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Original Research

Comparative Histomorphometric Analysis of the Proventriculus and Ventriculus of the Darag Philippine Native Chicken and Hubbard Redbro™

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Abstract

Background: Due to the increasing consumer's awareness of the impact of bird's welfare on meat quality and overall nutritional value, the global demand for chicken meat production has been gradually shifting to slow-growing breeds. However, comparative data on slow-growing commercial and native chicken breeds are scarcely available. **Methods:** Tissue samples of the proventriculus and ventriculus from *Darag* and Hubbard Redbro that were raised at different time periods were processed using standard staining technique for histomorphometric analysis. The measured histological parameters were then correlated with growth and selected production-related variables. **Results:** The proventricular deep gastric gland lobule area (DGGA) and depth (DGGD) as well as the ventricular tubular glands and lamina propria thickness (TGLPT) were comparable between breeds except during the 8th week period wherein *Darag* showed significantly higher TGLPT than Redbro. Interestingly, *Darag* exhibited a statistically significant strong positive correlation between DGGA and body weight, organ weight, and total feed consumption. Conversely, no significant correlation was found in Redbro strain. **Conclusion:** The findings of this study offer novel information regarding the histomorphological characteristics of the *Darag* chicken's stomach, which can potentially provide insights in further optimizing the current aspects of its management and farm practices.

Keywords: *Darag*, Histomorphometry, Proventriculus, Ventriculus, Philippine native chicken

1. Introduction

The Philippine native chicken is an important livestock resource in the country as it contributes to the supply of meat and eggs and considered as an income, savings, and insurance for rural Filipino farmers [1]. According to the chicken situation report released by the Philippine Statistics Authority (PSA) as of 30 September 2023, the native chicken population comprises the bulk of the 202.82 million total chicken population in the country accounting to 43.0% in comparison to the 34.7% and 22.3% share of the broiler and layer chickens, respectively [2]. This shows that the country's native chicken production is thriving and highly acceptable. Presently, there are many strains of native chicken in the country which include the *Banaba*, *Bolinao*, *Camarines*, *Paraokan*, *Boholano*, *ZamPen*, and *Darag* [3].

The *Darag* Philippine native chicken is an indigenous strain that is commonly raised in the Western Visayas area, specifically in the Island of Panay where it originated [2]. They are defined by their wheaten plumage color with hens having a penciled pattern and roosters displaying a plain pattern. They have medium to large single comb, white earlobes and skin color, orange irises, and mostly black shanks [4]. Adult males and females weigh around 1.30 and 1.00

kilograms, respectively, and are usually grown for 75-120 days to reach ready-to-slaughter age [3]. *Darag* chickens are usually preferred by rural Filipino farmers because of their lower rearing requirements compared to its commercial counterpart. They do not require specialized housing as they are normally grown free-range and are able to withstand the harsh natural environment without compromising their reproductive capability [5-6]. They are also relatively inexpensive as they do not need strict biosecurity measures and feeding requirements. Moreover, they are gaining popularity among local consumers as a healthier alternative due to their low cholesterol content not to mention their distinctive savory, gamey flavor [3].

At present, the demand for slow-growing chicken breeds like *Darag* and other native or indigenous chicken strains and some commercially available strains such as the Hubbard JA57, JA87, and Redbro has been consistently growing as influenced by the increasing knowledge of consumers on the impact of improved bird welfare on the nutritional and sensory characteristics of the meat [7-8]. Indeed, they are no longer just bred to cater a niche specialty market or serve as a meat source under a low-input system. The Redbro, which closely resembles *Darag*, is a hybrid broiler-type chicken that was developed by the Hubbard company to comply with the standards set forth by the Better Chicken Commitment (BCC) and Global Animal Partnership (GAP) to promote better welfare for chickens. It is reported to be more economical, yield better production performance, and produce a lower carbon footprint as compared to its older commercial counterpart [9-10].

However, despite their increasing popularity and utility, there is a marked scarcity of information to date on the comparative data of *Darag* and those slow-growing commercial strains especially in terms of the morphometry of their organs and its correlation with production and growth performance parameters. Recently, our group established a baseline information on the gross morphometry of the gastro- intestinal tract as well as the proportions of the lean, fat, and bone between *Darag* and Redbro thereby providing invaluable insights into the digestive capacity and nutrient utilization capability of these breeds [11]. In the present study, the comparative histomorphometric features of the stomach, particularly the proventriculus and ventriculus, of *Darag* and Redbro were investigated and then determined how these measured data relate to its growth and production performance. The findings of this work would provide better understanding of the histomorphological traits of *Darag* that will further aid in optimizing the current aspects of its management and farm practices.

2. Materials and Methods

2.1 Birds and Management

The birds used in the present study were provided by the completed "Genome-wide association study (GWAS) for growth and egg production traits of *Darag* native chicken" project of the Institute of Animal Science, College of Agriculture and Food Science (IAS-CAFS) which utilized 200 male day-old chicks consisting of 100 *Darag* and 100 Hubbard Redbro chicks [11]. The birds were raised for a total growing period of 15 weeks at the University Animal Farm of the IAS-CAFS (14.145485903561903, 121.25209186479026) and were housed under complete confinement system using elevated cages with the following pen dimensions: 4.0 ft (L) x 3.0 ft (W) x 2.5ft (H). They were maintained under a light exposure of 24 h for the first 7 days, 18 h from day 8 until day 21 followed by 12 hours for the remainder of the growing period until harvest. Environmental temperature, on the other hand, was kept at 31℃ from days 0 to 21, and then followed by 28℃ thereafter. The birds had free access to water and were fed with commercial booster feeds from 0 to 4 weeks of age, commercial starter feeds from 5 to 8 weeks of age, and commercial grower feeds from 9 weeks of age until harvest. All husbandry protocol and experimental procedures were carefully reviewed and approved by the University of the Philippines Los Baños Institutional Animal Care and Use Committee (UPLB-IACUC) with approval reference number, UPLB-2023-036 [11].

2.2 Sample Collection

Three (3) birds per group at different time periods (1, 2, 4, 6, and 8 weeks) were sacrificed via neck slaughtering wherein the trachea, esophagus, jugular vein, and carotid arteries were severed using a sharp knife [11]. A ventral coelomic incision was done to access the abdominal cavity, then the crop down to the rectum were collected. The proventriculus and ventriculus were carefully separated from any structures adhering to it, and the organ segments were then cut, weighed, and measured for total length. These segments were subsequently cut into smaller pieces and placed in a container with 10% neutral buffered formalin for at least 72 h.

2.3 Tissue Processing and Histomorphometric Examination

Tissue samples were subjected to routine paraffin technique and stained with hematoxylin and eosin (H&E). In detail, the formalin-fixed tissue samples were washed in running tap water and dehydrated in ascending concentrations of alcohol (70%, 80%, 90%, and 100%). These were then dipped in 100% xylene, embedded in paraffin, and cut into serial sections with 5 μm thickness using a microtome. For every four slides, one was stained with H&E in accordance with standard procedure. The sections were briefly deparaffinized in four changes of xylene for 5 min each, then were repeatedly immersed in a solution of 100% xylene and ethanol.

The slides were rehydrated by dipping it 10 times in decreasing concentrations of alcohol (100%, 90%, 80%, and 70%), followed by a 5 min rinsing over running water, and a 2 min staining with hematoxylin solution. Afterwards, the slides were dipped 5 times in 80% alcohol, stained for 5 to 10 min with eosin solution, and washed in ascending concentrations of alcohol. The slides were dipped in 100% alcohol, dipped 5 times in 100% xylene, and then soaked in two changes of xylene for 5 min each. Lastly, a drop of mounting medium was placed in the center of the slide and then covered with a clean coverslip.

The prepared H&E tissue sections were carefully examined using a compound light microscope (Amscope, China) to evaluate the histological and histomorphometric features of the proventriculus and ventriculus of both chicken strains that were collected at different time periods. Histomorphometric examination was carried out on the following parameters: the deep gastric gland lobule area (DGGA) and deep gastric gland lobule depth (DGGD) of the proventriculus and the combined tubular glands and lamina propria thickness (TGLPT) of the ventriculus. Using x10 magnification, the entire tissue section along with the micrometer was captured and was stitched together using the Photomerge feature of the Adobe Photoshop. DGGA, defined as the amount of surface that was covered by a lobule within the proventricular wall, was quantified by measuring at least 5 gland lobules. DGGD, defined as the distance from the wall of the gland to the tip of the epithelium of the gland, was quantified by measuring at least 5 depths from multiple lobules. Meanwhile, TGLPT, defined as the distance from the tip of the tubular gland to the base of the lamina propria, was similarly quantified by measuring the thickness of at least 5 different areas. Measurement of all parameters at each pre-determined time point was accomplished using Image J (Fiji software, National Institute of Health, Maryland, USA).

2.4 Correlation Analysis and Statistical Data

All data collected were analyzed using GraphPad Prism (San Diego, CA, USA). The mixed effects model was used to analyze the effect of age, strain, and their interaction on the different proventricular and ventricular parameters measured. The two-stage linear step-up method of Benjamini, Krieger, and Yekutieli was selected as the post hoc test to control the false positives that may be brought about by the mixed effects model. Furthermore, a two-tailed Pearson's correlation coefficient with a confidence interval of 95% was utilized to determine the relationship between the measured parameters and the animal's body weight, organ weight, and total feed consumption. All values with $P < 0.05$ were considered as significant, $P < 0.01$ were considered as highly significant, and $P < 0.001$ were considered as very significant.

3. Results

3.1 Proventricular DGGA and DGGD

The proventriculus, irrespective of the chicken strain, showed intact mucosal layers and prominent epithelial folds surrounding the centrally located lumen (Fig. 1A & 1B). The deep gastric glands, which are conspicuously occupying the bulk of the proventricular wall, presented some ovoid or pear-shaped lobules having multiple secretory tubules that are wrapped around with connective tissues and some muscle fibers (Fig. 1C). As shown in Fig. 1D, histomorphometric analysis of the proventricular DGGA did not significantly vary between *Darag* and Redbro at each of the examined time point. It was noted, however, that DGGA in *Darag* depicted a consistent upward trend starting at week 2 until week 8 whereas a steady increase was only observed in Redbro from week 1 to week 4. As for the DGGD, no statistically significant difference was likewise accounted between the two chicken strains throughout the experimental period (Fig. 1E).

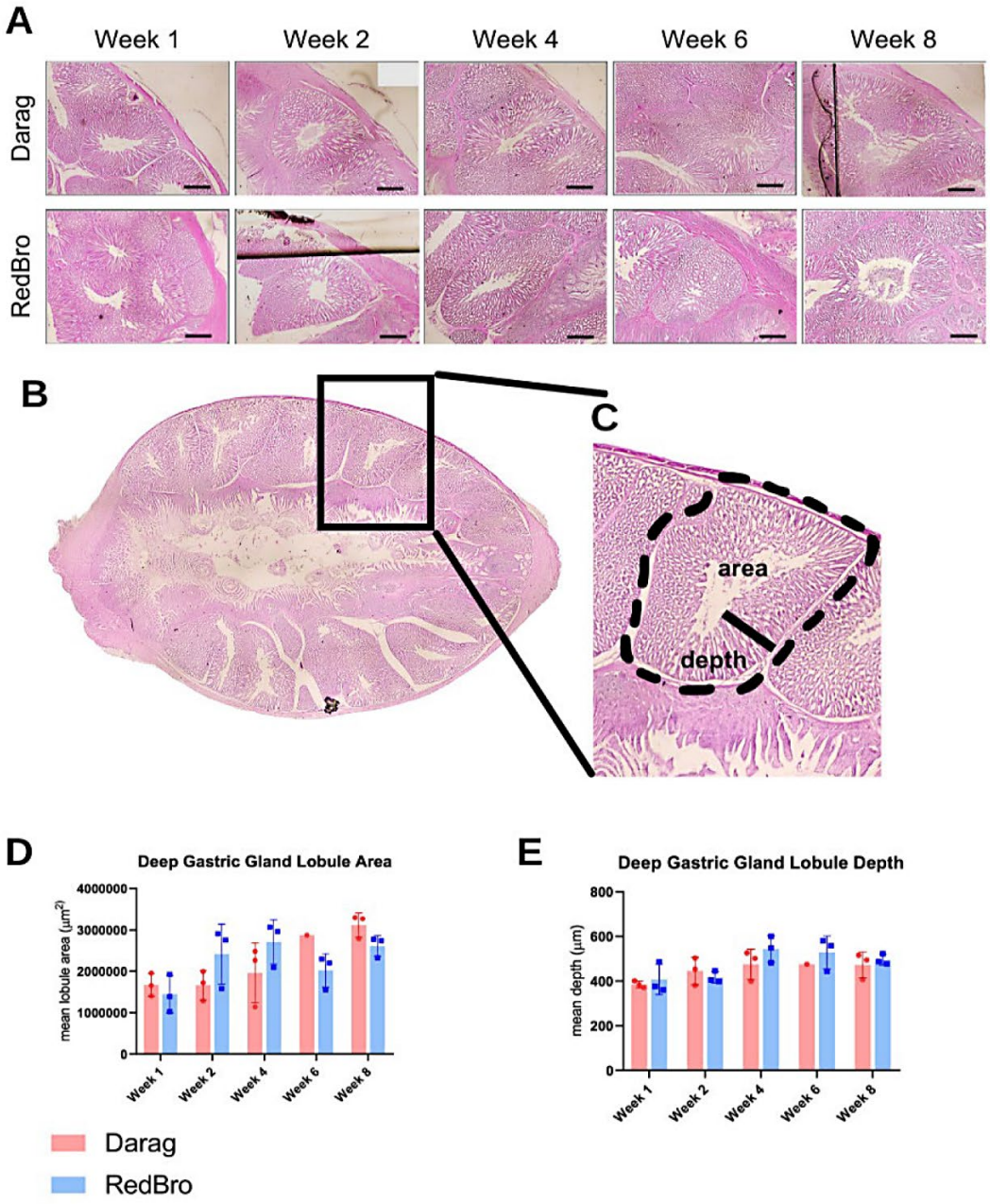

Fig. 1. Histomorphological findings of the proventriculus between the two chicken strains, *Darag* vs. Redbro show comparable parameters in all age points. (A) Representative images of the proventriculus sections from *Darag* and Redbro strains at weeks 1, 2, 4, 6, & 8. Scale bar = 500 µm. (B) Demonstrated is the whole proventriculus tissue section, and (C) the deep gastric gland lobule area (broken line) and depth (solid line). The bar graphs displaying (D) the mean deep gastric gland lobule area and (E) the mean deep gastric gland lobule depth of the proventriculus between the two groups at each examined time point. Data are shown as means ± standard deviation (SD) of 5 tissue sections per animal (n = 3 animals/group).

3.2 Ventricular TGLPT

Examination of the H&E sections of the ventriculus of both *Darag* and Redbro revealed a thick layer of keratinoid (cuticle) lining the mucosal tunic which presents columnar epithelium that are arranged in folds of varying height and glandular tubules that are divided by lamina propria. Also noticeable are the prominent muscular tunic consisting of the internal oblique, thick middle circular, and thin longitudinal

layers (Fig. 2A-2C). Histomorphometric analysis of the ventricular TGLPT, on the other hand, unveiled statistically comparable results between the two chicken strains across all examined time points with the exemption of week 8 wherein a significantly increased TGLPT was exemplified by *Darag* as opposed to Redbro.

