrotic gut lesions, reduced weight gain and feed conversion rates, heightened mortality rates, and increased susceptibility to other pathogens [2].

The management of avian coccidiosis involves the utilization of vaccines and antimicrobial medications such as coccidiostats and antibiotics, contributing to the enhancement of the immune system in birds. However, coccidiosis vaccination typically induces mild intestinal lesions and transient changes in performance in chickens as part of the normal immune response. These post-vaccination reactions are expected during the first and second oocyst cycling periods and are essential for the development of protective immunity. However, excessive reactions may occur if vaccine administration is uneven, oocyst cycling is poorly managed, or concurrent enteric pathogens are present, potentially leading to clinical coccidiosis or necrotic enteritis [3].

Despite decades of usage, coccidiosis vaccine outcomes are variable in practice. Several field trials and reviews have documented vaccination failures. A large-scale field trial involving ~900,000 chicks across three farms found that live vaccination sometimes leads to subclinical coccidiosis, with variations in lesion scores, oocyst shedding, and production index (PI), particularly when environmental or management conditions are suboptimal. Reviews of vaccine trials have noted that many experiments differ widely in design (challenge strain, timing, performance metrics), which makes comparing efficacy difficult and may mask failures or underperformances [4].

Antibiotic Growth Promoters (AGPs) and coccidiostats have also been used extensively to improve health, feed efficiency, and weight gain, thereby enhancing the quality of animal-derived products. Nevertheless, the inappropriate and overly frequent utilization of antimicrobials has resulted in the emergence and proliferation of antimicrobial resistance (AMR), the transmission of resistance factors from animals to humans through the presence of drug residues in meat and eggs, and an alteration in the balance of normal microflora [5]. Predictably, the persistent administration of drugs for addressing *Eimeria* infections creates substantial selection pressure that promotes the development of drug resistance [6].

Consequently, developing and providing functional dietary additives as antibiotic alternatives becomes imperative to reduce disease-related mortality and morbidity, maintain feed efficiency and good gut health status, and enhance immunity in poultry [7]. Proposed substitutes for AGPs encompass a range of options such as vaccines, bacteriophages, feed enzymes, plant extracts, organic or inorganic acids, and pro-, pre-, and symbiotic, among others. These alternatives need to demonstrate effectiveness not only in promoting healthy gut function but also in ensuring optimal performance in poultry. Furthermore, alternative agents should be accessible for farmers to utilize on a commercial scale and should be designed to minimize the risk of fostering bacterial resistance [8].

Prebiotics, dietary compounds that remain undigested by the host when consumed but support beneficial bacteria, are gaining significant attention as a promising candidate offering similar advantages. A strategy to mitigate the spread of foodborne pathogens involves incorporating prebiotics into the diet. When consumed, these prebiotics act as substrates for specific bacteria already present in the poultry gastrointestinal tract (GIT) that exhibit antagonistic effects against pathogens [9, 10]. Nurturing beneficial bacteria in the chicken's GIT triggers preventive mechanisms against pathogens through microbial metabolism. This, in turn, leads to changes in the microbial population of the GIT and subsequently enhances the overall health of chickens, as reflected in altered immune responses [9].

Beta-glucans are polysaccharides composed of D-glucose monomers, forming the structural components of cell walls found in yeast, fungi, algae, and cereal grains. ß-glucans contain three distinct glycosidic linkages ß-(1,3), ß-(1,4), and ß-(1,6). The effectiveness of ß-glucans in modulating the immune system varies due to structural differences and based on their origin [11]. Among these molecules, ß-1,3/1,6-glucan produced by a novel strain *Aureobasidium pullulans* has demonstrated the ability to enhance immune functions, particularly beneficial for growing chicks [12]. Acting as an immunomodulator, it may support the development of a healthy immune system.

Despite these known issues, there is limited data on how supplementing immunomodulatory compounds, such as ß-1,3/1,6-glucans, may mitigate vaccine failure in live *Eimeria* vaccination programs and extreme coccidiosis conditions. Although the immunomodulatory effects of ß-glucans have been extensively studied in the past, there is currently a lack of comprehensive information regarding the efficacy of the black yeast *Aureobasidium pullulans* in broiler chickens during live coccidiosis vaccination or extreme challenge conditions. The information obtained from this study will offer researchers essential data for optimizing prebiotics as a viable substitute for coccidiostat during live coccidiosis vaccination or field challenge.

2. Materials and Methods

2.1 Ethical Statement

This study was reviewed and approved by the University of the Philippines Los Baños Institutional Animal Care and Use Committee (UPLB IACUC) under approval reference number UPLB-2024-006 and protocol review number CAFS-2024-005.

2.2 Experimental Birds, House Management, and Diets

A total of one hundred eight, day old broiler chicks (Cobb500™) were acquired from a commercial hatchery (San Miguel Foods, Inc. Hatchery, Lecheria, Calamba City, Laguna). The chicks had a prior vaccination history for Newcastle disease (NCD) and Infectious Bronchitis (HB1 Mass Blen®) administered via spray, and Infectious Bursal Disease (Transmune®) administered subcutaneously (SQ). Upon arrival, the chicks were weighed. The broiler chickens were housed in an open-sided structure equipped with individual pens. Each pen consisted of a double-walled box measuring 76.2 cm x 57.15 cm x 39.37 cm and featured solid walls. To ensure a clean environment for the birds, completely dry rice hulls, disinfected with formalin prills prior to chick placement, were used as litter material. Additionally, plastic mesh sidings were installed around the pens to safeguard the birds from predators. A floor space allocation of 0.30 m^2 (1 ft^2) per broiler was provided, adhering to recommended space requirements. For brooding, the environmental temperature was initially set at 33-35°C, gradually decreasing until it reached 28-30°C by the end of the experiment. The humidity levels were kept at 60-70%. The lighting schedule entailed 23 hours of light and 1 hour of darkness during the initial week, subsequently reducing to 20 hours of light thereafter.

The birds were offered formulated booster (pre-starter) diets manually, and the birds had *ad libitum* access to both water and feed. All treatment pens were offered the same diet for 14 days. The composition of the booster (pre-starter) diet is presented in Table 1.

In this study, the selected ß-glucans, known as Polymune®, constituted a liquid supplement containing ß-1,3/1,6-glucan derived from a novel strain of *Aureobasidium pullulans*. The ß-glucans were incorporated into the drinking water, with a dosage of 1 mL per liter once a day (Day 1-14) for the 0.1% ß-glucan-treated group, while three times (3 mL/L) the said dosage was offered for the 0.3% ß-glucan-treated group as higher dose. To maintain freshness and cleanliness, the drinking solutions were diligently prepared twice every day at AM and PM, following the guidelines provided by the manufacturer. Daily maintenance involved washing the drinkers in each enclosure while avoiding the waterers of infected and uninfected groups from coming into contact and refilling them with the specified treatment consistently at the same time of day.

2.3 Experimental Design

The broiler chicks were placed into different pens under uniform environmental conditions,

Table 1. Ingredients and nutrient composition of the broiler booster (pre-starter) diet.