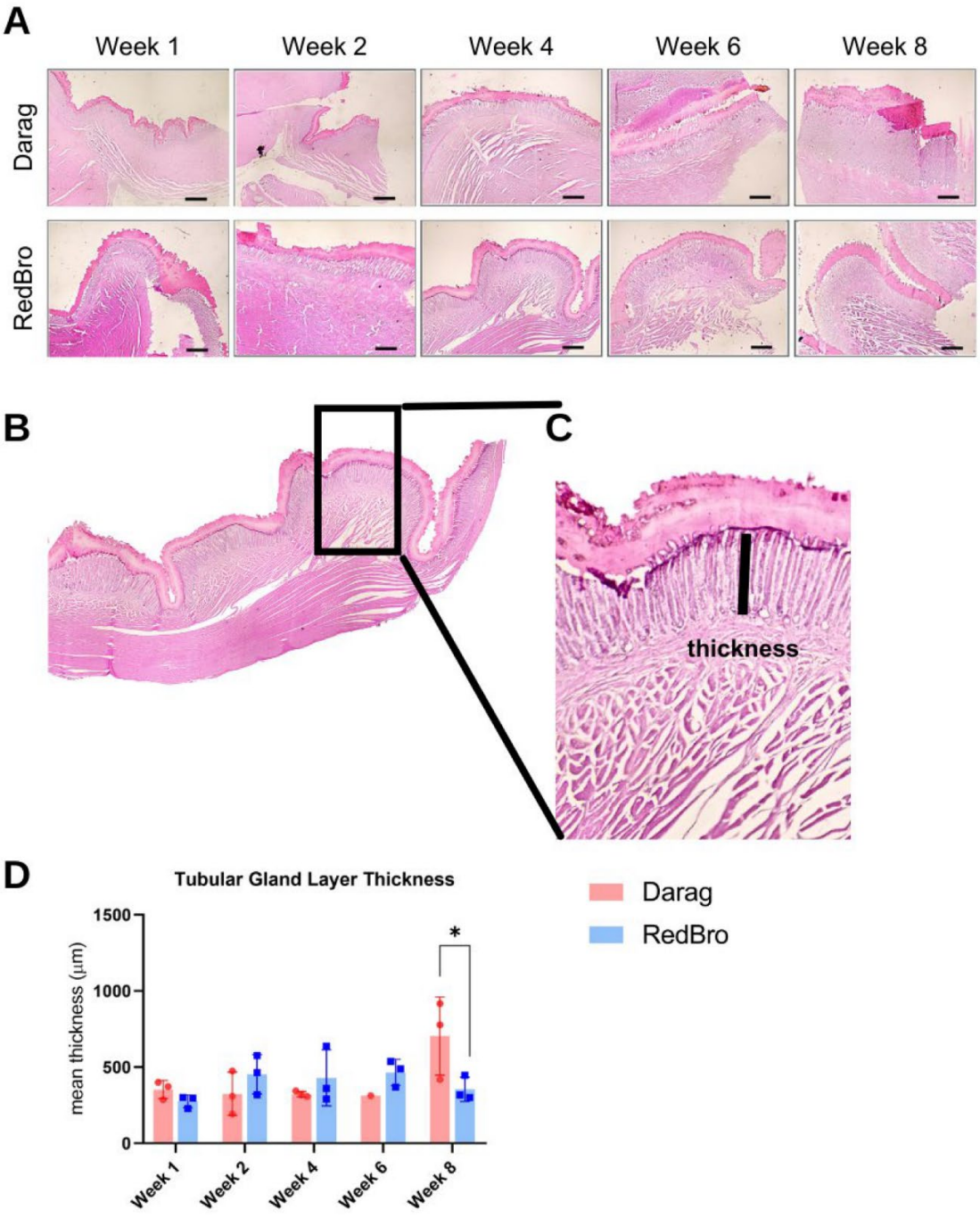

Fig. 2. Histomorphological findings of the ventriculus between the two chicken strains, *Darag* vs. Redbro show thicker tubular gland layers in the Darag strain. (A) Representative images of ventriculus sections from *Darag* and Redbro strains at weeks 1, 2, 4, 6, & 8. Scale bar = 500 µm. (B) Shown is the merged photo of the ventriculus. (C) The thickness of the tubular gland layer is demonstrated by the solid line. (D) The bar graph displaying the mean tubular gland layer and lamina propria thickness of the ventriculus between the two groups at each examined time point. Data are expressed as means ± standard deviation (SD) of 5 tissue sections per animal (n = 3 animals/group). * $P < 0.05$

3.3 Effects of Age and Strain on the Different Parameters, and their Interaction

Age had a highly significant effect on the proventricular DGGA and a significant effect on both proventricular DGGD and ventricular TGLPT. Interestingly, the interaction between age and strain only produced a highly significant effect on the variability of the mean ventricular TGLPT. Meanwhile, the strain did not appear to influence all examined parameters (Table 1).

3.4 Correlation between the different parameters and body weight

In sharp contrast to Redbro, the *Darag* strain exhibited a strong positive correlation between DGGA and body weight which achieved a high degree of statistical significance. The rest of the proventricular and ventricular parameters, regardless of chicken strain, did not yield any significant correlation with body weight (Table 2; Supplementary Table 1).

Table 1. Mixed-effects model test results for the effects of age and strain on the differences seen in each dependent variable.

Parameters	Age	Strain	Interaction
Proventriculus parameters			
DGGA	F (4, 18) = 6.0640 $P = 0.0028^{**}$	F (1, 18) = 0.0080 $P = 0.9294$	F (4, 18) = 2.9030 $P = 0.0513$
DGGD	F (4, 18) = 4.4330 $P = 0.0114^{*}$	F (1, 18) = 1.5990 $P = 0.2222$	F (4, 18) = 0.6521 $P = 0.6328$
Ventriculus parameter			
TGLPT	F (4, 14) = 3.5010 $P = 0.0351^{*}$	F (1, 4) = 0.0014 $P = 0.971$	F (4, 14) = 5.9600 $P = 0.0051^{**}$

* Effect is significant at $P < 0.05$; ** Effect is significant at $P < 0.01$; DGGA, Deep gastric gland area; DGGD, Deep gastric gland diameter; TGLPT, Tubular gland layer and lamina propria thickness

Table 2. Two-tailed Pearson correlation coefficients between the histomorphometric parameters of the proventriculus and ventriculus of *Darag* and Redbro and body weight.

Parameters		*Darag*	Redbro
Proventriculus			
DGGA	Pearson's Correlation	0.966^{**}	0.502
	Sig. (2-tailed)	0.008	0.389
	N	5	5
DGGD	Pearson's Correlation	0.702	0.663
	Sig. (2-tailed)	0.187	0.223
	N	5	5
Ventriculus			
TGLPT	Pearson's Correlation	0.764	0.173
	Sig. (2-tailed)	0.133	0.781
	N	5	5

**Correlation is significant at $P < 0.01$; DGGA, Deep gastric gland area; DGGD, Deep gastric gland diameter; TGLPT, Tubular gland layer and lamina propria thickness

3.5 Correlation between the different parameters and organ weight

In parallel with earlier findings on body weight, correlation analysis unveiled a highly significant strong positive relationship between proventricular DGGA and organ weight in *Darag* strain. Conversely, no significant correlation was determined between these factors in Redbro strain. Other measured parameters for both chicken strains did not appear to establish a significant correlation with organ weight (Table 3; Supplementary Table 2).

3.6 Correlation between the different parameters and total feed consumption

Among the different parameters measured, only the proventricular DGGA showed a statistically significant positive correlation with total feed consumption. This relationship, however, was notably observed in *Darag* alone but not in the Redbro strain (Table 4; Supplementary Table 3).

Table 3. Two-tailed Pearson correlation coefficients between the histomorphometric parameters of the proventriculus and ventriculus of *Darag* and Redbro and organ weight.

Parameters		*Darag*	Redbro
Proventriculus			
DGGA	Pearson's Correlation	0.968**	0.510
	Sig. (2-tailed)	0.007	0.380
	N	5	5
DGGD	Pearson's Correlation	0.725	0.819
	Sig. (2-tailed)	0.166	0.090
	N	5	5
Ventriculus			
TGLPT	Pearson's Correlation	0.663	0.354
	Sig. (2-tailed)	0.222	0.559
	N	5	5

**Correlation is significant at $P < 0.01$; DGGA, Deep gastric gland area; DGGD, Deep gastric gland diameter; TGLPT, Tubular gland layer and lamina propria thickness

Table 4. Two-tailed Pearson correlation coefficients between the histomorphometric parameters of the proventriculus and ventriculus of *Darag* and Redbro and total feed consumption.

Parameters		*Darag*	Redbro
Proventriculus			
DGGA	Pearson's Correlation	0.965**	0.685
	Sig. (2-tailed)	0.008	0.202
	N	5	5
DGGD	Pearson's Correlation	0.779	0.783
	Sig. (2-tailed)	0.121	0.117
	N	5	5
Ventriculus			
TGLT	Pearson's Correlation	0.674	0.337
	Sig. (2-tailed)	0.212	0.579
	N	5	5

**Correlation is significant at $P < 0.01$; DGGA, Deep gastric gland area; DGGD, Deep gastric gland diameter; TGLPT, Tubular gland layer and lamina propria thickness

4. Discussion

The gastro-intestinal tract morphometrics have been well established in literature to be intricately connected with the animal's digestive ability and nutrient absorptive capacity [12]. In relation to the bird's stomach, gross weight and dimensions (i.e. length, width, thickness) as well as the histomorphological features of the proventriculus and ventriculus have been extensively studied, as reported in diverse avian species including the ostrich [13], starling bird [14], red jungle fowl [15], turkey [16], Japanese quail [17], and even in commercial [18-20] and indigenous broiler chickens [21]. Recently, we have documented the comparative gross morphometry of the gastro-intestinal tract including the stomach tissues of the two slow-growing broiler strains, the *Darag* Philippine native chicken and a commercial strain, the Hubbard Redbro [11]. In the current study, the histomorphometric features of the proventriculus and ventriculus of these two chicken strains were further investigated to better understand the impact of genetic background on the morpho functional traits of the digestive organ. To our knowledge, our study belongs to the very few works that have attempted to investigate the histomorphometry of the gastric glands as majority have focused on determining the thickness of the layers or tunics of these gastric tissues.

Examination of the proventricular DGGA and DGGD revealed no significant difference between *Darag* and Redbro across all determined time periods. This coincided well with the gross morphometric findings which showed statistically comparable results on the normalized weight of the proventriculus between the two strains throughout the 8-week period [11]. On the other hand, the mixed effect model depicted a commensurate increase in both proventricular parameters with advancing age. These results essentially agree with the findings of Akter *et al.* (2018) [22] and Mehra and Kumar (2023) [23] using Cobb-500 broiler chickens. This is further supported by another study which accounted for the corresponding increase in the deep gastric gland diameter of the proventriculus as seen in Kadaknath fowl with increasing age [24]. The age-dependent increase in proventricular DGGA and DGGD may be attributed to increase in the number and size of the oxyntico-peptic cells lining the gland therefore resulting in higher digestive enzyme secretion and better digestion of ingesta [19]. This observation may also be explained in part by the fusion of two or more adjacently located glands as the bird ages thereby allowing formation of a large proventricular gland [24].

The ventricular TGLPT measured in both slow-growing chicken strains did not significantly deviate from one another during the first 6 weeks. However, a considerable increase in this parameter was notably observed on week 8 in *Darag* but not in the Redbro strain. This finding somewhat corroborated the result of the gross data except that significant difference in the normalized weight of the gizzard between the two strains was perceived as early as 3 weeks of age [11]. This apparent discrepancy implies that the expansion of other histological structures like the muscularis mucosa and the muscular layer might possibly occur in the ventriculus of *Darag* during these earlier growing periods wherein development of muscle fibers would be crucial in enhancing the muscle rhythm and digestive ability [12].

The mixed effect model provided confirmation that age, in combination with strain, truly influenced the variability of the mean ventricular TGLPT. Additionally, it showed that a linear relationship exists between TGLPT and age which is in consonance with previous studies [12,25]. The proportional increase in the thickness or height of the ventricular glands as a function of advancing age is critical for broilers and other avian species as this would consequently lead to an increase in the production of cuticle that is necessary for mechanical digestion. It has been previously shown that birds which are considered as insectivores, granivores, and herbivores possess a well-developed ventriculus with a prominently thick and abrasive cuticle layer [26-27]. Since the *Darag* native chicken breed has been particularly developed to be grown free-range and consume a variety of forages, insects, and grains instead of relying to commercial feeds like the Redbro strain, this would lend justification to the significantly greater ventricular TGLPT achieved during the 8th week of age. Moreover, it has been argued that availability of moderate amount of fiber in the diet of birds may enhance the size and development of the gizzard, which in turn could increase the flow of feed through this organ and allow higher contact between nutrients and digestive enzymes owing to the increase in the gastroduodenal refluxes [28].

Furthermore, it has been reported that fibrous material supplied by the range could enhance the production of insoluble non-starch polysaccharides (NSP) that may alter the development of gastro-intestinal tissues. In the case of the gizzard, this is typically reflected by a substantial increment in the size and weight of the tissue [20,29]. Future studies should incorporate analysis of the digestive secretions using histochemical staining methods [15,30-31] to gain better insights as to whether the significant increase in the size of the ventriculus along with its tubular glands in *Darag* would translate to more superior digestive function than in the Redbro strain.

Although gastric glands are integral component of the stomach as it facilitates efficient digestive process by elaborating essential enzymes and secretions that chemically act to reduce the complexity of ingested feeds and enhance the absorption of nutrients [32], the relationship between these structures and selected growth- and production-related variables are largely unknown. Therefore, in this present study, correlation analysis was finally performed between the measured proventricular and ventricular parameters and body weight, organ weight, and total feed intake in both slow-growing chicken strains. Results showed that *Darag*, in sharp contrast with the Redbro strain, demonstrated a statistically significant strong positive correlation between the proventricular DGGA and body weight, organ weight, and total feed intake. In support of our findings, Selim *et al.* [33] also found that birds fed post hatch had greater deep gastric gland lumen diameter along with greater mucosal fold and mucosal layer thickness of the proventriculus, versus the fasted group, therefore signifying that feed consumption exert an effect on the histomorphological structures of the proventriculus including its proventricular deep gastric gland [33].

On the other hand, the proventricular DGGD and ventricular TGLPT both failed to establish a substantial correlation with all the variables regardless of the chicken strain. These observations may be partly explained by the effect of intense selection pressure for desirable traits which tremendously disrupt the balance between growth performance and maturity of the organs [34]. As substantiated by previous works, the rapid increase in the muscle mass as especially observed in modern broiler chickens was associated with retarded growth of the digestive organs and presumably their associated gastric glands, when compared to unselected heritage lines of the same age [35-36]. Interestingly, our recent data unveiled a negative allometry for gastrointestinal growth in both chicken strains indicating that the growth of the gastro-intestinal tract proceeds at a relatively slower pace as compared to the increase in their body weight or muscle mass [11]. However, it should be noted that the ventricular TGLPT exemplified by *Darag* yielded a moderate to strong positive correlation with all the examined variables especially with respect to the body weight in contrast to the Redbro strain. In concordance with this, the *Darag* strain garnered a significantly higher normalized weight of the gizzard starting at week 3 until the end of the experimental period despite attaining a body weight that was more than 3-fold lower than those of the RedBro strain at any given point in time [11].

5. Conclusion

The findings of the present study essentially mirrored the gross morphometric findings showing comparable results among the measured proventricular and ventricular parameters between the two slow-growing broiler strains except for a significantly higher ventricular TGLPT exemplified by *Darag* during the 8th week period in contrast to the Redbro strain. Correlation analysis revealed that only the proventricular DGGA appears to correlate well with body weight, organ weight, and total feed intake as demonstrated by the statistically significant strong positive relationship in *Darag* but not in the Redbro strain. The novel information proffered by the present study may provide invaluable insights into further optimizing the current aspects of management and farm practices (i.e. feeding and nutrition) in *Darag* native chicken.

Availability of Data and Materials

All data are accessible to readers upon written request to the corresponding author.

Author Contributions

Conceptualization, M.J.M.D., V.A.M. and M.J.C.A.; Methodology, M.I.A.M.M., M.J.M.D., H.N.F.B. and M.J.C.A.; Investigation, M.I.A.M.M. and M.J.C.A.;

Writing – Original Draft, M.I.A.M.M., M.J.M.D. and M.J.C.A.; Writing – Review & Editing, M.I.A.M.M., M.J.M.D., V.A.M., H.N.F.B. and M.J.C.A.; Funding Acquisition, V.A.M.; Supervision, M.J.M.D., V.A.M. and M.J.C.A.; Resources, H.N.F.B. and V.A.M.

Ethics Approval and Consent to Participate

All experimental procedures adhered to pertinent international and local guidelines for the care and use of animals and were approved by the University of the Philippines Los Baños Institutional Animal Care and Use Committee with approval number UPLB-2023-036.

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Sequence Analysis of Thymidine-Kinase Encoding Gene of Koi Herpesvirus Infection Case in Malang and Batu City–Indonesia

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Abstract

Background: Koi Herpesvirus (KHV) infection evidently gave a serious impact to the ornamental fish industry in Indonesia. Measures have been managed to control the outbreak in local fisheries, however, the infection is somehow persistent due to presumptive broad contamination to native fishes, water body, and poor fishpond management. This study investigated the KHV infection cases occurrence in Malang and Batu city based on pathological changes found in deceased fish followed by partial thymidine kinase (TK) encoding gene analysis. **Methods:** Five and two koi fish were dissected to obtain gill tissue and preserved in non-buffered 10% formalin for histopathologic examination. Two gill tissues were performed polymerase chain reaction (PCR) and an amplicon with prominent specific band were sequenced using Sanger sequencing method. **Results:** The smaller fish presented lesser gills necrosis with multifocal whitish fibrinous exudate plaques and the larger fish tended to develop a vast area of necrosis. The amplicons partially covered about 410 base pairs (bp) of nucleotides of the total about 998 bp length nucleotides of TK encoding gene that matched to KHV 3 TK encoding gene sequences from NCBI Genbank. **Conclusions:** There was no distinct grouping of haplotypes based on KHV TK encoding gene sequences.

Keywords: Haplotype, Histopathology, Koi herpesvirus, Phylogeny, Thymidine kinase

1. Introduction

Known as high valuable ornamental and decorative fish for either outdoor or indoor ponds, koi fish has been extensively bred and contributed to Indonesia national commodity and income. During 2017 to 2018, the koi fish industry has culminated at 1.22 billion fish exported to so-called countries including the United States, United Kingdom, Australia, Japan, China, and Singapore. This indeed directly gave an impactful increase of income to Indonesia fisheries communities especially those who relied on ornamental fish's industry, reporting an increase of income from USD 21 million to USD 33 million in the last five years. Ornamental fish industry also apparently developed during the Covid-19 pandemic, giving alternative sources of revenue [1].

Though this koi fish industry seems promising for supporting national economic development, several infectious diseases including Koi Herpesvirus (KHV) infection have been reported deteriorating the fish health, giving a serious hit to koi population in the breeding ponds. These viruses evidently cause rapid death with high morbidity and mortality especially those that attack parent stocks leading to serious economic loss. Several KHV infection cases have been reported in many places in Indonesia since 2002 predominantly in Java Island including Blitar, Subang, and Cirata. In 2003, a similar case was reported in Lubuk Lingau, South Sumatra presenting a list of highly identical lesions to what were reported in Java. Considering the spread of infection, the contamination of KHV in a broader region, however, is not reported, is highly possible [2].

Several pathological lesions were reported associated with KHV infection though they were not pathognomonic merely in this infection. Fatal defects inducing systemic failure of organs were reported in kidneys and gills which alter the primary organ of fish osmoregulation. It might be associated with virus portal entry through superficial tissues including gills. Other studies suggested that fins may contribute as a primary portal of entry for KHV prior to reaching systemic organs [3]. In fact, gills were apparently the most reported organ presenting pathological lesions associated with KHV infection [4]. Analysis of pathological changes in organs of deceased fish due to KHV infection might help to provide symptomatic treatment and prophylactic therapy at the earliest time of infection.

Koi herpesvirus is a double stranded DNA virus (dsDNA) that causes infection in common carp (*Cyprinus carpio*) and koi carp (*Cyprinus carpio koi*). According to ICTV nomenclature, Koi herpesvirus is classified as *Cyvirus cyprinidallo3* belonging to the family of Alloherpesviridae, genus *Cyvirus*. It has an estimated 277 kbp genome size, greater than any herpesvirus, and encodes about 136 proteins. The primary protein of koi herpesvirus to infect the cell host is a glycoprotein. Glycoprotein mediates DNA virus injection to the nucleus for replication. The synthesis process of viral protein occurs in the nucleus. The protein will be transported to the Golgi apparatus to assemble its components and released into the environment [5]. At least, three major KHV lineages–American, Asian, and Europe– have been identified based on genotype to trace the transmission route according to their geographic origins.

Thymidine kinase is one of the important enzymes of koi herpesvirus that plays a critical role in replication and virulence. It is located within Open Reading Frame 55 of the koi herpesvirus genome. Thymidine kinase catalyzes the transfer of phosphate groups from ATP to thymidine, producing thymidine monophosphate (TMP). Thymidine monophosphate enters the nucleotide biosynthesis pathway, and undergoes several phosphorylation steps to become thymidine diphosphate (TDP) and eventually thymidine triphosphate (TPP). TTP is a key component of DNA synthesis, ensuring there are enough nucleotides available for koi herpesvirus replication. Thymidine kinase activity enables the virus to replicate more quickly and increase the viral load in the koi fish, leading to higher virulence. [6]

Herpesvirus infects many other animals, such as reptiles and mammals. They include in the subfamily Alphaherpesviridae—several symptom signs documented, such as stomatitis, conjunctivitis, and tumors. The analysis of herpesvirus from captive Chalonians in Europe during 2016 – 2020 [7]. With 4,797 samples, they found herpesvirus from captive chelonians 312 samples. Other herpes viruses infect cetaceans that include the subfamily Alphaherpesviridae and Gammaherpersviridae. The 966 tissues of cetaceans in Spain and 121 were tested positive [8]. It infected several tissues in cetaceans, such as the skin, urinary bladder, heart, and suprarenal glands.

Our study investigated the KHV infection cases occurrence in Malang and Batu city based on pathological changes found in deceased fish followed by partial thymidine kinase (TK) encoding gene analysis. As we could tell, there is no report of KHV detection performed in Malang and Batu City confirmed with polymerase chain reaction (PCR) and also we use thymidine kinase due to there are numerous references and gene databases, providing ample data for analysis. The genetic analysis of KHV TK sequences were aimed to predict the putative geographic origin of the KHV found in Malang and Batu City based on phylogenetic tree and haplotype network analysis.

2. Materials and Methods

2.1 Ethical Approval

This research has received ethical clearance from the Animal Care and Use Committee of Universitas Brawijaya (No. 085-KEP-UB-2023).

2.2 Study Period and Location

The study was conducted in June - July 2023 at the Laboratory of Veterinary Anatomic Pathology and Laboratory of Animal Disease Diagnostic, Faculty of Veterinary Medicine, Universitas Brawijaya.

2.3 Necropsy and Sample Collection

Five and two koi fish were submitted from local ponds in Malang and Batu City respectively in frozen condition. The fish were dissected in the Laboratory of Veterinary Anatomic Pathology, Faculty of Veterinary Medicine, Universitas Brawijaya to obtain gill tissues and other organs were inspected for any abnormality. All tissues predicted containing lesions were preserved in non-buffered 10% formalin for histopathologic examination. Those tissues were processed following histotechnique protocol in our laboratory.

2.4 Histopathology Preparation

The tissue processing was performed in routine protocol: fixation, trimming, dehydration, clearing, paraffin embedding and sectioning. Initially, the gill tissues were fixed in a 10% formalin for 24 hours. The tissues were sliced to approximately 0.5 cm thick. The tissues in the cassette were then dehydrated in series of ethanol with increasing concentration (70%, 80%, 85%, 90%, and 95%) followed by three immersions in absolute ethanol for one hour each. The clearing was done in xylenes three times for five minutes, five minutes, and 10 minutes, respectively. For paraffin embedding, the tissues were placed in melted paraffin wax three times for 60 minutes each inside an incubator set to 56°C. Embedding was completed by pouring liquid paraffin into metal molds to form tissue blocks. Finally, sectioning was performed using a microtome to cut the paraffin blocks into 5-micron thick sections, which were floated into a water bath and mounted onto glass slides.

For routine hematoxylin-eosin staining, following protocols were performed: deparaffinization with xylene three times for 20 minutes each, hydration in series of ethanol with decreasing concentrations for five minutes each, hematoxylin as primary staining for 15 minutes, decolorization with acid alcohol for four seconds, bluing in tap water for 20 minutes, counter staining with eosin for 10 minutes, dehydration with series of ethanol in increasing concentrations for five-seconds each, and finally clearing with xylene three times for 20 minutes each. At last, the slides were mounted with cover slip using Entellan® and observed under Olympus® CX-23 microscope using OptiLab® Advance Plus mounted camera. Pictures were taken in three magnifications: 40x, 100x, and 400x.

2.5 Genome Extraction

Six gill tissues were kept in a small bottle containing 70% ethanol representing specimens from Jakarta, Malang, and Batu City. The ethanol was evaporated by letting the tube open at room temperature for about 48 hours prior to DNA extraction performed. The DNA extractions were performed using the Wizard® Genomic DNA Purification Kit (Promega, USA) at Animal Disease Diagnostic (ADD) Laboratory, Faculty of Veterinary Medicine, Universitas Brawijaya following the manufactured protocol. The extracted DNA was then directly run for PCR without quantification or purification.

2.6 Amplification and Sequencing

We used a positive control for the diagnostic panel for KHV from Jakarta isolate that was previously confirmed through sequencing. The DNA isolates were put in PCR tubes and added PCR mix 5 µL (ThermoFischer) each tube for amplification. The 2.5 µL ddH2O were added in the tube and then DNA isolation samples 3 µL each tube. The two primers added on the PCR tubes were TK forward 1 µL (5'- GGG TTA CCT GTA CGA G – 3') and TK reverse 1 µL (5'- CAC CCA GTA GAT TAT GC -3') [9]. The suspensions were homogenized using a vortex mixer for 15 – 20 seconds. The amplifications were set up after a series of annealing temperature optimization in

following conditions: initial denaturation 95°C for three minutes and then 35 cycles of denaturation 95°C for 30 seconds, annealing 46°C for 30 seconds, elongation 72°C for 60 seconds and post elongation 72°C for five minutes. Amplicons were run in electrophoresis apparatus to visualize the amplicon bands. Amplicons with prominent specific bands were sequenced using Sanger sequencing method with Applied Biosystems® 3730XL with initial purification through gel extraction procedure.

2.7 Phylogenetic and Haplotype Analysis

Multiple sequence alignments were performed in MEGA 10.1.8 using ClustalW. The phylogenetic tree was constructed using maximum likelihood as statistical method with bootstrap method (1000 times replication) for test of phylogeny and Tamura 3-parameter (T92) for substitution model. Haplotype analysis was conducted using DnaSP v6.12.03 and NETWORK 10.2.0.0 to create haplotype network. For the median joining network analysis, we included TK sequences from NCBI Genbank with full coverage of our sequences consisting sequences from United Stated (NC_009127.1, HM347096.1, DQ657948.1, EU932923.1, MT914509.1, MK987087.1, MK987090.1, MK733801.1, MK733802.1), United Kingdom (HM347097.1, AB375389.1), Indonesia (HM347098.1, HM347099.1, HM347101.1, HM347102.1, HM347103.1, HM347104.1, HM347106.1, HM347107.1, HM347108.1, HM347109.1, HM347110.1, HM347111.1, HM347112.1, HM347113.1, KX544843.1, KX544844.1, KX544845.1, KX544846.1, KX544847.11, KX544848.1, HM347098, HM347110, HM347112), Iran (KX609546.1, KP280047, KT290517, KT364393, KX609547), Mexico (KY703866.1, KY703857.1, KY703858.1, KY703863.1, KY703867.1), China (JQ247182.1, JN180630.1, MG755640.1, KY703859.1, KY703860.1, JQ247183.1, KJ627438.1, KX777255.1, MK260013.1), Iraq (MW928743.1, MK817063.1), India (MZ475945.1), Israel (AJ535112.1, AB375386.1), Netherlands (AB375387.1, AB375388.1), Japan (AB458384.1), Poland (KX544842.1), Belgium (MG925485.1, MG925486.11, MG925487.1, MG925488.1, MG925489.1, MG925490.1, MG925491.1), Croatia (MN913971.1) and unknown origin (KP343683.1, KP343684.1, DQ177346.1, AP008984.1, AB375385.1, AB375390.1, AB375391.1). We also included a sequence from KHV 1 (DQ177346.1) as outgroup comparison.

2.8 Genetic Distance Analysis

We estimated the genetic distance among sequences from the NCBI Genbank database consisting of KHV 1, 2, and 3 to our specimens. We collected representative sequences from several distant regions in the world categorized as KHV 1, 2, and 3. The KHV 1 group sequences consist of KT587200.1 (Brazil), NC_019491.1 (Japan), KU672601.1 (USA), and KU672599.1 (UK). The KHV 2 group sequences consist of MN201961.1 (China), NC_019495.1 (China), and JQ815364.1 (Japan). The KHV 3 group sequences consist of MN913972.1 (Croatia), ON340600.1 (Czech), MK987097.1 (USA), KT364393.1 (Iran), MG755640.1 (China) and HM347101.1 (Jakarta). The distance estimation was computed in MEGA 11 using bootstrap method 1000 times as variance estimation method and Tamura-3 parameter as substitution model.

3. Results

3.1 Gross and Histopathological Findings

The most prominent and distinguished lesion in all collected specimens was located in gills where necrotic areas cover almost all lamellae racks with severe branchitis and occasional haemorrhage (Figure 1). Grossly, there were marked multifocal extensive pale white discolorations in the primary gill lamellae with thickening lamella tips presuming lamella epithelial hyperplasia. Complete whitening of gills might be associated with prolonged icy preservation, putrefaction, or decreased blood perfusion to the gills due to hypostasis. We found that the smaller and younger fish presented lesser gills necrosis with multifocal whitish fibrinous exudate plaques. In contrast, the larger fish tend to develop a vast area of necrosis before being found deceased. There is no specific anatomic alteration deemed as pathological change in other organs. Figure 1A and 1B showed multifocal plaques of exudates in the surface of secondary lamellae of younger fish which were lesser than what were encountered in adult one. Figure 1C and 1D presented a more extensive discoloration of

Figure 1. Gross changes of koi fish gills infected with KHV presented extensive white to yellow discoloration with no marked demarcation (asterisks). Note that various degrees of suspected gill necrosis were present in pre-adult fish from Malang City (A-B) and adult fish from Jakarta (C) and Batu City (D).

gill lamellae in adult fish which might indicate a broader tissue necrosis. The difference in the area of necrosis depends on the external factor, one of which is age-related differences in the immune system development. Younger fish tend to have an immature immune system compared to adult fish. This could affect how koi fish responds to viral infection. Younger fish might exhibit a weaker immune response, while adult fish might mount a stronger immune response, leading to more extensive necrosis.

Histopathology examination revealed severe damage of secondary lamellae of gills architecture (Fig. 2). Extensive haemorrhages were present along the primary lamellas indicated by elongated clusters of nucleated erythrocytes. Either primary or secondary lamella architecture apparently underwent necrosis with abundant inflammatory cell infiltration. Rupture and fusion of secondary lamellae were distinct especially in area adjacent to necrotic tissues.

3.2 Molecular Detection and Genetic Analysis of TK Encoding Gene

The amplified amplicons using suggested primers resulted in products sitting near to the 400 bp marker as depicted in Figure 3. Lane 1 and 2 were amplicons from KHV extracted Jakarta, lane 3 to 5 were from Malang, and lane 6 was from Batu City. Multiple bands were observed in lane 1, 4, and five while smears occurred in all amplicons except lane 6 which template was extracted in separate time. A single band in each lane confirmed as a fragment of TK encoding gene based on Sanger sequencing was present in various intensity indicating the amount of viral DNA extracted and load of virion in tissue collected might vary. Interestingly, amplicons in lane 1 and 4 showed multiple bands almost in exact size compared to markers which apparently was not present in other lane.