Item	Booster (Day 1 - 14)
Ingredient (%)	
Yellow Corn	55.24
Soybean Meal US HP	38.71
Coconut oil	2.07
Limestone	1.1
Monodicalcium Phosphate	2.3
Vitamin Premix	0.05
Mineral Premix	0.05
Refined Iodized Salt	0.3
L-Methionine	0.18
Analyses Nutrient Content (%)	
Crude Protein	20.50
Crude Fat	4.90
Crude Fiber	1.93
Ash	6.73
Moisture	11.27

following a completely randomized design in a 2 x 3 factorial arrangement with coccidiosis infection and ß-glucan dosage as factors and pen as the experimental unit. The experiment included two vaccinated treatments, three distinct water treatments, and three replicate pens arranged randomly for each treatment. Each pen housed six birds (n=108).

Treatment groups:

A. **Negative Control:** No ß-glucan treatment and no live coccidiosis vaccination.
B. **Coccidiosis Vaccinated Control:** With coccidiosis live vaccination but without ß-glucan treatment.
C. **0.1% β-glucan-treated Group:** With ß-glucan treatment at 0.1% concentration and no coccidiosis live vaccination
D. **0.1% β-glucan and Coccidiosis Vaccination:** With ß-glucan treatment at 0.1% concentration and coccidiosis live vaccination.
E. **0.3% β-glucan-treated Group:** With ß-glucan treatment at 0.3% concentration and no coccidiosis live vaccination
F. **0.3% β-glucan and Coccidiosis Vaccination:** With ß-glucan treatment at 0.3% concentration and coccidiosis live vaccination.

2.4 Growth Performance

Morbidity and mortality were assessed daily. Each replicate pen underwent weekly evaluations to measure and compute various growth parameters, including broiler body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR). Body weight gain (BWG) was calculated by subtracting the initial weight at the start of the week from the weight recorded at the end of the week. Feed intake (FI) was

determined by subtracting the remaining feed in the feeder from the amount initially provided on a given day. Finally, the FCR was computed at the end of the week by dividing the total feed consumed by the experimental birds by their total weight gain.

2.5 *Eimeria* spp. Vaccination

On the specified day 7, each bird in the 3 replicate pens for each ß-glucan supplementation level underwent administration of a 1 mL (18x of recommended dose to also replicate clinical coccidiosis challenge) of live attenuated *E. acervulina, E. maxima, E. mitis, E. praecox*, and *E. tenella* vaccine (EVANT®), manually using a syringe into the oral cavity [13, 14]. These designated pens were labeled as the "vaccinated groups." The formulation of the undiluted vaccine used is presented in Table 2.

Table 2. Formulation of each 7 µL (dose) of undiluted vaccine (EVANT®).

Item	Number of sporulated oocysts at the time of blending *in vitro*
Eimeria acervulina (003 strain)	332 – 450
Eimeria maxima (013 strain)	196 - 265
Eimeria mitis (006 strain)	293 - 397
Eimeria praecox (007 strain)	293 - 397
Eimeria tenella (004 strain)	276 – 374

The process of preparing the diluted vaccine suspension included mixing the 7 mL vaccine with 223 mL water and 50 mL solvent, resulting in a combined volume of 280 mL. Initially, the solvent vial was shaken to ensure uniformity, and its contents were then mixed with clean room temperature water into an appropriate container. Subsequently, the vaccine vial was shaken, and its contents were added to the previously prepared solution. To prevent any potential cross-contamination, the non-vaccinated and vaccinated birds were housed in two distinct yet identical pens, ensuring uniformity in terms of pen size, temperature, and humidity conditions.

2.6 Measurement of Relative Weights of Digestive Organs

At the end of the brooding period (14 days), two broilers per replicate were selected based on the average body weight within each group. Subsequently, these chosen birds were weighed and euthanized through alcohol euthanasia. The gastrointestinal tract (GIT) and organs were then carefully excised. Measurements of the small intestine and caeca lengths of individual birds were taken using a ruler. Additionally, the proventriculus, gizzard, and liver of each bird were weighed [15]. The relative weights of these organs were calculated as percentages of the live weight and expressed accordingly.

2.7 Measurement of Relative Weights of Immune Organs

The same 36 broiler chickens (2 per replicate pen) selected for digestive organ measurement were also used for the evaluation of immune organs. The thymus, spleen, and Bursa of Fabricius were carefully extracted from each carcass and individually weighed. The relative weights were calculated by dividing the weight of each organ (in grams) by the BW of the bird (in kilograms) [16].

A bursameter, a flat plastic ruler with eight calibrated holes ranging from narrowest (1) to widest (8), was used to measure the size of the Bursa of Fabricius [17]. The corresponding diameters per bursameter score are shown in Table 3.

Table 3. Bursameter score and corresponding diameters [16].

Score	Diameter (mm)
1	3.50
2	6.50
3	9.50
4	13.00
5	16.00
6	19.00
7	22.50
8	25.50

2.8 Tissue Section Preparation (Histopathology)

On day 14 (seven days post-vaccination), six broiler chickens per treatment group were euthanized by cervical dislocation. Samples of the ileum and liver were collected and fixed in 10% neutral buffered formalin for at least 24 hours. The tissues were processed following standard histological procedures, including dehydration, paraffin embedding, and sectioning into 5 µm-thick slices. Sections were stained with hematoxylin and eosin for microscopic evaluation. Intestinal lesions were assessed by measuring 20 villi per bird under 10× magnification, with 20× and 40× magnifications used to confirm alterations. Liver samples were examined in 10 fields per bird at 10× magnification using an optical microscope (AmScope T120B-5M, California, USA).

Lesions were scored blindly using the I See Inside (ISI) methodology [18] a system currently in the process of patenting (INPI BR 1020150036019). The ISI method is based on assigning a numeric score of alteration to histological and macroscopic findings. An impact factor (IF) ranging from 1 to 3 is defined for each alteration according to its expected effect on organ functional capacity, supported by prior literature and background research. Severe alterations such as necrosis receive the highest IF (3), as they completely compromise cell function. In addition to IF, the extent of each lesion is evaluated based on intensity or observed frequency relative to non-affected organs or tissues, with scores ranging from 0 (absence of lesion or frequency) to 1 (alteration up to 25% of the area or frequency), 2 (alteration affecting 25–50% of the area or frequency), and 3 (alteration affecting more than 50% of the area or frequency). The final ISI index is calculated by multiplying the extent score by the impact factor for each alteration, then summing the values of all alterations per sample. This approach provides a quantitative measure of gut and liver health by accounting for both the severity and biological relevance of observed lesions.

2.9 Heterophil: Lymphocyte Ratio

At 10 and 14 days of age (3- and 7-days post vaccination), fresh blood samples were obtained from two birds housed in each replicate pen to assess the heterophil-to-lymphocyte ratio. During the blood collection procedure, a gauge 23, 1-inch needle syringe was utilized to withdraw 1.5 mL of blood from the wing vein. Subsequently, the blood samples were promptly transferred to 3 mL ethylene diamine tetra acetic acid (EDTA) tubes (Kingmed) to prevent clotting.

A drop of blood was placed onto a slide, creating a single-cell layer blood smear. The smears were air-dried and subsequently stained using the Giemsa staining set. After staining, the slides were rinsed with distilled water and allowed to air-dry. Following preparation, the slides with blood smears were examined under a microscope at high-power magnification (400x). A total of 100 leukocytes, comprising both granular (heterophils) and non-granular (lymphocytes) cells, were counted to

determine the heterophil-to-lymphocyte ratio [19]. The H/L ratio was calculated by dividing the number of heterophils by the number of lymphocytes in these 100 leukocytes.

2.10 Oocyst per Gram (Modified McMaster Fecalysis) and Realtime PCR of *Eimeria* spp.

Fresh fecal samples were collected per experimental unit (pen as the experimental unit) on days 6, 12, 13, and 14. Samples were refrigerated until analysis. For the Modified McMaster egg counting technique, fecal samples were mixed with 26 mL of Sheather's solution. The suspension was transferred into a McMaster chamber using a pipette, and oocysts were counted under a microscope at 10× objective magnification. The total number of oocysts per gram (OPG) was calculated using the following formula: OPG = Total oocyst count × 25. Concurrently, fecal samples from the treatment and control groups were sent to Hipra Diagnostic Laboratory Philippines for molecular detection of *Eimeria* spp. using a polymerase chain reaction (qPCR) developed at the Institute for Animal Health (Compton, UK) to specifically detect *E. acervulina*, *E. maxima, E. mitis, E. praecox and E. tenella* [20]. Genomic DNA from fecal samples were extracted using an automated extraction protocol on the QIAcube system (Qiagen, Hilden, Germany), following the manufacturer's instructions. Quantitative PCR was performed using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany). PCR cycling conditions consisted of an initial denaturation at 95 °C for 20 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Cycle threshold (Ct) values of ≤38.5 cycles were considered positive, while Ct values greater than 38.5 cycles were considered negative.

2.11 Statistical Analysis

Data were analyzed using a completely randomized design in a 2 × 3 factorial arrangement (three ß-glucan levels × two vaccination statuses). Based on historical data for BW ($\sigma = 0.08$ kg), a priori power analysis indicated that this design provides approximately 91% power ($\alpha = 0.05$, two-sided) to detect a 5% difference in BW between vaccination treatments. Growth performance, organ weights, H/L ratio, and oocyst per gram (OPG) counts were analyzed by two-way ANOVA using the General Linear Model (GLM) procedure in SAS® Studio (SAS Institute Inc., Cary, NC, USA). Post hoc comparisons were conducted using Tukey's HSD test when significant effects were detected. Statistical significance was set at $P < 0.05$, and actual P-values were reported where applicable. For histopathological evaluations, data were expressed as mean ± standard error of the mean (SEM). Normality was assessed using the Shapiro–Wilk test. Two-way ANOVA followed by Tukey's test was performed using GraphPad Prism version 8.0.1 (GraphPad Software, Boston, MA, USA). On graphs and tables, error bars represent SEM. Different superscripts or asterisks indicate significant differences between groups, as detailed in the figure or table legends.

3. Results

3.1 Growth Performance

3.1.1 Body Weight

Table 4 presents the BWG of broiler chickens at day 7 (pre-vaccination) and day 14 (7 days post-vaccination), supplemented with different levels of ß -glucans, and subjected to either live coccidiosis vaccination or no vaccination. At day 14, a significant difference in BWG was observed between vaccinated and non-vaccinated birds ($P = 0.002$). No significant differences were detected among ß-glucan levels at either day 7 or day 14 ($P > 0.05$), and no significant interaction between ß-glucan supplementation and vaccination was found ($P = 0.056$).

3.1.2 Feed Conversion Ratio

FCR of broiler chickens on day 7 (pre-vaccination) and day 14 (7 days post-vaccination), both with and without coccidiosis vaccination, and supplemented with ß-glucans at different levels (Table 4). At both day 7 and day 14, no significant differences in FCR were observed among the varying levels of ß-glucan supplementation and vaccination conditions, with no notable variations in their combined effects ($P > 0.05$).

3.1.3 Mortality

Mortality rates of broiler chickens on day 7 (pre-vaccination) and day 14 (7 days post-vaccination), both with and without coccidiosis vaccination, supplemented with ß-glucans at different levels (Table 4). No significant differences in mortality rates were observed among ß-glucan treatment groups on day 7 (pre-vaccination) ($P > 0.05$). Notably, no mortalities were recorded throughout the post-vaccination period (day 7 onwards). There were no signs of diarrhea observed in all treatment and control groups.

Table 4. Body Weight Gain, FCR, and mortality (d 7 & 14) of broilers chickens supplemented with ß-Glucans (0%, 0.1%, 0.3%) under both vaccinated and unvaccinated conditions with live coccidiosis.

	Body Weight Gain (g)			Feed Conversion Ratio (feed / gain)			Mortality Rates (%)		
		Day 7	Day 14		Day 7	Day 14		Day 0-7	Day 8-14
Main Effects									
Beta glucan level	0%	98.54	210.18	0%	1.09	1.03	0%	0.154	0
	0.10%	92.1	193.46	0.10%	1.44	1.29	0.10%	0.772	0
	0.30%	87.77	203.27	0.30%	1.31	0.94	0.30%	0.772	0
Coccidiosis vaccination	with	-	189.02[b]	with	-	1.17	with	-	0
	without	-	215.59[a]	without	-	1	without	-	0
Source of Variation									
Beta glucan level		0.434	0.173		0.138	0.129		0.54	-
Coccidiosis vaccination		-	0.002		-	0.231		-	-
Beta glucan level x Coccidiosis vaccination		-	0.056		-	0.285		-	-
Interactive Effects									
With coccidiosis	0%	-	193.28	0%	-	1.02	0%	-	0
	0.10%	-	192.89	0.10%	-	1.53	0.10%	-	0
	0.30%	-	180.89	0.30%	-	0.97	0.30%	-	0
Without coccidiosis	0%	-	227.09	0%	-	1.05	0%	-	0
	0.10%	-	194.03	0.10%	-	1.05	0.10%	-	0
	0.30%	-	225.64	0.30%	-	0.91	0.30%	-	0

[ab] within the same column, means with different superscripts are significantly differed ($P < 0.05$).
"-"indicates the absence of vaccination at this time point, hence no value is recorded.

3.2 Gut Health and Morphometry

3.2.1 Digestive Organ Weights and Relative Weights

Table 5 present the relative digestive organ morphometry of broiler chickens, comparing those with and without coccidiosis vaccination, and supplemented with ß-glucans at different levels (0%, 0.1%, 0.3%). It specifically summarizes the weights and relative weights of the proventriculus, gizzard, and liver in the broiler chickens, as well as the weights, relative weights, and lengths of the duodenum, jejunum, ileum, and caeca on day 14.

Among the digestive organs measured, statistical analysis revealed a significant difference (P = 0.010) solely in the relative weight of the liver (Table 6). This difference appeared to be primarily driven by the coccidiosis vaccination, with ß-glucan dosage having no statistically significant effect (P > 0.05). In essence, the relative weights of the proventriculus and gizzard were not significantly impacted by either ß-glucan supplementation, coccidiosis vaccination, or the interactive effects of both factors. While not statistically significant, the relative weights of the proventriculus and gizzard tended to be numerically higher in the coccidiosis-vaccinated birds compared to the non-vaccinated groups.

Measurements of the small intestine and caeca showed significant differences, primarily in the relative weights of the duodenum, jejunum, ileum, and the weight of the caeca, with coccidiosis vaccination being the main influencing factor (P < 0.05). Additionally, ileum length was significantly affected by b ß-glucan levels (P = 0.014). Similar to the liver findings, coccidiosis-vaccinated broiler chickens exhibited higher relative weights compared to unchallenged groups.