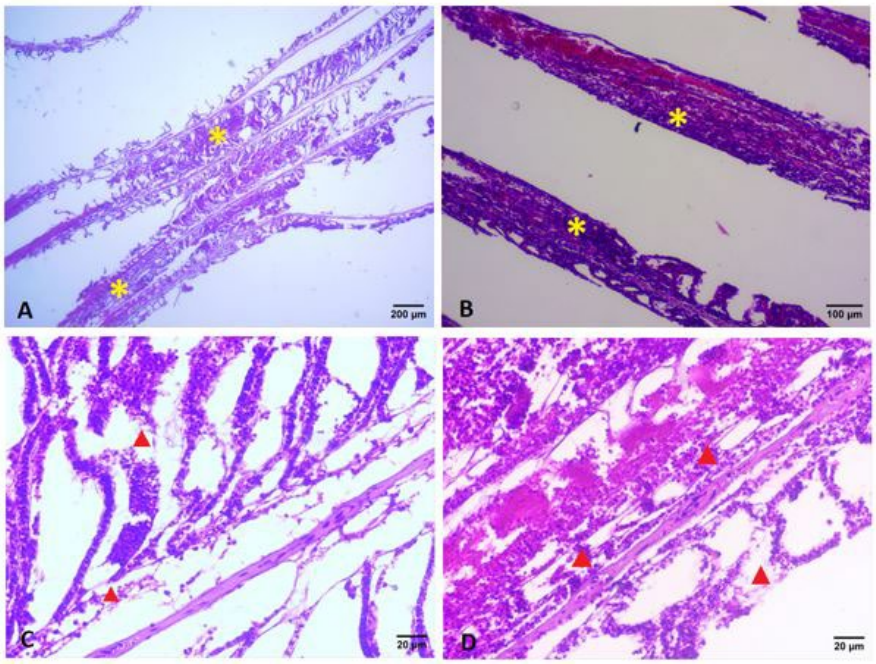

Figure 2. Necrotizing and hemorrhagic branchitis with secondary lamellar fusion (yellow asterisks) were found in all fish gills with white to yellow discoloration (Figure 2A, 40x magnification; Figure 2B 100x magnification). Extensive necrosis of primary and secondary lamellae (red arrow heads) messed the gill architecture to be tissue debris (Figure 2C-D, 400x magnification).

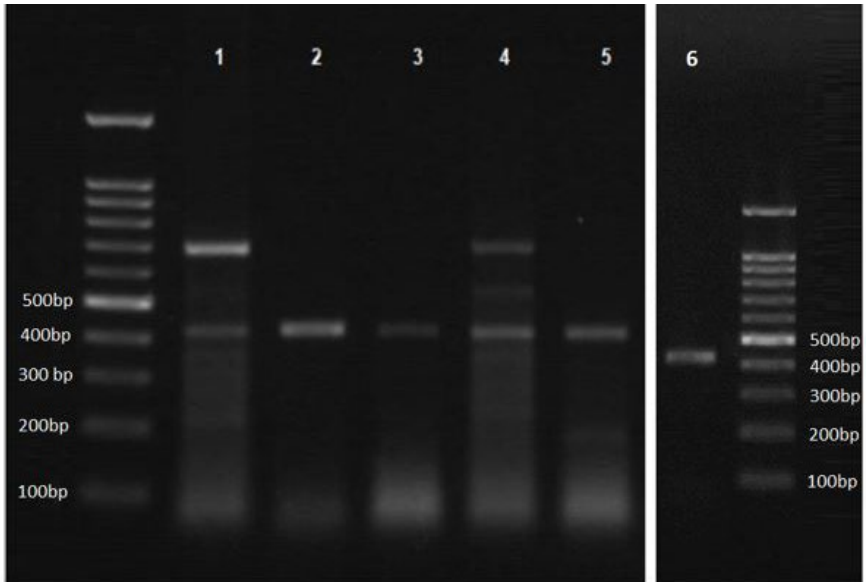

Figure 3. Presentation of TK encoding gene amplicons with 100 bp marker as reference in electrophoresis gel. Respectively from left to right lane were specimens from Jakarta (lane 1,2), Malang (lane 3,4,5) and Batu (lane 7). The desired TK encoding gene amplicons were located near to the 400 bp marker band.

We aligned the representative sequences of KHVs infecting koi fish in Jakarta, Malang, and Batu City with thirty sequences of TK encoding genes archived in NCBI Genbank. Our amplicons partially covered about 410 base pairs (bp) of nucleotides of a total about 998 bp length nucleotides of TK encoding gene that matched to KHV 3 TK encoding gene sequences from NCBI Genbank. These amplicons cover the nucleotide region from 358 to 458 of complete TK encoding genes. Our metaanalysis based on phylogenetic analysis and genetic distance among sequences

concluded that only slight genetic distance (0.00-0.04) observed among them indicating a high conserved gene even from about a decade ago archived sequences. The phylogram of our amplicons compared to NCBI Genbank TK encoding gene sequences is presented in Figure 4. Our analysis in haplotype diversity based on TK encoding gene sequence presented a major cluster grouping Indonesia, United State, Poland, Netherland, Iran, Israel, Japan, United Kingdom, Belgium, some of Mexico and China sequences into a single haplotype (Figure 5). Several sequences from Mexico, China, and Iran were grouped to separate smaller haplotype groups. The genetic distance estimation among KHV 1, 2, 3 and our specimens also presented a distinct distance especially between KHV 3 group and other groups (Table 1). The genetic distance between KHV 3 group (where our specimens were included) and KHV 2 group ranges from 0.65 to 0.68 and

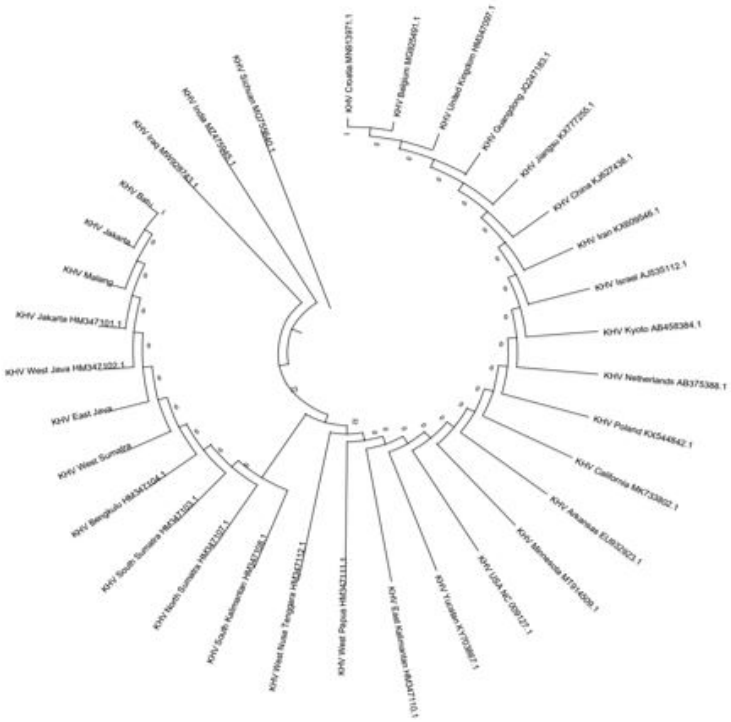

Figure 4. Phylogram of partial sequences of TK encoding gene of KHV 3 compiled from this study and archival sequences in NCBI Genbank.

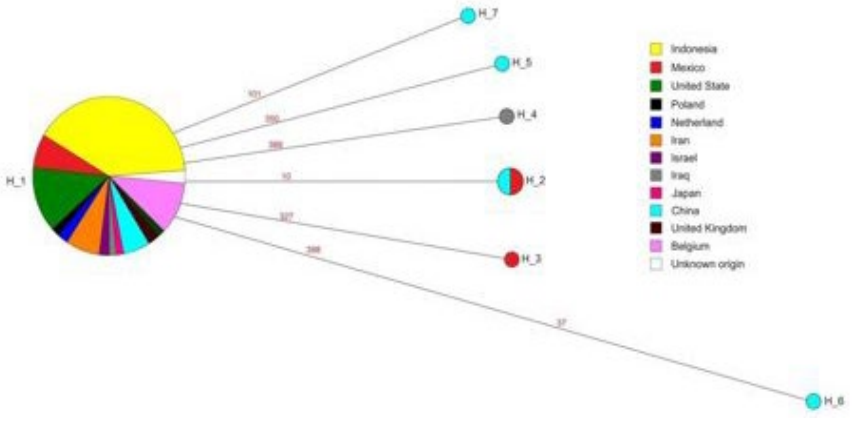

Figure 5. Haplotype Analysis of TK1 Encoding Gene

apparently were more closely related genetically to KHV 1 (0.58-0.59). The KHV 1 and 2 showed more distant relatedness rather than to KHV 3 (0.71-0.73).

Marked pathological changes were not present in other internal organs in our cases, though theoretically, the viruses must be able to travel to all organs [12]. This might be due to the lower load of KHV in other organs which was not capable of creating grossly noticeable lesions. The breeding ground in gill tissues might also induce anoxic death before other organs developed lesions. Pivotal organ that we prioritized to be inspected was kidneys since these organs provide osmoregulation ability for fish to maintain their fluid homeostasis within [13]. Freshwater fishes tend to uptake more water from the environment due to higher cytoplasmic fluid osmotic pressure than freshwater around, allowing the solvent passively diffuses to the interior part of fishes' body cavity. On a regular basis, kidneys, gills, and integuments maintain the water transport to tackle this osmotic dynamic of fluid between inner and outer body environment [14]. In case of compromised renal function, a failure of regulating the water volume in the fish body might happen followed by passive diffusion of solvent from the outer environment to fishes' body cavity. This flooding solvent creates distension inside the body cavity and shapes the fish body to be swelling tear drop-like presentation called dropsy syndrome [15]. Though this symptom might not be specific to KHV clinical signs, any renal damage inducing fluid build-up in the body cavity will end in this presentation. There was no evidence of body distension or fluid build-up in the body fish cavity in our cases, indicating that perhaps the fish died not due to systemic organ failure but rather to acute anoxic condition due to gill tissue necrosis.

Table 1. Genetic distance estimation of KHV 1, 2, 3 from NCBI Genbank database and our specimens

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	KHV_Jakarta_HM347101.1															
2	KHV_Malang	0.00														
3	KHV_Batu	0.00	0.00													
4	KHV_Jakarta	0.00	0.00	0.00												
5	KHV_3_China_MG755640.1	0.00	0.00	0.00	0.00											
6	KHV_3_Iran_KT364393.1	0.00	0.00	0.00	0.00	0.00										
7	KHV_3_USA_MK987097.1	0.00	0.00	0.00	0.00	0.00	0.00									
8	KHV_3_Czech_ON340600.1	0.00	0.00	0.00	0.00	0.00	0.00	0.00								
9	KHV_3_Croatia_MN913972.1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00							
10	KHV_2_Japan_JQ815364.1	0.67	0.67	0.67	0.67	0.66	0.67	0.67	0.67	0.67						
11	KHV_2_China_NC_019495.1	0.67	0.67	0.67	0.67	0.66	0.67	0.67	0.67	0.67	0.00					
12	KHV_2_China_MN201961.1	0.65	0.65	0.65	0.65	0.64	0.65	0.65	0.65	0.65	0.00	0.00				
13	KHV_1_UK_KU672599.1	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.73	0.73	0.73			
14	KHV_1_USA_KU672601.1	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.71	0.71	0.71	0.02		
15	KHV_1_Japan_NC_019491.1	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.73	0.73	0.73	0.00	0.02	
16	KHV_1_Brazil_KT587200.1	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.71	0.71	0.71	0.03	0.01	0.03

4. Discussions

Our pathological examination presented identical findings to all reported KHV cases and supported that the viruses might harbour through gill tissues as initial breeding ground. Gill secondary lamellae epithelial cells apparently play a role as harbouring port of KHV to enter the systemic circulation. Assuming a similar mode of replication to other endotheliotrophic herpesviruses [10], KHV might find the shortest route to access endothelial cells to start initial replication at the site of infection. The overload volume of replicated virion parts and assembly might distend the endothelial cells causing cell lysis while virion release occurred and triggered haemorrhages to what was typically found in acute KHV-infected fishes. Soon the ischemic condition developed, most of gill tissues were degenerated and underwent necrosis leaving white anaemic dead tissues appearance as present in most gill necrosis events. The discoloration area might vary based on load virus or chronicity of the infection, but our findings showed that the adult fish tend to present wider extensive and multilamellar gill necrosis compared to the juveniles. Several anthropogenic interventions such as freezing might affect the coloration of gill tissues due to icing [11], however, typical discoloration of gill necrosis remained distinguishable.

The TK encoding gene of KHV 3 sequences evidently provide a conserved region with low potential of mutation rate and nucleotide motive diversity. Amplification of TK encoding genes from our specimens showed high similarity to KHV 3 based on obtained partial sequence alignment to the NCBI Genbank database. This might indicate that there is no meaningful point mutation happening in at least the last decade since the oldest TK encoding gene of KHV 3 reference published in the Genbank database among regions worldwide. It is supported by low genetic distance among sequences, however, full-length TK encoding gene sequence alignment of the latest cases might provide a more comprehensive dynamic of TK sequence nucleotides. This feature also makes TK encoding gene fitting as a marker for KHV detection including for discrimination of KHV 1, 2, and 3. We found that there is no distinct grouping of haplotype based on KHV TK encoding gene sequences of KHV 3, supported by the phylogram that placed our sequences in a single monophyletic cluster with KHV isolates from Indonesia. Six haplotypes were separate from the large cluster one including TK sequences from twelve countries; however, considering the genetic distance and nucleotide diversity, this might not significantly affect the viral properties or impact to the hosts [16].

The presence of a conserved encoding gene might indicate the existence of a conserved protein as a target for a detection system or an antigen to generate antibodies or a vaccine. However, specificity and sensitivity of the developed KHV test or experimental vaccine employing the TK encoding gene are not largely explored. Several experimental vaccines such as live attenuated vaccines, inactivated vaccines, and live recombinant vaccines have been developed though there is only one commercial live attenuated KHV vaccine produced by a company in Israel that is applied to common carp. However, the vaccine efficacy was reduced in small carp less than 50 g due to virulence factor [17].

Other studies find effective and efficient vaccines for KHV with deletion in some open reading frames or employing recombinant proteins encapsulated in various materials. A Study by Klafack *et al*. (2022) [16], used a wild-type koi herpesvirus with alginate-encapsulated and the deletion of ORF150. The KHV is capable of interfering with the signal cascade in innate immunity and the vaccine boosted the gene expression of NF-κB as a signal for releasing pro-inflammatory cytokines such as IL-1 and IL-6. Furthermore, it could call out the immune system like macrophages from the blood vessel (monocyte form) and evolve in the tissue to eliminate the virus. A study by Huang *et al*. (2021) [18], used ORF81 of KHV delivered in a chitosan-alginate capsule. This experimental vaccine substantially induced IgM production with KHV-neutralizing activity and provided 85% protection. A study by Hu *et al*. (2020) [19], used ORF149 of KHV packed in a carbon nanotube medium that gave a protective effect of 81.95% against KHV challenge.

Genomic recombination is not yet reported among herpesvirus strains, however it is not improbable considering the nature of viral genome dynamics. Currently, the recombination rate of herpesvirus strains in the family Alloherpesviridae is low. We did not find any articles to support the recombination in strains belonging to the family Alloherpesviridae. However, recombination has occurred in the family Alphaherpesviridae. There is evidence of recombination in Equine Herpesvirus 1 (EHV-1) and EHV-4 [20]. Both herpes viruses are genetically similar, but they have different pathogenesis. A study by Vaz *et al*. (2016) [19], found that recombination events were observed in EHV-4 and only one event was observed in Equine EHV-1. The recombination rate of herpesvirus strains is low because the genome of herpesvirus is DNA. It has low nucleotide substitution and is stable in normal conditions.

The success of vaccination is indicated by the ability to induce humoral immunity. Humoral immunity prevents infection by blocking the virus from entering the host cells and neutralizing the epitope of the virus [21]. The TK encoding gene, a part of KHV genome, has been used to determine KHV infection and has become a highly sensitive diagnostic tool for KHV. In this research, we found that there are similarities among KHV genome sequences from Indonesia, Iran, and China using the gene encoding thymidine kinase. Thus, we assume that using the TK encoding gene could be a good candidate for vaccine development. Thymidine kinase protein can stimulate both cell-mediated immunity through T lymphocytes, mediated by dendritic cells, and humoral immunity through B lymphocytes mediated by internalization of thymidine kinase protein. Cell-

mediated immunity has two main functions, eliminating infected cells by cytotoxic T lymphocytes (CD8+) and promoting the formation of long-live antibodies within the assistance of helper T lymphocytes (CD4+). Humoral immunity is responsible for producing long-live antibodies in collaboration with helper T lymphocytes. However, it needs further investigation to illuminate the protective capability of the vaccine against KHV.