3.2.2 Histopathology Scores of the Intestine and Liver

The histopathological scores for both the ileum and liver are shown in Figure 1. Panel (A) presents the Total I See Inside (ISI) histological scores for ileum histopathology, while panel (B) shows the ISI scores for liver histopathology. Vaccinated groups supplemented with ß-glucan (0.1% and 0.3%) exhibited significantly higher ISI scores compared to the non-vaccinated and non-supplemented groups, indicating increased histological lesions. The combination of ß-glucan supplementation and vaccination resulted in an additive increase in histological lesions. The ISI lesion scores of the ileum revealed clear differences among treatments. Birds receiving no ß-glucan supplementation and no coccidiosis vaccination exhibited minimal scores (2.0 ± 1). Supplementation with 0.1% ß-glucan alone resulted in a slight increase in ileal lesion scores (3.0 ± 1), whereas the combination of 0.1% ß-glucan with

Table 5. Summary of digestive organ weights and relative weights in broilers chickens supplemented with ß-Glucan (0%, 0.1%, 0.3%) under both vaccinated and unvaccinated conditions with live coccidiosis.

Weights and Relative Weights of Digestive Organs							
		Proventriculus		Gizzard		Liver	
		wt, g	RW, %	wt, g	RW, %	wt, g	RW, %
Main Effects							
Beta glucan level	0%	3.38	0.8941	16.59	4.42	15.81	4.19
	0.1%	2.92	0.8167	16.59	4.61	14.56	4.11
	0.3%	3.23	0.9100	16.81	4.73	14.98	4.15
Coccidiosis vaccination	with	3.05	0.9011	16.36	4.82	15.49	4.51[a]
	without	3.31	0.8461	16.97	4.36	14.54	3.79[b]
Source of Variation							
Beta glucan level		0.079	0.167	0.982	0.595	0.659	0.970
Coccidiosis vaccination		0.120	0.198	0.581	0.079	0.515	0.010
Beta glucan level x Coccidiosis vaccination		0.206	0.689	0.221	0.246	0.343	0.555
Interactive Effects							
With coccidiosis	0%	3.33	0.9266	17.60	4.85	16.77	4.65
	0.1%	2.92	0.8635	15.30	4.54	15.53	4.57
	0.3%	2.90	0.9132	16.18	5.06	14.17	4.31
Without coccidiosis	0%	3.42	0.8616	15.58	3.99	14.85	3.72
	0.1%	2.93	0.7699	17.88	4.68	13.58	3.65
	0.3%	3.57	0.9068	17.43	4.41	15.80	4.00

wt means weight and RW means relative weight.
[ab] within the same column, means with different superscripts are significantly differed ($P < 0.05$).

Table 6. Summary of weights, relative weights, and lengths of gastrointestinal tract of broiler chickens supplemented with ß-Glucan (0%, 0.1%, 0.3%) under both vaccinated and unvaccinated conditions with live coccidiosis.

Weights, Relative Weights, and Lengths of GI tract													
		Duodenum			Jejunum			Ileum			Caeca		
		wt, g	RW, %	L, cm	wt, g	RW, %	L, cm	wt, g	RW, %	L, cm	wt, g	RW, %	L, cm
Main Effects													
	0%	7.7	2.05	19	8.5	2.26	49.08	6.08	1.62	43.01[a]	3.93	1.04	10.88
Beta glucan level	0.10%	7.57	2.11	18,75	8.94	2.49	46.21	5.97	1.64	42.75[a]	3.12	0.86	10.12
	0.30%	7.81	2.2	18.83	8.38	2.36	46.08	4.88	1.38	35.25[b]	3.18	0.89	10.12
Coccidiosis vaccination	with	7.54	2.22[a]	18.5	9.07	2.67[a]	46.92	5.69	1.68[a]	42.31	2.91[b]	0.85	10.22
	without	7.84	2.02[b]	19.22	8.15	2.08[b]	47.33	5.59	1.41[b]	38.37	3.92[a]	1.01	10.53
Source of Variation													
Beta glucan level		0.887	0.427	0.977	0.64	0.178	0.5	0.115	0.169	0.016	0.181	0.366	0.152
Coccidiosis vaccination		0.455	0.041	0.457	0.08	<0.001	0.859	0.843	0.03	0.101	0.014	0.122	0.393
Beta glucan level x Coccidiosis vaccination		0.701	0.936	0.473	0.188	0.294	0.377	0.513	0.324	0.823	0.215	0.11	0.311
Interactive Effects													
	0%	7.7	2.13	17.83	9.58	2.66	50.08	6.12	1.73	44.42	3.65	1	10.42
With coccidiosis	0.10%	7.5	2.2	19	9.3	2.73	43.67	5.67	1.68	44.25	2.12	0.62	9.92
	0.30%	7.42	2.33	18.67	8.32	2.6	47	5.3	1.64	38.25	2.95	0.92	10.33
	0%	7.7	1.96	20.17	7.42	1.85	48.08	6.05	1.5	41.6	4.22	1.07	11.33
Without coccidiosis	0.10%	7.63	2.01	18.5	8.58	2.25	48.75	6.27	1.6	41.25	4.12	1.1	10.33
	0.30%	7.42	2.08	19	8.45	2.13	45.17	4.47	1.13	32.25	3.42	0.86	9.92

wt means weight, RW means relative weight, and L means length.
[ab] within the same column, means with different superscripts are significantly differed ($P < 0.05$).

coccidiosis vaccination markedly increased scores (24.0 ± 5–6). Similarly, birds supplemented with 0.3% ß-glucan without vaccination showed moderate scores (17.0 ± 3), which further increased with vaccination (25.0 ± 2). A comparable ISI score pattern was observed in liver histology. The control group without ß-glucan or vaccination showed minimal hepatic lesions (1.0 ± 1), whereas ß-glucan supplementation combined with vaccination produced pronounced hepatic immune responses (18.0 ± 4 to 19.0 ± 3). Birds receiving ß-glucan without vaccination exhibited lower liver lesion

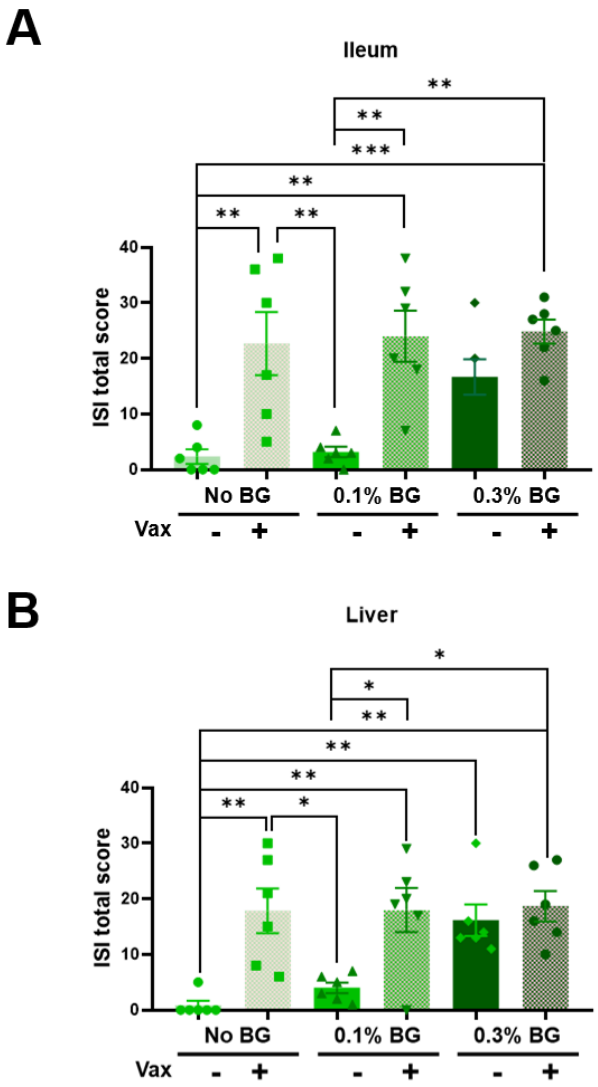

Figure 1. Intestinal and liver histopathology scores of broiler chickens supplemented with ß-glucan (0%, 0.1%, 0.3%) under both vaccinated and unvaccinated conditions with live *Eimeria* spp. Vaccine. Bars represent the mean ± SE, and asterisks indicate statistical significance: () $P < 0.05$, () $P < 0.01$, () $P < 0.001$

scores (4.0 ± 1 for 0.1%; 16.0 ± 3 for 0.3%) compared to their vaccinated counterparts.

3.3 Immune Response

3.3.1 Immune Organ Weights and Relative Weights

Table 7 summarizes the immune response of broilers with and without coccidiosis vaccination, across varying ß-glucan levels (0%, 0.1%, 0.3%). It includes data on the weights and relative weights (compared to body weight) of the thymus, spleen, and Bursa of Fabricius at day 14.

The results revealed significant weight reductions only in the thymus and Bursa of Fabricius of birds vaccinated with coccidiosis ($P < 0.05$). While not statistically significant, the spleen weight also showed a similar decreasing trend. Importantly, there were no significant differences observed in immune organ weight due to varying ß-glucan levels or its interaction with coccidiosis vaccination.

3.3.2 Heterophil to Lymphocyte Ratio (H:L Ratio)

Table 8 summarizes the immune response of broilers with and without coccidiosis vaccination,

Table 7. Summary of immune organ weights and relative weights in broilers chickens supplemented with ß-Glucan (0%, 0.1%, 0.3%) under both vaccinated and unvaccinated conditions with live coccidiosis.

Weights and Relative Weights of Immune Organs								
		Thymus		Spleen		Bursa		
		wt, g	RW, %	wt, g	RW, %	wt, g	RW, %	Bursa-meter
Main Effects								
Beta glucan level	0%	1.62	0.4291	0.4500	0.1203	0.9583	0.2511	4.83
	0.1%	1.52	0.4235	0.4750	0.1332	0.8167	0.2282	4.67
	0.3%	1.45	0.4045	0.4333	0.1215	0.9667	0.2685	4.92
Coccidiosis vaccination	with	1.37[b]	0.4062	0.4278	0.1264	0.77[b]	0.2276	4.56
	without	1.69[a]	0.4319	0.4778	0.1235	1.06[a]	0.2709	5.06
Source of Variation								
Beta glucan level		0.680	0.889	0.768	0.682	0.557	0.621	0.803
Coccidiosis vaccination		0.046	0.558	0.295	0.828	0.032	0.207	0.121
Beta glucan level x Coccidiosis vaccination		0.965	0.946	0.959	0.826	0.704	0.789	0.756
Interactive Effects								
With coccidiosis	0%	1.48	0.4153	0.4167	0.1324	0.78	0.2154	4.67
	0.1%	1.35	0.4023	0.4500	0.1224	0.75	0.2208	4.50
	0.3%	1.27	0.4010	0.4167	0.1287	0.78	0.2467	4.50
Without coccidiosis	0%	1.75	0.4429	0.4833	0.1143	1.13	0.2869	4.83
	0.1%	1.68	0.4447	0.5000	0.1340	0.88	0.2356	5.00
	0.3%	1.63	0.4081	0.4500	0.1182	1.15	0.2902	5.33

wt means weight and RW means relative weight.
[ab] within the same column, means with different superscripts are significantly differed ($P < 0.05$).

Table 8. Heterophil and lymphocyte counts, and heterophil to lymphocyte ratio (d10 and 14) of broiler chickens supplemented with ß-Glucan (0%, 0.1%, 0.3%) under both vaccinated and unvaccinated conditions with live coccidiosis.

Heterophil, Lymphocyte, and Heterophil: Lymphocyte Ratio							
		Day 10			Day 14		
		H	L	H: L	H	L	H: L
Main Effects							
Beta glucan level	0%	25.17	70.33[b]	0.3750	26.25[a]	71.67	0.3676[a]
	0.1%	26.33	71.50[ab]	0.3615	25.28[a]	72.33	0.3550[a]
	0.3%	25.83	72.25[a]	0.3488	24.42[b]	73.08	0.3346[b]
Coccidiosis vaccination	with	26.11	71.50	0.3660	27.00[a]	70.89[b]	0.3816[a]
	without	25.44	71.22	0.3576	23.83[b]	73.83[a]	0.3232[b]
Source of Variation							
Beta glucan level		0.102	0.023	0.059	0.002	0.180	0.005
Coccidiosis vaccination		0.132	0.610	0.335	<0.001	<0.001	<0.001
Beta glucan level x Coccidiosis vaccination		0.183	0.818	0.487	0.1848	0.667	0.234
Interactive Effects							
With coccidiosis	0%	27.17	70.50	0.3860	28.00	70.00	0.4006
	0.1%	26.17	71.83	0.3646	27.50	70.67	0.3897
	0.3%	25.00	72.17	0.3473	25.50	72.00	0.3544
Without coccidiosis	0%	25.50	70.17	0.3639	24.50	73.33	0.3346
	0.1%	25.50	71.17	0.3585	23.67	74.00	0.3202
	0.3%	25.33	72.33	0.3503	23.33	74.17	0.3149

[ab] within the same column, means with different superscripts are significantly differed ($P < 0.05$).

across varying ß-glucan levels (0%, 0.1%, 0.3%). It presents the differential white blood cell counts (heterophils, lymphocytes, and their ratio) at days 10 and 14.

Analysis revealed distinct influences of ß-glucan and coccidiosis vaccination on leukocyte subpopulations depending on the sampling time point. Day 10 data (3 days post-vaccination)

indicated a significant effect of ß-glucan level on lymphocyte counts ($P < 0.05$), with a notable increase observed. Conversely, day 14 (7 days post-challenge), significant differences were observed in heterophil counts and the heterophil-to-lymphocyte (H:L) ratio, mainly due to the ß-glucan level and coccidiosis vaccination ($P < 0.05$). Additionally, a significant difference in lymphocyte counts was noted on this day, mainly due to the effects of the coccidiosis vaccination ($P < 0.001$). There were no significant changes in heterophil counts, lymphocyte counts, or the H:L ratio due to the interactive effects of ß-glucan level and coccidiosis vaccination.