5. Conclusions

We reported that there was no distinct grouping of haplotypes based on KHV TK encoding gene sequences. The TK encoding gene sequences evidently provide conserved regions with a low mutation rate and nucleotide motif diversity. The presence of a conserved encoding gene theoretically fits as a target for a detection system or as an antigen to generate antibodies or vaccines. This study strengthens that TK encoding gene is plausible as a candidate for marker either in multistrain KHV detection and or vaccine development.

Availability of Data and Materials

The data are already included as part of the submitted manuscript.

Author Contribution

Conceptualization, A.B.H.; Methodology and Investigation, A.B.H., G.W.A., N.A.C., H.P.U., D.F.R., and D.R.Z.N.; Writing – Original Draft, A.B.H. and G.W.A.; Writing – Review & Editing, A.B.H. and G.W.A.

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Conflict of Interest

The authors declare no conflict of interest.

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(Original Research)

First Report on Antibiotic-Resistant *Pseudomonas* Species Isolated from Tilapia Aquaculture in Sampaloc Lake, San Pablo City, Laguna, Philippines

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Abstract

Background: Tilapia aquaculture in Sampaloc Lake significantly contributes to the livelihood and food security of the communities in San Pablo City, Laguna. Early detection of pathogens and antibiotic-resistant bacteria that could potentially affect tilapia production and the health of the consumers is essential to avoid outbreak of infections. **Methods:** The study identified 16 selected bacterial strains from tilapia fish in Sampaloc Lake through a polyphasic taxonomic approach in bacterial identification. **Results:** The bacterial strains are Gram-negative, aerobic, non-fermentative bacteria, and most produce fluorescent green pigment. Through 16S rRNA gene sequencing, they were identified as *Pseudomonas* species, particularly *P. entomophila* (n=2), *P. guariconensis* (n=3), *P. mosselii* (n=6), *P. oryzihabitans (n=1) P. soli* (n=2) and *P. wadenswilerensis* (n=2). They all exhibited resistance against Ceftazidime (30µg) and most were resistant to about two to four out of the eight antibiotics tested through the Kirby-Bauer disk diffusion method. *Pseudomonas mosselii* were described as a fish pathogen; thus, the addition of their antibiotic resistance could serve as threat in Tilapia aquaculture. **Conclusion:** The result of the study could facilitate the formulation of evidence-based mitigating measures and policies aimed at safeguarding public health ensuring the socio-economic stability of Sampaloc Lake, including its resources.

Keywords

AMR, *Pseudomonas*, Pathogen, Food safety

1. Introduction

Several published studies have been done in other parts of the Philippines on the detection of pathogens in clinical settings, but the microbiological state of aquaculture has never been fully monitored with respect to emerging pathogens. Fish and fish products are considered a fundamental part of the human diet due to their high nutritional value. The incidence, associated mortality, and negative economic repercussions make food-borne diseases to be considered a major public health challenge worldwide. Contamination by pathogenic microorganisms at any stage in the food chain may lead to poor food safety [1]. This also highlights the potential of these pathogens to be transmitted to the marketplace, where they pose a threat to consumers. Isolation of pathogenic microorganisms from environmental samples such as meat products has been done by the proponent in the past. In

fact, a paper on the prevalence of *Escherichia coli* O157:H7, the first publication on the particular strain in the Philippines, had been authored by the proponent in 2013 [2]. These pathogens may be present in the water and may pose a threat to the overall health. Water and foodborne pathogen contamination in ambient water bodies and related diseases are a major health concern throughout the world. Pathogen contamination is a serious issue, making its recognition and understanding essential [3].

The Seven Lakes of San Pablo, situated in the bustling city of San Pablo, were formed through a phreatic eruption of Mt. San Cristobal, where steam-heated eruptions formed crater-like depressions that were eventually filled with rainwater, resulting in the formation of the seven lakes. The resulting seven lakes are now economically important as a source of food, in aquaculture, and as tourism spots. However, these activities are known to have impacted the microbiological state, especially on the prevalence of emerging and potentially zoonotic pathogens in other known lakes. One of the well-known bacterial species that are pathogenic for humans, animals, and aquatic organisms belongs to the genus *Pseudomonas* [4]. They are regarded as one of the most common bacterial infectious agents of aquaculture, such as Tilapia, and have been reported to cause stress-related diseases in fish, especially under farming conditions [5]. It was estimated that up to 50% of farms fish are lost due to bacterial infection before their marketing. *P. aeruginosa* is autochthonous to fish; however, under stressful conditions, this may cause diseases such as ulcerative syndrome and hemorrhagic septicemia, which may cause big losses in farmed fish, such as tilapia. Moreover, detection of *Pseudomonas* species (spp.) is necessary for quick diagnosis, outbreak prediction, and mitigation measures in aquaculture. Polyphasic approaches, especially with the use of rapid molecular and conventional biochemical tests, will aid in the quick, reliable, and accurate prevalence determination, isolation, and characterization of bacterial pathogens such as Pseudomonads. Attempts for detection of *Pseudomonas* spp. including the opportunistic *P. aeruginosa* were considered not only for its economic impact but also for its public health importance. Consumption of raw fish and its byproducts may cause *Pseudomonas* infection in humans [6]. Furthermore, typical colonies of *P. aeruginosa* from fish are closely related to those isolated in hospitals causing disease in humans [7].

Addressing the microbiological status of the Tilapia aquaculture in Sampaloc Lake by means of sound policy grounded on evidence will ensure food and health safety standards, ultimately benefiting the economic status of the people and the lake's economic potential. Detection of potentially pathogenic microbes such as *Pseudomonas* spp. will serve as an indication of the impacts of human activities on the lake and, at the same time, assess the microbiological safety of the aquaculture. Pertinent intervention programs and policies to address this problem of water quality and environmental impact can be formulated through science communication and participatory and collaborative approaches among the research, community, and local leaders.

2. Materials and Methods

2.1 Study Area and Sampling Sites

Sampaloc Lake is one of the seven crater lakes in San Pablo City, Laguna, located near the town proper. It has four clusters of aquacultures following the 14% lake surface area limit recommended in the Laguna Lake Development Authority (LLDA) Memorandum Circular 2017-03 [8]. It is surrounded by residential and commercial areas as well as a hospital. The study collected tilapia fish samples, particularly from the fish pens and fish cages in Sampaloc Lake. Sampling points were established in the aquaculture, particularly near the residential areas (Site 1), near the San Pablo City District Hospital (Site 2), and near the lake outlet (Site 3), where exposure to anthropogenic activities is relatively high, as shown in Figure 1.

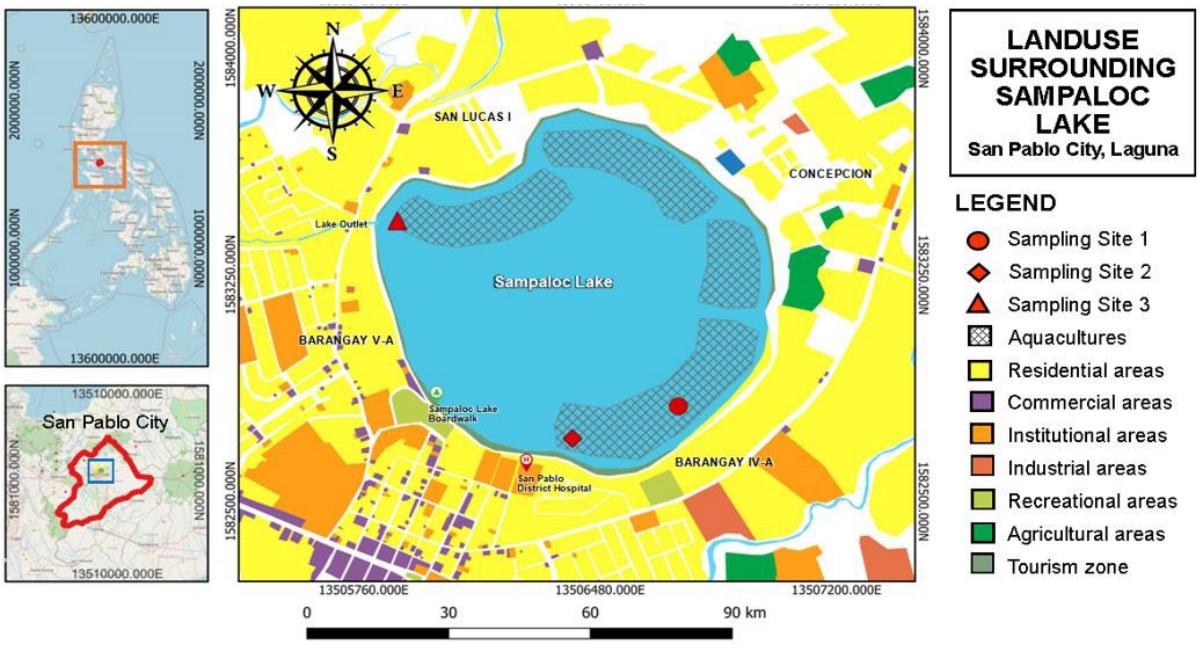


Figure 1. Study area and sampling site in Sampaloc Lake, San Pablo City, Laguna.

2.2 Sample Collection and Preparation

In the Philippines, explicit guidelines for determining sample sizes in aquaculture studies, such as those conducted by Tanyag *et al.*, 2021 [9], are not established. Internationally, the World Organization for Animal Health (WOAH) recommends sample sizes of 30, 60, and 150 fish to detect diseases with minimum expected prevalences of 10%, 5%, and 2%, respectively [10]. However, strictly following these standards can be challenging due to feasibility constraints, including laboratory capacity and budget limitations. Instead, the study used purposive sampling technique to potentially detect antimicrobial resistant (AMR) *Pseudomonas* spp. from the tilapia aquaculture that were highly exposed to anthropogenic activities. A total of twelve fish samples were collected from the sampling sites during the dry season (n=7) which were collected in December 2023, and in the wet season (n=5) in July 2024. Specifically, during the dry season, fish samples were collected randomly from the three aquacultures: two from Site 1, three from Site 2, and two from Site 3. Due to the unavailability of tilapia fish from the previous sampling sites during the collection time in wet season, five individuals that were grown both in Site 1 and Site 2 were obtained. Using a nylon fishing net they were immediately transferred individually into sterile polyethylene bags. The samples were placed in bins containing enough crushed ice to maintain the fresh state of the samples and to promote slow bacterial growth for microbiological analysis [11]. Samples were transported immediately to the laboratory and then processed within two hours after collection.

Gills, flesh, and guts of each Tilapia fish were obtained through dissection and then homogenized per sample using mortar and pestle on a sterile polyethylene bag. Pre-enrichment was done by transferring 25 g of homogenized samples to 225 mL of Tryptic Soy Broth (TSB) for the propagation of *Pseudomonas* spp. and incubated at 30°C for 24 hours. After the incubation, serial dilution was performed tenfold up to 10^{-5} using 1% peptone water as diluent and spread plated in duplicates on Pseudomonas CFC Agar or Pseudomonas Agar Base (Thermo Fisher Scientific, Oxoid) supplemented with cetrimide, fucidin, and cephalosporin (CFC). It is a selective culture medium that promotes the growth of *Pseudomonas* spp. from meat, water, and environmental samples. It consists of 16 g of gelatin peptone, 10 g of casein hydrolysate, 10 g of potassium sulfate, 1.4 g of magnesium chloride, 11 g of agar, and 10 mL of glycerol per liter. It is supplemented with 10 mg

of cetrimide, 10 mg of fucidin, and 50 mg of cephalosporin per liter to inhibit the growth of other microorganisms other than *Pseudomonas* spp. The inoculated petri dishes were incubated at room temperature (20-25°C) for 24 hours, wherein green, blue-green, yellow, and brown pigments were observed after incubation.

2.3 Isolation of *Pseudomonas* spp.

According to the manufacturer's description of Pseudomonas CFC Agar, it is a selective medium used for the isolation of *Pseudomonas* spp. wherein the colonies that may grow are suspected to be the members of the said genus. Moreover, presumptive *Pseudomonas* spp. colonies were characterized on the said selective medium as colonies having soluble and agar-diffusing colored pigments such as pyocyanin (blue green), pyorubin (red), and pyomelanin (brown), or with fluorescence under UV light after the incubation of 24-48 hours at 25°C. Isolated colonies that were observed with such characteristics were streaked for isolation and purified on the same selective medium. However, those that did not produce pigments were streaked and purified on Plate Count Agar (PCA) for further characterization. Isolate codes were established in the format of lake name initials: Sampaloc (SP), season of sampling (D: dry season or W: wet season), site number (1-3), type of sample (F: fish), number of sample, and the number in order of isolation. Fish samples in wet season were picked in between Site 1 and Site 2, thus site numbers were not used.

2.4 Phenotypic Characterization

As reference for phenotypic characterization, *P. aeruginosa* BIOTECH 1335 was purchased from the Philippine National Collection of Microorganisms (PNCM) in National Institute of Molecular Biology and Biotechnology (BIOTECH). Characterization of the cell and cultural morphology of the bacterial isolates were performed describing their Gram-reaction, cell shape, colony color, elevation, texture, shape and margin. Moreover, fluorescence of the isolated bacterial isolates was observed under the ultra-violet light having a wavelength of 302-365 nm using MaestroGen UV Transilluminator. Fluorescent isolates were considered as putative members of *Pseudomonas* spp.

2.5 Differentiation of *Pseudomonas* spp. from other Gram-negative bacteria

Although the culture medium that was used was selective for *Pseudomonas* spp., few enteric and Gram-negative bacteria other than the species of *Pseudomonas* may grow on Pseudomonas CFC Agar appearing as non-fluorescent, pigmented, or nonpigmented colonies. The Triple Iron Sugar (TSI) test was performed to differentiate putative *Pseudomonas* spp. from other Gram-negative bacteria based on the ability to ferment sugars; glucose, lactose and sucrose. As *Pseudomonas* spp. are non-fermenting bacteria with some species that can produce Hydrogen sulfide (H_2S) aerobically, they can be distinguished from other Gram-negative, facultative anaerobic and fermentative bacteria when inoculated on TSI agar and incubated at 37°C for 24 hours. *Escherichia coli* BIOTECH 1095, *Salmonella typhimurium* BIOTECH 1826 and *P. aeruginosa* BIOTECH 1335 were used as the control organisms. *P. aeruginosa* were expected to have K/K or alkaline over alkaline and no gas production on TSI agar referring to not being able to ferment and produce acids or gas from the sugars contained in the culture medium [11]. Black precipitates on TSI agar may possibly be observed on the surface and refers to H_2S production due to the formation of H_2S from ferric ammonium citrate and sodium thiosulfate. The use of *E. coli* and *S. typhimurium* as controls, which are both members of Enterobacteriaceae, could clearly differentiate *Pseudomonas* spp. from their reaction on TSI agar. *E. coli* was expected to have A/A or acid over acid reaction (yellow slant, yellow butt, gas positive, and no H_2S produced) while *S. typhimurium* would show red slant, yellow butt, gas positive and H_2S produced. For the detection of *Pseudomonas* spp. among the isolates, no change in color, no gas production and with or without black precipitates on the surface of the agar must be observed as the same reaction of *P. aeruginosa* on TSI agar. Those bacterial isolates that were observed having similar reaction to that in *E. coli* and *S. typhimurium* were excluded in the study and referred them as members of the Enterobacteriaceae, a family of gram negative, non-spore-forming and fermentative bacteria

commonly found in the gut [13]. *Pseudomonas* spp. are aerobic in nature and most are oxidase positive (except *P. luteola and P. oryzihabitans*) and catalase positive [14], thus the tests for oxidase and catalase were essential to further narrow down the number of bacterial isolates targeting *Pseudomonas* spp.