Figure 2 and Table 9 present the summary of oocyst per gram (OPG) counts in broiler chickens supplemented with ß-glucan (0%, 0.1%, 0.3%) under both challenged and unchallenged conditions. Groups A, C, and E (unchallenged) exhibited zero OPG counts across days 6, 12, 13, and 14, while groups B, D, and F (challenged) showed zero counts on day 6 but demonstrated increased OPG counts beginning on day 12.

Table 10 presents the detection of genetic material from five *Eimeria* species *(E. acervulina, E. maxima, E. tenella, E. mitis, E. praecox)* in Treatment B, D, and F on days 12, 13, and 14. The results indicate whether genetic material was detected (POS) or not detected (NEG). When positive, the quantity is categorized as low (+), moderate (++), or large (+++) based on the Ct values. Fecal samples collected on days 12, 13, and 14

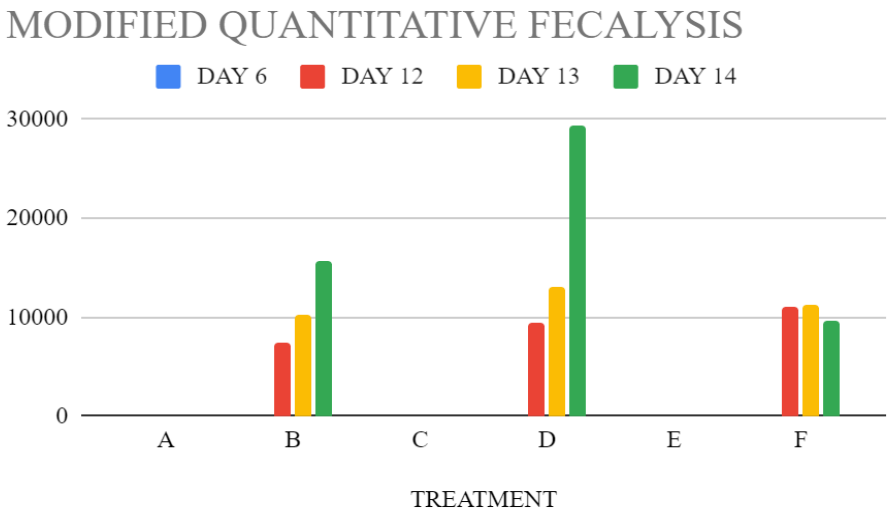

Figure 2. Summary of total mean oocyst counts (OPG) in broiler chickens supplemented with ß-glucan (0%, 1%, 3%) under vaccinated and unvaccinated conditions at days 6, 12, 13, and 14.

Table 9. Effects of coccidiosis vaccination on mean oocyst counts (OPG) in broiler chickens supplemented with ß-glucan (0%, 1%, 3%) on days 12, 13, and 14.

Modified Quantitative McMaster Fecalysis			
	Day 12	Day 13	Day 14
N	6	6	6
Mean	4675	5797	9107
Median	3742	5167	4804
Standard Deviation	5247	6415	11857
Minimum	0	0	0
Maximum	11083	13158	29383

Table 10. Summary of qPCR to detect *Eimeria* spp. vaccinated broiler chickens supplemented with ß-glucan (0%, 1%, 3%) on days 12, 13, and 14 with corresponding average Ct value (n=3).

Eimeria species	Day 12			Day 13			Day 14		
	B	D	F	B	D	F	B	D	F
E. acervulina	+++ (Ct 24.8)	+++ (Ct 24.9)	+++ (Ct 23.5)	+++ (Ct 26.6)	+++ (Ct 26.2)	+++ (Ct 25.4)	+++ (Ct 26.1)	+++ (Ct 27.6)	+++ (Ct 26.6)
E. maxima	NEG	NEG	NEG	+ (Ct 34.6)	++ (Ct 32.9)	++ (Ct 33.0)	++ (Ct 34.5)	++ (Ct 31.3)	++ (Ct 33.7)
E. tenella	NEG	NEG	NEG	+ (Ct 34.3)	NEG	++ (Ct 31.5)	++ (Ct 36.5)	NEG	NEG
E. mitis	+++ (Ct 26.0)	+++ (Ct 26.6)	+++ (Ct 25.3)	+++ (Ct 26.7)	+++ (Ct 25.8)	+++ (Ct 26.8)	+++ (Ct 27.0)	+++ (Ct 29.2)	+++ (Ct 28.1)
E. praecox	+++ (Ct 25.0)	+++ (Ct 26.8)	+++ (Ct 26.4)	+++ (Ct 26.0)	+++ (Ct 24.4)	+++ (Ct 25.9)	+++ (Ct 23.1)	+++ (Ct 24.5)	+++ (Ct 24.9)

Legend
Reference values (Ct): POS < 38.5
NEG: No genetic material of the tested pathogens was detected.
POS (+): A low quantity of genetic material of the tested pathogens was detected.
POS (++): A moderate quantity of genetic material of the tested pathogens was detected.
POS (+++): A large quantity of genetic material of the tested pathogens was detected.

B = Coccidiosis Vaccinated Control
D = 0.1% ß-glucan and Coccidiosis Vaccination
F = 0.3% ß-glucan and Coccidiosis Vaccination

revealed species-specific responses: *E. acervulina* and *E. mitis* maintained high loads across all groups, while *E. maxima* and *E. tenella* exhibited delayed detection. Notably, 0.1% ß-glucan suppressed *E. tenella* but not *E. maxima*, whereas 0.3% ß-glucan correlated with elevated *E. praecox* loads.

Statistical analysis revealed a highly significant main effect of challenging condition ($P < 0.0001$), indicating a strong impact of experimental infection on OPG counts. In contrast, the main effect of ß-glucan dosage level was not significant ($P = 0.2492$), suggesting that different supplementation levels did not significantly influence OPG outcomes. Similarly, the interaction between dosage level and challenging condition was not significant ($P = 0.2492$). Overall, the means for the challenged groups were significantly higher compared to the unchallenged groups, further emphasizing the substantial effect of experimental infection.

4. Discussion

Live *Eimeria* vaccination in broilers has been shown to reduce the severity of intestinal lesions and oocyst shedding, and to improve production indices under field conditions [21]. Pages *et al.* (2025) [22] conducted safety profiles of a live attenuated and two live non-attenuated coccidiosis vaccines administered at overdose (10x) in chickens, following the standard model established in the European Pharmacopeia (Ph. Eur.) monograph. To replicate the extreme challenge condition and vaccination in field (tropical environment), we opted to use attenuated live coccidiosis vaccination (x18 recommended dose). In a study by Wang *et al.* (2019), clinical coccidiosis was induced by giving 20x the regular dose of commercial *Eimeria* vaccines in challenged broiler chicks [14]. In this study, the reduction in BWG observed among *Eimeria* spp. vaccinated broiler chickens confirmed that live coccidiosis vaccination exerted a detrimental effect on early growth performance. This outcome is consistent with previous reports [23], which documented significantly lower BW in coccidiosis-infected chicks compared to unchallenged controls. Similarly, growth depression following vaccination was observed [24], likely attributable to intestinal epithelial damage that impairs nutrient absorption [25, 26]. In addition, studies have suggested that exceeding the recommended doses of coccidiosis vaccines may further impair weight gain by triggering heightened immune activation

[27]. Although live vaccines are generally safe and effective, the increased immune stimulation induced by higher doses demands greater metabolic resources, which may detract from growth performance [28, 29]. Conversely, not all studies align with the present findings. De Sabate *et al.* (2001) reported no significant differences in BW between vaccinated and non-vaccinated birds, a discrepancy that may be explained by compensatory growth mechanisms [30]. Mathis (1999) described how a temporary reduction in growth post-vaccination could be followed by accelerated growth later in the production cycle [31]. Variability in study designs, including broiler strain, rearing conditions, and methods of inducing coccidial infection, as noted by McDougald and Reid (1991), may also contribute to the differences observed across studies [32]. This study did not include a coccidiostat-treated positive control group because the primary objective was to evaluate the interaction effects of ß-glucan supplementation and coccidiosis vaccination. The inclusion of a chemoprophylactic group could have confounded interpretation by altering gut microbiota composition and immune activation of the experimental birds.