2.6 Presumptive Test for *Pseudomonas aeruginosa*

All the isolated bacteria with the ability to produce fluorescent pigments and did not ferment glucose or produce H_2S were tagged as the possible putative strains of *Pseudomonas* spp. To further differentiate Pseudomonads and determine if there are possible strains of *P. aeruginosa* among the isolates, incubation at 42°C of all putative *Pseudomonas spp.* isolates were performed in duplicates, as *P. aeruginosa* is the only species of *Pseudomonas* that could grow well at 42°C [14]. Suspected *P. aeruginosa* isolates were inoculated in acetamide broth in duplicates to confirm their identity following UNI EN ISO 16266:2008. As the said species can deaminate acetamide into ammonia that results in an increase of pH thus the change of color from yellow to purplish red is indicative of positive results for the identification as *P. aeruginosa* [15]. After denoting some of the phenotypic characteristics of the genus *Pseudomonas* on the isolated bacteria, they were streaked on nutrient agar as stock culture in plates or slants and preserved 40% glycerol stocks and stored in the freezer at -18°C.

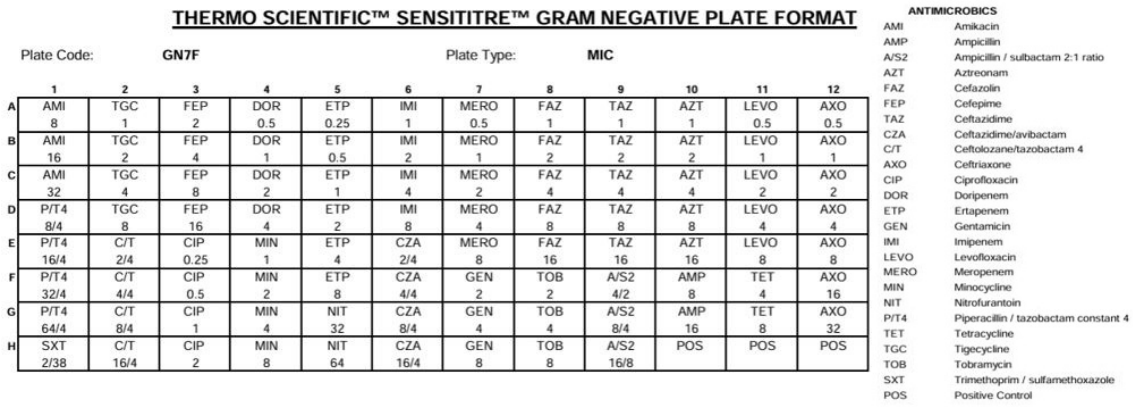
2.7 Antibiotic Susceptibility Test

All the isolated putative *Pseudomonas* spp. were screened in triplicates for their susceptibility and resistance to different antibiotics following the Kirby-Bauer Disk Diffusion (KBDD) method [16]. Selected antibiotics that were used were according to the 2024 Clinical and Laboratory Standards Institute (CLSI) guidelines for primary testing panel and routine reporting of antibiotic susceptibility test for *P. aeruginosa* [17] (Table 1).

Table 1. Antimicrobial agents and zone diameter breakpoints used for Kirby-Bauer Disk Diffusion antimicrobial susceptibility test.

Antibiotic Class	Antimicrobial agent	Disk Content	Interpretive Categories and Zone Diameter Breakpoints, nearest whole millimeter (mm)		
			Susceptible	Intermediate	Resistant
ß-lactam combination agents	Piperacillin-tazobactam	100/10 µg	≥21	15-20^	≤14
Cephems	Ceftazidime	30 µg	≥18	15-17^	≤14
	Cefepime	30 µg	≥18	15-17^	≤14
Carbapenems	Imipenem	10 µg	≥19	16-18^	≤15
	Meropenem	10 µg	≥19	16-18^	≤15
Aminoglycosides	Tobramycin	10 µg	≥15	13-14^	≤12
Fluoroquinolones	Ciprofloxacin	5 µg	≥25	19-24^	≤18
	Levofloxacin	5 µg	≥22	15-21^	≤14

For the precise and accurate acquisition of minimum inhibitory concentration (MIC) values of the antibiotics against the AMR *Pseudomonas* spp., Sensititre™ MIC Plate System (Thermo Fisher Scientific Inc.) that was provided by Scientific Biotech Specialties, Inc. (SBSI) was used in a single run following the manufacturer's protocol and guidelines using the GN7F Sensititre MIC Plate (Fig. 2). It is an automated broth microdilution antibiotic susceptibility test (AST) system that was found to be comparable to a reference broth dilution method. The MIC breakpoints (µg/mL) and interpretation based on CLSI 2024 were shown in Table 2. Results were viewed digitally with interpretations: green-colored wells indicate that the bacteria are "susceptible" to the antibiotics, while yellow indicates



THERMO SCIENTIFIC™ SENSITITRE™ GRAM NEGATIVE PLATE FORMAT

Plate Code: GN7F Plate Type: MIC

	1	2	3	4	5	6	7	8	9	10	11	12
A	AMI 8	TGC 1	FEP 2	DOR 0.5	ETP 0.25	IMI 1	MERO 0.5	FAZ 1	TAZ 1	AZT 1	LEVO 0.5	AXO 0.5
B	AMI 16	TGC 2	FEP 4	DOR 1	ETP 0.5	IMI 2	MERO 1	FAZ 2	TAZ 2	AZT 2	LEVO 1	AXO 1
C	AMI 32	TGC 4	FEP 8	DOR 2	ETP 1	IMI 4	MERO 2	FAZ 4	TAZ 4	AZT 4	LEVO 2	AXO 2
D	P/T4 8/4	TGC 8	FEP 16	DOR 4	ETP 2	IMI 8	MERO 4	FAZ 8	TAZ 8	AZT 8	LEVO 4	AXO 4
E	P/T4 16/4	C/T 2/4	CIP 0.25	MIN 1	ETP 4	CZA 2/4	MERO 8	FAZ 16	TAZ 16	AZT 16	LEVO 8	AXO 8
F	P/T4 32/4	C/T 4/4	CIP 0.5	MIN 2	ETP 8	CZA 4/4	GEN 2	TOB 2	A/S2 4/2	AMP 8	TET 4	AXO 16
G	P/T4 64/4	C/T 8/4	CIP 1	MIN 4	NIT 32	CZA 8/4	GEN 4	TOB 4	A/S2 8/4	AMP 16	TET 8	AXO 32
H	SXT 2/38	C/T 16/4	CIP 2	MIN 8	NIT 64	CZA 16/4	GEN 8	TOB 8	A/S2 16/8	POS	POS	POS

ANTIMICROBICS

AMI	Amikacin
AMP	Ampicillin
A/S2	Ampicillin / sulbactam 2:1 ratio
AZT	Aztreonam
FAZ	Cefazolin
FEP	Cefepime
TAZ	Ceftazidime
CZA	Ceftazidime/avibactam
C/T	Ceftolozane/tazobactam 4
AXO	Ceftriaxone
CIP	Ciprofloxacin
DOR	Doripenem
ETP	Ertapenem
GEN	Gentamicin
IMI	Imipenem
LEVO	Levofloxacin
MERO	Meropenem
MIN	Minocycline
NIT	Nitrofurantoin
P/T4	Piperacillin / tazobactam constant 4
TET	Tetracycline
TGC	Tigecycline
TOB	Tobramycin
SXT	Trimethoprim / sulfamethoxazole
POS	Positive Control

Figure 2. Thermo Fisher Sensititre GN7F Plate (Reproduced with permission from Scientific Biotech Specialties, Inc.).

Table 2. Antimicrobial agents and the minimum inhibitory breakpoints used in Sensititre broth microdilution susceptibility testing system.

Antibiotic Class	Antimicrobial agent	Interpretive Categories and MIC Breakpoints, µg/mL		
		Susceptible	Intermediate	Resistant
ß-lactam combination agents	Piperacillin-tazobactam	≤ 16/4	32/4–64/4^	≥ 128/4
	Ceftazidime-avibactam	≤ 8/4	–	≥ 16/4
	Ceftolozane-tazobactam	≤ 4/4	8/4^	≥ 16/4
Cephems	Ceftazidime	≤ 8	16^	≥ 32
	Cefepime	≤ 8	16^	≥ 32
Monobactams	Aztreonam	≤ 8	16^	≥ 32
Carbapenems	Doripenem	≤ 2	4^	≥ 8
	Imipenem	≤ 2	4^	≥ 8
	Meropenem	≤ 2	4^	≥ 8
Aminoglycosides	Tobramycin	≤ 1	2^	≥ 4
	Amikacin	≤ 16	32^	≥ 64
Fluoroquinolones	Ciprofloxacin	≤ 0.5	1^	≥ 2
	Levofloxacin	≤ 1[1]	2^	≥ 4

"intermediate" and red means that the bacteria are "resistant." The exact MIC values can be provided by the said system, eliminating the potential for manual reading errors. The isolates that were reported as AMR were then subjected to molecular identification.

2.8 Identification via 16S rRNA Gene Sequencing and Phylogenetic Analysis

The DNA of the AMR isolates were extracted using Vivantis DNA Extraction Kits following the manufacturer's instructions and guidelines. NanoDrop spectrophotometer and agarose gel electrophoresis were used to evaluate the quality and quantity of the isolated DNA. Amplification through polymerase chain reaction (PCR) of the 16S ribosomal RNA gene were performed using the primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT). PCR amplicons were sent to Kinovett Scientific Solutions Co. for 16S rRNA gene Sanger sequencing using 785F (GGATTAGATACCCTGGTA) and 907R (CCGTCAATTCMTTTRAGTTT). The obtained sequences were assembled using Bioedit v7.7 and analyzed through NCBI Basic Local Alignment Search Tool (BLAST) database with parallel identification using Pseudomonas Genome Database to determine if the isolates belong to Pseudomonads. Sequence alignment and construction of the phylogenetic tree were performed using MEGA 11 revealing its tentative species identity and the closest species related to the unknown bacterial isolates.

3. Results

3.1 Characterization of Bacterial Isolates

Upon the targeted isolation of *Pseudomonas* spp., a total of 151 bacterial isolates were initially obtained from the fish samples (dry season, n = 96; wet season, n = 55). All the bacterial isolates were subjected to fluorescence observations using a MaestroGen UV Transilluminator at 302-365 nm. Eventually, out of 151 bacterial isolates, only 21 bacterial isolates have observed fluorescent colonies. Figure 3 shows two of the fluorescent bacterial isolates that were initially considered as putative *Pseudomonas* spp. Fluorescence of the isolates varies from green (Fig.3B) to blue-green (Fig. 3C) colored fluorescence with diffusible pigments in color yellow-green or brown on Pseudomonas agar base supplemented with cetrimide, fucidin, and cephalosporin (Pseudomonas CFC Agar).

Through the TSI test, as shown in Figure 4. A, the suspected *Pseudomonas* spp. isolates were determined with having no fermentation reactions as in *P. aeruginosa* BIOTECH 1335 (labeled as PA) and distinct reaction from the other control organisms from Enterobacteriaceae, *E. coli* BIOTECH 1095 (EC) and *S. typhimurium* BIOTECH 1826 (ST). Fermentation, gas and H_2S production were observed on 127 isolates as shown on TSI agar reaction in Fig. 4.B. and were suspected of being members of Enterobacteriaceae or other fermentative Gram-negative bacteria. These isolates were then excluded from the experiment, leaving a total of 24 suspected *Pseudomonas* spp. consisting of 21 fluorescent and 3 non-fluorescent bacterial isolates.

All the 24 presumptive *Pseudomonas* isolates were incubated at 42°C to determine if there were bacterial strains belonging to *P. aeruginosa*, since it is the well-known species of *Pseudomonas* that could grow well at the said temperature. The results revealed that all bacterial strains were able to grow at 42°C, thus they were all subjected to further presumptive tests, specifically, acetamide utilization test. Moreover, only one strain had a positive result for acetamide utilization which was the Isolate SPD-S3F1-15 and was considered presumptively as a strain of *P. aeruginosa.*

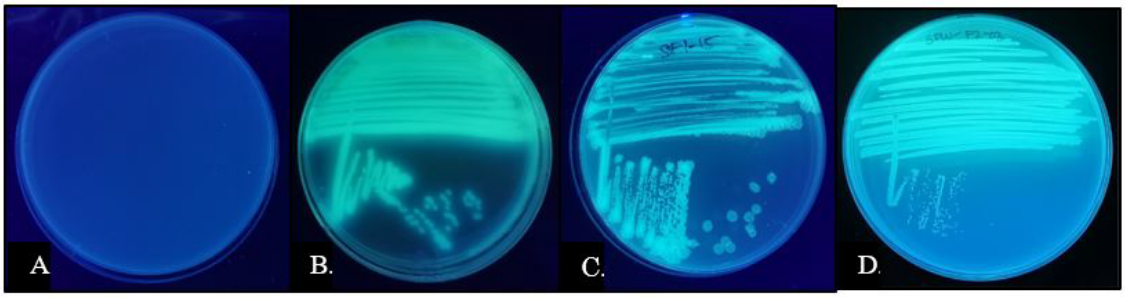


Figure 3. Fluorescent bacterial isolates from Tilapia fish. (**A**)Negative control, Pseudomonas CFC Agar; (**B**) *P. aeruginosa* BIOTECH 1335; (**C**) Isolate SPD-S3F1-15 and (**D**) Isolate SPW-F2-03.

Figure 4. Determination of carbohydrate fermentation and H_2S production using TSI agar. (**A**) Suspected *Pseudomonas* spp. isolates showing no change in color on TSI agar with EC: *E. coli* BIOTECH 1095, ST: *S. typhimurium* BIOTECH 1826, and PA: *P. aeruginosa* BIOTECH 1335, as control organisms. (**B**) Suspected members of Enterobacteriaceae showing acid, gas and H_2S production as products of fermentation reaction.

3.2 Screening for Antibiotic Resistance

The KBDD test was performed and revealed that most of the isolated putative *Pseudomonas* strains showed resistance against two to four antibiotics out of the eight antibiotics tested including cefepime (CPM) piperacillin/tazobactam (PIT), meropenem (MR), and all were found to be resistant to ceftazidime (CAZ). The isolates SPD-S1F1-06 and SPD-S2F1-13 have the highest multiple antibiotic resistance (MAR) index of 0.50, as shown in Table 3, with both having an antibiotic resistance pattern of CAZ-CPM-PIT-MR. The most frequent resistance detected was toward CAZ-PIT-MR, where eight (33%) were observed to have similar antibiotic resistance as shown in Table 4. While the least frequent resistance was with the CAZ-CPM-PIT antibiotic resistance pattern observed only on SPW-F3-02. All isolates were observed as susceptible to tobramycin (TOB), ciprofloxacin (CIP), levofloxacin (LEV) and imipenem (IMP) in KBDD test.

Table 3. Antibiotic resistance profile and multiple antibiotic resistance (MAR) index of the *Pseudomonas* spp. isolates from Tilapia fish.