The absence of significant differences in FCR among varying levels of ß-glucan supplementation and coccidiosis vaccination, as well as their combined effects, suggests that neither treatment notably impaired feed efficiency during the early stages of broiler development. However, the numerically higher FCR observed with increasing ß-glucan supplementation, particularly at the 0.1% inclusion level, may be attributed to the relatively low energy contribution of ß-glucans and the energy diverted toward immune system activation [11]. While ß-glucans are known to enhance innate immunity [33], they do not appear to significantly improve BWG despite promoting feed intake [34]. Interestingly, increasing the ß-glucan level to 0.3% resulted in a slight improvement in FCR, aligning with observations that yeast cell wall extracts containing ß-glucans may help mitigate performance declines in coccidia-vaccinated birds [35]. The slight numerical increase in FCR observed in vaccinated birds can be explained by the negative effects of coccidiosis vaccination on nutrient absorption or the compensatory increase in feed intake to meet immune system demands [25]. Supporting this, it was reported that vaccination-induced immune responses and mild infection symptoms could temporarily impair feed efficiency [36]. Moreover, the pathophysiological effects of *Eimeria* infection, which disrupt nutrient absorption in the small intestine, likely contribute to reduced growth and worsened FCR [37]. Despite these trends, the differences observed did not reach statistical significance. This agrees with findings that dietary ß-glucan supplementation has no significant effects on BWG and FCR [34]. Similar observations were made showing that ß-glucan supplementation does not adversely affect bird performance regardless of the health status or environmental conditions [38, 39]. In contrast, others documented a significant interaction between ß-glucan supplementation and vaccination [2], while some reported improved feed efficiency with yeast ß-glucan supplementation at later stages of growth [35]. Disparities across studies may be attributed to differences in broiler genetics, housing conditions, ß-glucan type and source, or vaccination protocols. Environmental factors, particularly housing conditions, likely influenced the responses observed in this study. The open-sided housing during the summer season may have exposed broilers to heat stress, a known stressor that reduces feed intake and negatively affects growth performance [40]. Modern broiler strains, genetically selected for rapid growth and high feed intake, are particularly susceptible to heat stress, which disrupts nutrient intake and diverts energy toward thermoregulation [41]. As a result, performance impairments linked to environmental stressors may have masked or compounded the potential effects of ß-glucan supplementation and vaccination on FCR.

No significant differences in mortality rates were observed among the ß-glucan treatment groups during the pre-vaccination period. Mortality during the first week appeared unrelated to ß-glucan supplementation, with environmental factors such as heat stress temperatures reaching up to 36.4°C in the open-sided housing and predation likely contributing to early losses, particularly in the group receiving 0.1% ß-glucans. The absence of mortality across all groups during the second week suggests a potential immunostimulatory effect of ß-glucans, supporting enhanced resistance to infections [42]. Furthermore, despite administering the coccidiosis vaccine at a dosage higher than the recommended level, the absence of mortality demonstrated its margin of safety in birds. This aligns with findings showing that vaccinated

flocks exhibit lower mortality rates compared to their unvaccinated counterparts [43]. Vaccination not only confers protection against coccidiosis but also mitigates the growth depression typically associated with infection, thereby improving overall flock health and survival outcomes [44].

The observed increase in the relative weight of the liver in coccidiosis-vaccinated birds is noteworthy and may be attributed to the immune response triggered by the vaccination. This aligns with reports of a similar increase in liver weight following *E. maxima* infection [45]. However, contrasting findings, which reported a decrease in liver weight with *E. tenella* infection, suggest that the specific *Eimeria* species involved may play a key role in modulating liver response [46, 47]. It is possible that the heightened immune activation following vaccination could lead to temporary hyperplasia in the liver, particularly among Kupffer cells, as observed by Moryani *et al*. (2021), which may explain the increase in liver weight in the vaccinated birds [48].

The increased relative weights of the duodenum, jejunum, ileum, and caeca observed in the coccidiosis-vaccinated birds suggest an immune system response in the GIT. These findings are in line with studies showing an increase in the relative weight and length of the duodenum following vaccination [49], although the effects on the jejunum and ileum were less pronounced. These findings differ from reports of an increase in relative weight, length, and content of intestinal sections with increasing coccidia dose [50]. The increase in relative organ weights could reflect immune system stimulation, where the body allocates resources to the development of immune cells and antibody production. This hypothesis is supported by earlier work highlighting the role of immune activation in influencing intestinal morphology [51]. Interestingly, the increase in relative weight in vaccinated birds may also involve subtle changes in intestinal morphology, such as an increase in villus size, to improve nutrient absorption. The significant effect of ß-glucan supplementation on ileum length suggests that ß-glucans may influence gut development, which may support better intestinal health and immune modulation. However, further research is needed to elucidate the specific mechanisms underlying these changes. In future studies, histomorphometry of the duodenum, jejunum, and ileum is recommended.

The significantly increased ISI histopathological scores observed in the ileum and liver of unvaccinated broilers supplemented with higher dose (0.3%) ß-glucan may be associated with heightened immune activity or other health issues which need further investigation. This finding suggests that a higher ß-glucan dose could elicit a more pronounced local and systemic immune response. Nevertheless, this interpretation requires further validation. To confirm whether the observed liver lesions are related to the elevated ß-glucan supplementation, the determination of serum biomarkers such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) is recommended. Multiple studies support the histological improvements associated with dietary ß-glucan supplementation, particularly under *Eimeria* challenge or vaccination. At the intestinal level, ß-glucans improve gut morphology by increasing villus height, villus:crypt ratios, and goblet cell numbers, which expands the absorptive surface area and strengthens mucosal defense [52]. Fluctuations in adrenocorticotropic hormone levels under stress [53] can significantly influence the ISI histopathological scores.

The observed reduction in thymus and Bursa of Fabricius weights following coccidiosis vaccination is consistent with previous reports, suggesting an immune system response to the vaccine challenge. Leung *et al*. (2019) similarly noted higher thymus weights in uninfected birds compared to those infected with *Eimeria*, indicating that immune activation following challenge may lead to tissue involution [54]. This is in line with the findings that greater small lymphocyte populations in healthier birds are associated with larger lymphoid organs, suggesting that stress or immune activation may reduce organ size [55]. The significant decrease in Bursa of Fabricius weight further supports this, with others proposing that stress-induced corticosteroid production following infection or vaccination may lead to bursal atrophy [56]. Given the Bursa of Fabricius' critical role as a primary lymphoid organ [5], its reduced weight at day 14 could reflect a transient suppression of immune activity, possibly linked to the immune system's prioritization of responding to the coccidial challenge. This is supported by the decrease in both relative weights and scores of the Bursa of Fabricius after vaccination, although scores across all treatments remained within the healthy range,

suggesting no pathological damage had occurred. Contrary to the present findings, some reported increased bursal development in coccidiosis-infected birds, attributed to the proliferation of *E. tenella* within the Bursa of Fabricius itself [47]. Studies indicate that *E. tenella* may reside in the Bursa of Fabricius, a crucial immune organ in broilers [57, 58]. Infection can trigger an early immune response, as evidenced by increased bursa cell numbers and elevated CD3-T lymphocytes [59]. The inconsistencies in results regarding immune organ weight and coccidiosis may be due to factors such as the severity and dosage of coccidiosis, which can overwhelm the immune system, *Eimeria* species specificity, and the age and health status of the birds. Overall, the monitoring should be extended until harvest date is recommended in future studies. Omara *et al.* (2021) found that 0.1% yeast-derived ß-glucan elevated expression of immune genes in spleen, thymus, and Bursa of Fabricius in broilers challenged with *Eimeria* spp., indicating immune priming and regulation [60]. In vaccinated broilers, yeast cell wall/ß-glucan supplementation has also been shown to increase mucosal IgA, modulate cytokine responses in the ileal mucosa, and reduce oocyst shedding [34][61]. They upregulate tight junction proteins (occludin, claudins) and mucin-2 expression, thereby reducing gut permeability and limiting pathogen translocation [41].

The heterophil-to-lymphocyte (H:L) ratio, a well-established indicator of stress in poultry [62], was evaluated at two time points: day 10 (3 days post-vaccination) and day 14 (7 days post-challenge). Several factors can influence the H:L ratio, including bird age, the severity of infection, and feed additive composition [63, 12]. Heterophils are associated with innate immunity and microbial infection control, whereas lymphocytes primarily mediate antibody production [5]. Studies suggest that elevated doses of coccidiosis vaccines might enhance the immune response in poultry by stimulating stronger cell-mediated immunity, which is critical for combating intracellular pathogens like *Eimeria* [42]. Research also indicates that live attenuated coccidiosis vaccines, which utilize weakened *Eimeria* strains, are designed to be safe and effective even at higher dosages, promoting a protective immune response in poultry with minimal adverse effects [44, 72]. Across all treatment and control groups, the H:L ratios fell outside the generally accepted healthy reference range of 0.5–4.0 [64]. Borges *et al.* (2004) similarly reported H:L values between 0.25 and 0.43 in heat-stressed birds, which may offer a useful comparative reference [65]. The observed increase in lymphocyte counts at day 10 following ß-glucan supplementation is consistent with previous studies showing similar lymphocyte increases in chicks fed with 1,3-1,6 ß-glucan [66]. ß-glucan can mimic pathogen-associated molecules, binding to pattern recognition receptors (PRRs) on white blood cells, and trigger an immune cascade, enhancing overall immune responses [67]. Nonetheless, discrepancies exist, as others found no differences in white blood cell or lymphocyte counts in broilers supplemented with ß-glucan, likely due to variations in ß-glucan type, structure, or dosage [68]. At 7 days post-vaccination, increased heterophil counts and elevated H:L ratios aligned with previous observations in *E. tenella* infections [69], which also noted significant lymphocyte declines post-infection. The rise in heterophils could reflect an intensified immune response against *E. tenella* [69, 70]; however, since heterophils also elevate during stress [62], these results require cautious interpretation. Together, the increased heterophil and lymphocyte counts suggest an active immune response in coccidiosis-vaccinated birds. The elevated H:L ratio further supports this, indicating a dual response targeting both infection and tissue damage [71].

qPCR analysis confirmed that *Eimeria acervulina*, *E. mitis*, and *E. praecox* were consistently detected at high levels (Ct < 27.0) across all vaccinated groups from day 12 through day 14, indicating successful initial cycling of vaccine-derived oocysts. *E. maxima* was detected at low to moderate levels (Ct ≈ 31–34) beginning on day 13, while *E. tenella* was only detected in the vaccinated control and 0.3% ß-glucan group at moderate levels (Ct ≈ 31–36) on day 13. No mortality was observed, confirming that vaccine cycling occurred without excessive pathogenic challenge under the litter floor rearing conditions.

The absence of significant reductions in oocyst counts among broilers supplemented with ß-glucans suggests limited direct anticoccidial activity under the conditions of this study. Although ß-glucans are well-documented for their immunomodulatory effects stimulating both innate and adaptive immune responses in poultry [72], these effects may not necessarily lead to a

reduction in *Eimeria* spp. oocyst shedding. This could be attributed to the nature of immune priming, where ß-glucans enhance immune readiness rather than directly inhibit protozoal replication. The timing of supplementation relative to the challenge, as well as differences in ß-glucan source, purity, and dosage, may also influence the outcome [2]. Future studies should include comparative evaluations of AGPs, including coccidiostats, to fully understand how they differ from ß-1,3/1,6-glucan**.**

In contrast to the present findings, some reported that whole yeast cell products reduced fecal oocyst counts during an *Eimeria* challenge, accompanied by increased macrophage nitric oxide production and proinflammatory cytokine gene expression indicating a stronger immunological activation [73]. However, Ott *et al.* (2018) found that broilers supplemented with ß-glucans shed significantly more oocysts than those given antibiotics, and oocyst counts were not significantly different from control birds, while non-challenged groups showed no detectable oocysts [2]. Taken together, these findings suggest that the mixed effects of ß-glucans may be more produced lesion severity (0.3% dose) in ileum and liver rather than in directly suppressing oocyst shedding. The persistently high oocyst counts in all challenged groups suggest that any immunoprotective effects of ß-glucans were not robust enough to interfere with the parasite's reproductive cycle during the timeframe of this study.

5. Conclusions

In conclusion, the observed significant differences in BWG, gut morphometry and immune responses are linked to the main effects, namely the coccidiosis vaccination status and the level of ß-glucans, acting independently rather than interactively. The absence of a significant interaction between ß-glucans and coccidiosis suggests their effects may be separate. While higher-dose coccidiosis vaccination may have reduced mortality rates and potentially strengthened cell-mediated immunity, as suggested by the observed H:L ratio range, it also had negative consequences. These included decreased BWG, disruption of gut health, and a weakened immune response, evident in lower BWG, gut damage, and stunted immune organ growth. Additionally, vaccination presence was the sole factor associated with significant differences in oocyst shedding, with ß-glucan supplementation showing no direct effect under the study conditions. Meanwhile, dietary ß-glucan inclusion exhibited promise in enhancing immune cell activity by increasing lymphocyte counts and subtly influencing ileum length, suggesting a potential role in gut development.

Availability of Data and Materials

All data are available in this study

Author Contributions

Conceptualization, S.I.C.; Data curation, R.E.A.R., A.L.T., A.G.P.V., K.M.N., S.I.C., and M.J.C.A.; Formal analysis, S.I.C., M.J.C.A., and R.E.A.R.; Writing – Original Draft R.E.A.R.; Writing, R.E.A.R., and S.I.C.

Ethics Approval and Consent to Participate

Not applicable

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Conflict of Interest

The authors declare no conflict of interest.

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