Pseudomonas Isolates	Inhibitory zone diameter to the nearest millimeter (mm) and interpretation								Drug Susceptibility Result			MAR Index (R/n=8)
	Antibiotic Class											
	Cephems		ß-lactam combination agents	Amino-glycosides	Fluoroquinolones		Carbapenems					
	CAZ	CPM	PIT	TOB	CIP	LE	IPM	MR	S	I	R	
P. aeruginosa BIOTECH 1335	10^R	20^S	21^S	27^S	38^S	35^S	31^S	28^S	7	0	1	0.13
SPD-S1F1-06	10^R	12^R	12^R	25^S	31^S	28^S	35^S	13^R	4	0	4	0.50
SPD-S2F1-13	10^R	14^R	10^R	26^S	31^S	29^S	32^S	13^R	4	0	4	0.50
SPD-S2F1-14	11^R	16^I	13^R	28^S	33^S	30^S	34^S	15^R	4	1	3	0.38
SPD-S3F1-04	11^R	15^I	12^R	26^S	32^S	29^S	33^S	16^I	4	2	2	0.25
SPD-S3F1-06	8^R	21^S	17^I	27^S	33^S	30^S	34^S	18^I	5	2	1	0.13
SPD-S3F1-15	11^R	17^I	17^I	26^S	32^S	30^S	34^S	13^R	4	2	2	0.13
SPW-F1-01	0^R	22^S	15^I	27^S	31^S	30^S	32^S	20^S	6	1	1	0.13
SPW-F1-02	0^R	22^S	15^I	26^S	31^S	29^S	33^S	10^R	5	1	2	0.25
SPW-F1-03	0^R	24^S	15^I	28^S	31^S	28^S	30^S	9^R	5	1	2	0.25
SPW-F1-04	0^R	25^S	14^R	26^S	31^S	29^S	31^S	14^R	5	0	3	0.38
SPW-F1-05	0^R	20^S	12^R	28^S	31^S	28^S	34^S	15^R	5	0	3	0.38
SPW-F1-06	0^R	21^S	14^R	25^S	31^S	27^S	32^S	18^I	5	1	2	0.25
SPW-F1-10	0^R	16^I	10^R	27^S	29^S	26^S	33^S	10^R	5	0	3	0.38
SPW-F2-01	0^R	20^S	11^R	27^S	29^S	28^S	34^S	17^I	5	1	2	0.25
SPW-F2-02	7^R	15^I	10^R	27^S	31^S	28^S	34^S	19^S	5	1	2	0.25
SPW-F2-03	8^R	27^S	15^I	26^S	31^S	28^S	32^S	8^R	5	1	2	0.25
SPW-F2-07	0^R	20^S	11^R	27^S	34^S	28^S	31^S	18^I	5	1	2	0.25
SPW-F2-08	11^R	21^S	14^R	29^S	31^S	28^S	35^S	21^S	6	0	2	0.25
SPW-F3-02	0^R	12^R	8^R	27^S	31^S	30^S	34^S	18^I	4	1	3	0.38
SPW-F4-01	0^R	20^S	12^R	25^S	31^S	30^S	32^S	12^R	5	0	3	0.38
SPW-F4-04	0^R	18^S	9^R	25^S	30^S	28^S	35^S	9^R	5	0	3	0.38
SPW-F4-05	0^R	19^S	10^R	24^S	31^S	29^S	30^S	8^R	5	0	3	0.38
SPW-F4-08	0^R	19^S	11^R	28^S	31^S	31^S	36^S	18^I	5	1	2	0.25
SPW-F5-06	0^R	21^S	13^R	27^S	31^S	28^S	33^S	11^R	5	0	3	0.38

CAZ, Ceftazidime 30 µg (S= ≥18, I= 15-17^, R= ≤14); CPM, Cefepime 30 µg (S= ≥18, I= 15-17^, R= ≤14); PIT, Piperacillin-tazobactam 100/10 µg (S= ≥21, I= 15-20^, R= ≤14); TOB, Tobramycin 10 µg (S= ≥15, I= 13-14^, R= ≤12); CIP, Ciprofloxacin 5 µg (S= ≥25, I= 19-24^, R= ≤18); LE, Levofloxacin 5 µg (S= ≥22, I= 15-21^, R= ≤14); IPM, Imipenem 10 µg (S= ≥19, I= 16-18^, R= ≤15); MR, Meropenem 10 µg (S= ≥19, I= 16-18^, R= ≤15).

Table 4. Antibiotic resistance pattern of the isolated *Pseudomonas* spp. strains.

Antibiotic Pattern	Frequency (n = 24)	Rate (%)
CAZ	2	8.3
CAZ-MR	5	20.8
CAZ-PIT	6	25
CAZ-CPM-PIT	1	4.1
CAZ-PIT-MR	8	33
CAZ-CPM-PIT-MR	2	8.3
Total	24	100

Based on the antimicrobial susceptibility profile of the isolates on KBDD test, 16 isolates were selected considering their multidrug resistance pattern and their source of isolation. Surprisingly, upon subjecting them in the determination of MIC using Sensititre™ MIC Plate System, contrasting results were observed between the qualitative AST (KBDD), and the quantitative AST (broth microdilution via Sensititre) The isolates that were resistant to CAZ, CPM, PIT, and MR in the KBDD test were susceptible to the said antibiotics in the Sensititre MIC Test (Table 5). Although most isolates have a clear-edged zone of clearing, inner colonies termed as subpopulations in the KBDD test were observed in few of the isolates, specifically in the zone of inhibition for CAZ, CPM, and MR, as seen in Fig. 5. These subpopulations were

Table 5. Antibiotic resistance profile of the *Pseudomonas* spp. isolates from Tilapia fish using Sensititre broth microdilution AST system..

Isolates from Tilapia Fish	Minimum inhibitory concentration (µg/mL) and interpretation on GN7F Sensititre Plate														Drug Susceptibility Result		
	Antimicrobics																
	AMI	AZT	FEP	TAZ	CFZAVI	C/T	CIP	DOR	GEN	IMI	LEVO	MR	P/T	TOB	S	I	R
SPD-S1F1-06	≤8^{S}	16^{I}	4^{S}	2^{S}	≤2^{S}	≤2^{S}	≤0.25^{S}	1^{S}	≤2^{S}	>8^{R}	≤0.5^{S}	2^{S}	16^{S}	≤4^{S}	12	1	1
SPD-S2F1-13	≤8^{S}	16^{I}	4^{S}	2^{S}	≤2^{S}	≤2^{S}	≤0.25^{S}	1^{S}	≤2^{S}	≤1^{S}	≤0.5^{S}	4^{S}	64^{I}	≤4^{S}	12	2	0
SPD-S2F1-14	≤8^{S}	16^{I}	4^{S}	2^{S}	≤2^{S}	≤2^{S}	≤0.25^{S}	1^{S}	≤2^{S}	2^{S}	≤0.5^{S}	2^{S}	16^{S}	≤4^{S}	13	1	0
SPD-S3F1-04	≤8^{S}	16^{I}	4^{S}	2^{S}	≤2^{S}	≤2^{S}	≤0.25^{S}	1^{S}	≤2^{S}	2^{S}	1^{S}	2^{S}	32^{I}	≤4^{S}	12	2	0
SPD-S3F1-06	≤8^{S}	>16^{R}	4^{S}	4^{S}	4^{S}	≤2^{S}	≤0.25^{S}	≤0.5^{S}	≤2^{S}	≤1^{S}	1^{S}	2^{S}	32^{I}	≤4^{S}	12	1	1
SPD-S3F1-15	≤8^{S}	>16^{R}	≤2^{S}	2^{S}	≤2^{S}	≤2^{S}	≤0.25^{S}	1^{S}	≤2^{S}	≤1^{S}	≤0.5^{S}	2^{S}	16^{S}	≤4^{S}	13	0	1
SPW-F1-04	≤8^{S}	>16^{R}	≤2^{S}	2^{S}	≤2^{S}	≤2^{S}	≤0.25^{S}	1^{S}	≤2^{S}	≤1^{S}	≤0.5^{S}	4^{S}	16^{S}	≤4^{S}	13	0	1
SPW-F1-05	≤8^{S}	16^{I}	8^{S}	2^{S}	≤2^{S}	≤2^{S}	≤0.25^{S}	1^{S}	≤2^{S}	≤1^{S}	1^{S}	2^{S}	16^{S}	≤4^{S}	13	1	0
SPW-F1-10	≤8^{S}	16^{I}	4^{S}	4^{S}	≤2^{S}	≤2^{S}	≤0.25^{S}	1^{S}	≤2^{S}	≤1^{S}	≤0.5^{S}	1^{S}	16^{S}	≤4^{S}	13	1	0
SPW-F2-02	≤8^{S}	>16^{R}	4^{S}	2^{S}	≤2^{S}	≤2^{S}	≤0.25^{S}	1^{S}	≤2^{S}	2^{S}	≤0.5^{S}	2^{S}	16^{S}	≤4^{S}	13	0	1
SPW-F2-03	≤8^{S}	16^{I}	≤2^{S}	2^{S}	≤2^{S}	≤2^{S}	≤0.25^{S}	4^{S}	≤2^{S}	≤1^{S}	≤0.5^{S}	4^{S}	32^{I}	≤4^{S}	12	2	0
SPW-F3-02	≤8^{S}	16^{I}	4^{S}	2^{S}	≤2^{S}	≤2^{S}	≤0.25^{S}	1^{S}	≤2^{S}	2^{S}	≤0.5^{S}	4^{S}	16^{S}	≤4^{S}	13	1	0
SPW-F4-01	≤8^{S}	>16^{R}	≤2^{S}	4^{S}	4^{S}	≤2^{S}	≤0.25^{S}	1^{S}	≤2^{S}	≤1^{S}	≤0.5^{S}	8^{I}	16^{S}	≤4^{S}	12	1	1
SPW-F4-04	≤8^{S}	16^{I}	4^{S}	2^{S}	4^{S}	≤2^{S}	≤0.25^{S}	1^{S}	≤2^{S}	≤1^{S}	≤0.5^{S}	4^{S}	≤8^{S}	≤4^{S}	13	1	0
SPW-F4-08	≤8^{S}	16^{I}	4^{S}	2^{S}	≤2^{S}	≤2^{S}	≤0.25^{S}	≤0.5^{S}	≤2^{S}	≤1^{S}	≤0.5^{S}	2^{S}	≤8^{S}	≤4^{S}	13	1	0
SPW-F5-06	≤8^{S}	>16^{R}	≤2^{S}	4^{S}	4^{S}	≤2^{S}	≤0.25^{S}	1^{S}	≤2^{S}	≤1^{S}	1^{S}	4^{S}	16^{S}	≤4^{S}	13	0	1

AMI, Amikacin (S= ≤16, I= 32^, R= ≥64); **AZT,** Aztreonam (S= ≤8, I= 16^, R= ≥32); **FEP**, Cefepime (S= ≤8, I= 16^, R= ≥32); **TAZ**, Ceftazidime 30 µg (S= ≤8, I= 16^, R= ≥32); **CFZAVI**, Ceftazidime-avibactam (S= ≤8/4, R=≥16/4); **C/T**. Ceftolozane/Tazobactam (S= ≤4/4, I= 8/4^, R=≥16/4); **CIP**, Ciprofloxacin (S= ≤0.5, I= 1^, R= ≥2); **DOR**, Doripenem (S= ≤2, I= 4^, R= ≥8); **GEN**, Gentamycin (S= ≤4, I= 8^, R= ≥16); **IMI**, Imipenem (S= ≤2, I= 4^, R= ≥8); **LEVO**, Levofloxacin (S= ≤4, I= 8^, R= ≥16); **MR**, Meropenem (S= ≤2, I= 4^, R= >8); **P/T**, Piperacillin-tazobactam (S= <16/4, I= 32/4-64/4^, R=>128/4); **TOB**, Tobramycin (S= <15, I= 13-14^, R= >12)

probably not detected in the Sensititre MIC test. Increased MIC on aztreonam were also observed in most of the isolates that were not tested in disk diffusion assay. Moreover, in this study, the results of both methods were essential in providing information to determine the antimicrobial resistance profile of the isolated *Pseudomonas* spp. Additionally, the isolates that underwent the Sensititre MIC Test were resistant to other antibiotics tested but were not included in CLSI such as ampicillin, tetracycline, ertapenem, nitrofurantoin, ampicillin/sulbactam, cefazolin, trimethoprim/sulfamethoxazole and ceftriaxone (Supplementary Fig. 1 and 2).

Upon deliberation of the phenotypic characteristics of all isolated bacteria, 16 bacterial isolates were decided as representatives to be subjected for molecular identification through 16S rRNA gene sequencing. Sequences received from Kinovett Scientific Solutions Co. were analyzed using BLASTN and revealed the species' hit name described in percentage similarity. Phylogenetic trees, specifically neighbor-joining trees, were also constructed (see Supplementary Fig. 3 to Fig.8). It was used to determine the closest related species alongside the percentage similarity produced in NCBI BLAST. Table 6 shows the result of the bioinformatics analysis for the identification of the selected isolates up to the species level. Sequences were submitted and registered to NCBI Sequence

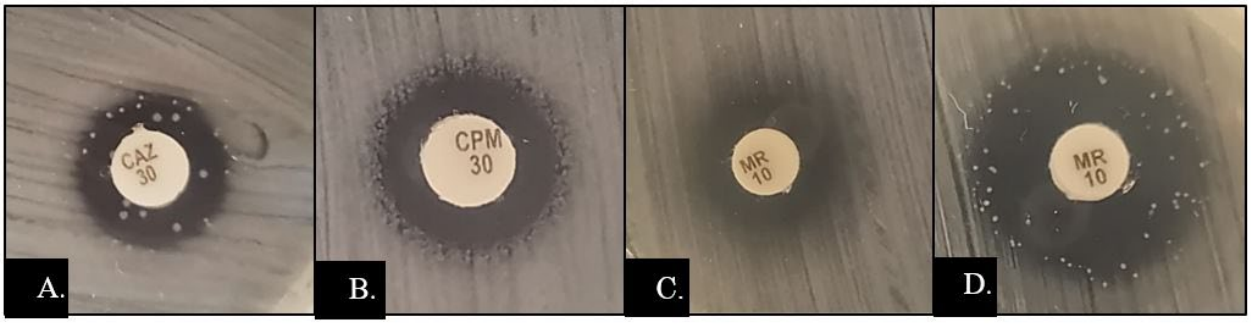


Figure 5. Inner colonies in the zone of clearing. (A) Isolate SPD-S3F1-15, ceftazidime. (B) Isolate SPD-S1F1-06, cefepime and (C) meropenem. (D) Isolate SPD-S3F1-06, meropenem.

Table 6. Partial identity of the selected antibiotic-resistant bacterial isolates from Tilapia fish.

Isolate Code	Species Hit Name	Percentage Similarity
SPD-S1F1-06	*Pseudomonas mosselii*	99.88%
SPD-S2F1-13	*Pseudomonas mosselii*	99.76%
SPD-S2F1-14	*Pseudomonas mosselii*	99.76%
SPD-S3F1-04	*Pseudomonas mosselii*	99.88%
SPD-S3F1-06	*Pseudomonas guariconensis*	99.52%
SPD-S3F1-15	*Pseudomonas guariconensis*	99.40%
SPW-F1-04	*Pseudomonas wadenswilerensis*	99.69%
SPW-F1-05	*Pseudomonas mosselii*	99.84%
SPW-F1-10	*Pseudomonas soli*	100%
SPW-F2-02	*Pseudomonas entomophila*	99.83%
SPW-F2-03	*Pseudomonas oryzihabitans*	99.82%
SPW-F3-02	*Pseudomonas soli*	99.70%
SPW-F4-01	*Pseudomonas guariconensis*	99.27%
SPW-F4-04	*Pseudomonas mosselii*	99.08%
SPW-F4-08	*Pseudomonas entomophila*	99.83%
SPW-F5-06	*Pseudomonas wadenswilerensis*	99.84%

Read Archive (SRA) with BioProject ID PRJNA1237605.

4. Discussion

Nile tilapia (*Oreochromis niloticus*) is the second most farmed fish in the Philippines due to the abundance of freshwater environments that these fishes can be cultured [18]. However, microbial contamination of these aquatic food sources also occurs due to the pollution brought upon by anthropogenic activities to the aquacultures and therefore serves as reservoirs of pathogenic bacteria. In this study, the presence of antibiotic-resistant *Pseudomonas* spp. in Nile tilapia fish from the aquaculture of Sampaloc Lake, San Pablo City, Laguna was investigated along with the determination of the antibiotic resistance pattern of the isolates. *Pseudomonas* spp. is part of the normal gut microflora of healthy Nile tilapia fish but eventually causes infections when such hosts become immunocompromised [19]. *Pseudomonas* spp. have been described as one of the most common bacterial infectious agents of cultured fish that causes stress-related diseases such as ulcerative syndrome and hemorrhagic septicemia, especially under farming conditions [20][21]. *Pseudomonas putida* is also an opportunistic pathogen that infects Nile tilapia and induces ascites, exophthalmia and ulcers in the body [22]. The diversity of *Pseudomonas* spp. in Nile Tilapia fish was described in the study of Duman *et al*., 2021, wherein they have found out that the maximum isolation rate of *Pseudomonas* from liver, kidneys and spleen in Nile tilapia are 35%, 30% and 21.25%, respectively [5]. They also determined the major pathogenic species in Nile tilapia are *P. aeruginosa. P. putida* and as well as *P. angulliseptica* which is the highly pathogenic one with 100% mortality rate. However, there are chances that only few *Pseudomonas* spp. can be isolated from live fishes and the dominant species belongs to the family *Enterobacteriaceae*, specifically *E. coli* and *Citrobacter freundii* due to the slightly anaerobic nature of their internal organs [19][23]. In the study of Dissassa *et al*. in 2022 [24], species of *Vibrio, Salmonella, Aeromonas,* and *Escherichia,* which belong to Enterobacteriaceae, dominated the bacterial population in the gut of live Nile tilapia fish samples by about 90%. It was also observed here in this study, wherein only 24 *(*15.89%) of the initial 151 bacterial isolates were identified as species of *Pseudomonas*. The majority, 127 (84.11%), were suspected as members of the facultative anaerobic and fermentative family of bacteria, Enterobacteriaceae, based on the result of TSI test, a biochemical test that distinguish non-fermentative and fermentative bacteria. The targeted isolation of putative *Pseudomonas* spp. from Nile tilapia fish here in this study was solely based on pigment production, fluorescence expression, production of oxidase, and having no ability of fermentation. Fluorescence of the isolated bacteria in this study varies from green to blue-green colored fluorescence, having weak to strong intensity, and with diffusible pigments varying in color yellow-green or brown. Diffusible green pigment production is one of the distinguishing characteristics of *Pseudomonas* spp. Their fluorescence may vary depending on the diffusible pigments or siderophores they can produce. Pyoverdine is the well-known yellow-green, fluorescent pigment produced by the members of the genus *Pseudomonas* such as *P. putida* and *P. fluorescens* during iron-limiting conditions and may appear as blue-green fluorescence under UV light [17]. Synthesis of other pigments aside from pyoverdine, such as pyocyanin (blue) and pyorubin (red brown), commonly found in *P. aeruginosa*, may alter the color of fluorescence into intense yellow-green or bluish coloration. Growth conditions also affect the production of fluorescent pigments and may appear weak [25]. Species of nonfluorescent groups also exist in *Pseudomonas,* including *P. stutzeri, P. mendocina, P. alcaligenes, P. pseudoalcaligenes* and *P. luteola* [26].

The distribution of the isolated *Pseudomonas* strains from Nile tilapia fish in this study varied greatly on the temporal variation during the sampling of Nile tilapia fish. The Nile tilapia fish were sampled in two different seasons in the Philippines, dry season and the wet season. Only six putative *Pseudomonas* strains (0.25%) were initially isolated from the Nile tilapia fish collected in the dry season and 18 bacterial strains (75%) were isolated during the wet season. This could be attributed to the environmental factors that affect bacterial contaminations in the lake waters specifically in Sampaloc Lake. Lakes are large water-filled inland basins which are prone to contaminants from effluents, as these water bodies usually only have one, or no outlet at all, and have slow-moving waters which usually remain stagnant [27]. High abundance of *Pseudomonas*

within a particular freshwater environment was associated with storm waters as they were considered as contaminants in the runoffs from different water sources [28][29]. It was observed in the study of Ebohon *et al*. in 2023 [30], wherein the percentage population density of *P. aeruginosa* in the surface waters of Lagos lagoon in Nigeria, was the highest at the peak of the rainy season due to the increased runoffs that could contaminate the water environment [30]. Bacterial colonization can be observed in the skins, gills and the digestive tract of fish when they are exposed to contaminated waters [31]. The aquatic environments such as lakes are hotspots of the spread of antimicrobial resistance due to the biological and chemical pollutants from anthropogenic activities [32].

Multidrug-resistant (MDR) *Pseudomonas* were also reported from Nile tilapia fish according to previous investigations. In the study of Ayoub *et al*. (2021), about 27.9% of the isolated bacteria from infected Nile tilapia in El-Abassa fish farm in Egypt belongs to the genus of *Pseudomonas* identified as *P. aeruginosa. P. fluorescens* and *P. angulliseptica* [33]. All the isolated species were found to be resistant against at least five different antibiotics and considered as MDR bacteria. In this study, screening the antibiotic resistance was performed using the qualitative KBDD antibiotic susceptibility test for the 24 putative *Pseudomonas* isolates and the quantitative MIC determination using Sensititre™ MIC Plate System for the 16 selected AMR isolates. As described in the results, all the 24 strains were resistant to CAZ, and some showed resistance to CPM, PIT and MR in the KBDD method. However, when the selected 16 AMR isolates were subjected to MIC determination tests using the Sensititre™ MIC Plate System, they were susceptible to all the antibiotics found to showed resistance in the disk diffusion method, showing contradictory results between the two methods. Although the results were erratic, it was also found in the study of Luc *et al*. (2015), where a comparison between disk diffusion and broth microdilution methods using TREK Sensititre MIC was performed [34]. In their study, the two methods showed very major errors in aztreonam resistance among *P. aeruginosa* strains. Major errors were also seen mainly in three different antibiotics: CAZ, MR, and PIT. They concluded that *P. aeruginosa* have inner colonies inside the zones of clearing, which were termed as subpopulations (shown as resistant in the KBDD test), which cannot be detected in TREK Sensititre MIC. According to Palladini *et al*., [35] the broth microdilution method was found to be more restrictive than agar disk diffusion [35]. Nevertheless, the result of the KBDD tests were still considered as it follows the standard protocol in screening for antibiotic susceptibility of bacteria.

Ceftazidime belongs to the class of third-generation cephalosporin antibiotics used to treat numerous bacterial infections, including *P. aeruginosa* infections. As described by Ramsay *et al*. 2023, ceftazidime resistance is complex and multifactorial due to genetic mutations and alterations that can contribute to resistance in ceftazidime [36]. For instance, a mutation to the *dac*B gene in *P. aearuginosa* increased the MIC to 16-fold, and if combined with a mutation in the *amp*C gene, it became fully resistant to ceftazidime. Some mutations also cause additive effects in lipopolysaccharide synthesis and other metabolic functions. The resistance to ceftazidime is frequently associated with the resistance to carbapenem antibiotics such as meropenem. Although they do not share mutations in the same set of genes, changes in metabolic functions such as increased efflux pump could be associated with the increased resistance of *P. aeruginosa* to meropenem [35]. Acquired ß-lactamase genes such as *bla*VIM can also be pointed out as the main source for meropenem resistance [37].

Antibiotic resistance in *Pseudomonas* spp. occurs in different mechanisms. Intrinsic resistance to multiple antibiotics of *Pseudomonas* spp. is a major threat that confers virulence and pathogenicity to any of its hosts. Intrinsic or innate resistance is a naturally occurring characteristic of *Pseudomonas* spp. as it is chromosomal and genetically controlled, which influence the structural and physiological characteristics of the microorganism [38]. Acquired resistance can also be exhibited by *Pseudomonas* spp. through adaptive mutation or via horizontal gene transfer [39]. Adaptive mutational resistance caused by spontaneous mutations occurs due to the prolonged exposure to antibiotics that induce selective pressure and lead to evolution [40]. Moreover, they may also have adaptive resistance mechanisms, wherein the presence of stress factors and antibiotics induces the regulatory genes to facilitate the expression of antimicrobial resistance genes. Unlike adaptive mutational

resistance, it is not permanent and becomes inactive in the absence of external stimuli such as stress factors and antibiotics [41]. Determination of the antibiotic resistance mechanisms of the isolated bacterial isolates in this study was not performed; thus, further studies are required for complete profiling of their antibiotic resistance.

Upon application of 16S rRNA gene sequencing to the 16 selected bacterial isolates, the current study confirmed the presence of bacterial species belonging to the *P. putida* group, specifically *P. entomophila* (n=2), *P. guariconensis* (n=3), *P. mosselii* (n=6), *P. oryzihabitans (n=1) P. soli* (n=2) and *P. wadenswilerensis* (n=2), in fresh Tilapia fish from the aquaculture of Sampaloc Lake, San Pablo City, Laguna. Most of the identity of *Pseudomonas* spp. were originally isolated from soil environments, and *P. mosselii* was the only species isolated from human and fish samples. *P. mosselii* was reported and described as a fish pathogen in Tilapia aquaculture in Mexico. Sick fish exhibited lethargy, erratic swimming, skin discoloration, scale loss, lateral blindness, red or opaque eyes, and/or exophthalmos [42]. Recently, cases of opportunistic infection by *P. mosselii* were reported in immunocompromised patients in India during the time of the pandemic when SARS-CoV-2 infection was combined with other diseases such as diabetes, hypertension, chronic kidney disease, and heart disease [43]. It was isolated from the respiratory samples of the patients and contributed to the increasing symptoms of pneumonia. Currently, there are no reports yet on the transmission of *P. mosselii* infections from fish to humans or fish kill events caused by *P. mosselii* in the Philippines. Another bacterial strain partially identified as *P. oryzihabitas* was also isolated in this study. This species of *Pseudomonas* was first isolated from rice (*Oryza sativa*) and clinical specimens in 1985 [44] and recognized as a potential nosocomial pathogen, especially in immunocompromised host [45] causing bacteremia, sepsis, peritonitis, and endophthalmitis [46-48]. An outbreak of *P. oryzihabitans* pseudobacteremia occurred in 2014 in an emergency room of a tertiary hospital in Korea [45]. Bacterial cultures of *P. oryzihabitans* were isolated from the blood samples of patients. It was presumably due to the contaminated saline gauze canister that was not aseptically prepared. Another species of *Pseudomonas* that is currently tagged as an emerging multidrug-resistant and opportunistic pathogen is *P. guariconensis*. It was first isolated and described in 2013 from rhizospheric soil in Venezuela [49]. Some clinical cases were associated with *P. guariconensis* infection, such as the necrotizing fasciitis of a 67-year-old man in the United Kingdom [50] and asymptomatic bacteriuria of a 63-year-old woman in Seoul, Korea [51]. Although there was still no record of infections by *P. oryzihabitans* and *P. guariconensis* in fish, consumption of fish contaminated with the said opportunistic pathogen could potentially harm its consumers, especially those immunocompromised individuals or with underlying conditions. The other species of *Pseudomonas* partially identified in the study (*P. entomophila*, *P. soli,* and *P. wadenswilerensis*) have no record yet of being pathogens in fish and in humans, as they are common inhabitants of soil and water. Even though they do not usually cause diseases, they can be a reservoir of antimicrobial resistance genes that can be transferred horizontally to other species of bacteria [52].

In terms of spatial distribution of the detected AMR *Pseudomonas* strains, calculation of their prevalence could not be made as the number of isolated strains varied temporally and the number of samples per sampling site were not the same for the two seasons. Moreover, the isolates with the highest MAR index having CAZ-CPM-PIT-MR resistance pattern and partially identified as *P. mosselii*, were isolated in the dry season from Site 1 and Site 2 which are near the residential areas and near the San Pablo City District Hospital, respectively. This could possibly be attributed to the anthropogenic activities around these areas that might influence the expression of antimicrobial resistance in bacteria. The primary reason for the development of antibiotic resistance is the inappropriate application and disposal of antibiotics, which have been extensively abused by the community through their misuse or overuse to treat various human, plant, or animal infections [53]. Antimicrobial resistance has been recognized as a global threat that diminishes the efficacy of common antibiotics against broad bacterial infections. It was estimated that by the year 2050, about 10 million deaths per year will be recorded globally if the issue was given inappropriate action [54]. In the Philippines, several pathogenic bacteria that developed antimicrobial resistance and became multidrug resistant (MDR) bacteria have been isolated and detected from clinical settings as well as from the environment. An MDR *E. coli* has been isolated from the water and fecal

sources in Laguna Lake that exhibits resistance to 14 antibiotics out of 16 antibiotics tested [55]. *Vibrio* spp. harbored from two of the top cultured fishes in the Philippines, *Chanos chanos* (milk fish) and *O. niloticus* (tilapia), was detected and found resistant to ampicillin, tetracycline, kanamycin and polymyxin B [56]. In the 15-year (2004-2018) report of antimicrobial resistance monitoring of *Salmonella* by the Department of Health, most of the *Salmonella* serotypes were found resistant to ampicillin, cefotaxime and ciprofloxacin combinations [57]. Up to this day there have been no records yet of MDR *Pseudomonas* spp. in the water environments or aquaculture in the Philippines according to the review of antimicrobial resistance in Southeast Asian water environments conducted by Siri *et al.* in 2023 [58]. The emergence of MDR bacteria set an alarm for another possible wave of pandemic in the future other than the viral diseases such as COVID-19.

To combat such threat, the "Philippine National Action Plan on Antimicrobial Resistance: One Health Approach" (PNAP) was developed in the partnership of the Department of Health (DOH), WOAH, and the Food and Agriculture Organization of the United Nations (FAO). The national action plan aims to strengthen the AMR awareness, infection prevention and control as well as to minimize the antimicrobial use and its effect in the environment, animals and human as a one health approach [59]. To address the AMR issues in the agricultural sector specifically in aquaculture, the Philippine Bureau of Fisheries and Aquatic Resources (BFAR) actively participated in the said action plan focusing on the enhancement of the surveillance, measurement, regulation and control of antibiotic use in the aquaculture through the implementation of Good Aquaculture Practice (GAqP) among the aquaculture farmers. In the 2020 status of the said plan, 68% of the surveyed 84 registered fish farmers from all over the Philippines determined that they were not using antimicrobials in their aquaculture operations while 32% used probiotics and chemicals such as disinfectants and antibiotics. Moreover, there has been no detection of antibiotics in shrimp or fish meat from the registered farms. In contrary to the said results, antibiotic residues were detected from the Nile tilapia cultured in the lakeshore Barangays of Los Baños, Laguna, wherein all fifty-four fish samples from different barangay contained residues of tetracycline and ceftiofur [60]. Furthermore, 83% and 9% of the fish samples were found to have quinolone and florfenicol residues, respectively. Detection of such antibiotics in fish poses health risks and concerns about the development of antimicrobial resistance. Moreover, the detection of AMR *Pseudomonas* spp. in the tilapia aquaculture of Sampaloc Lake, San Pablo City, Laguna suggests that stronger surveillance and monitoring of our aquaculture is in dire need of attention to prevent and control the possible spread of infections caused AMR bacteria in the environment, other animals and to humans.

5. Conclusions

The study detected antibiotic-resistant *Pseudomonas* spp. from the Tilapia aquaculture in Sampaloc Lake, San Pablo City, Laguna, and were partially identified as *P. entomophila, P. guariconensis*, *P. mosselii*, *P. oryzihabitans, P. soli,* and *P. wadenswilerensis,* which all belong to the *P. putida* group. Based on the KBDD method, all the isolated bacteria exhibit resistance to Ceftazidime 30 µg and to other antibiotics, with the most frequent pattern being CAZ-PIT-MR (33%). The highest MAR index was observed in two of the isolates with a 0.50 MAR index, both having a resistance pattern of CAZ-CPM-PIT-MR. Furthermore, the majority of the identified isolates (40%) were partially identified as strains of *P. mosselii*, which was described as a pathogen in tilapia fish. The isolates with the highest MAR index were also identified as strains of *P. mosselii*. The detection of these antibiotic-resistant bacteria serves as evidence of the current microbiological state of the Tilapia aquaculture in Sampaloc Lake. Thus, the study could be considered in the formulation of policies for improving proper handling, managing, and operation of the Tilapia aquaculture to ensure food safety and the overall health of the lake and its resources, as well as the health of the community of San Pablo City, Laguna.

Availability of Data and Materials

All data are available in this study

Author Contributions

Conceptualization, R.J.D.F., C.P.R., and J.G.J.; Methodology, R.J.D.F., C.P.R., and J.G.J.; Investigation, R.J.D.F. and C.P.R.; Writing – Original Draft, R.J.D.F. and C.P.R.; Writing – Review & Editing, R.J.D.F.

Ethics Approval and Consent to Participate

The current research has followed the accepted principles of ethical conduct by committee of the University of the Philippines Los Baños Animal Care and Use Committee (IACUC) for the sampling and microbiological analysis of tilapia fish.

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Conflict of Interest

The authors declares no conflict of interest.

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Original Article

A Coprological Investigation on Gastrointestinal Parasites of Wild Boars (*Sus scrofa*) from Hatay Province, Türkiye

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Abstract

Background: Research on disease surveillance in wildlife species is of fundamental importance for understanding the epidemiology of zoonotic diseases. **Methods:** Fecal samples were collected from 24 wild boars in the districts of Antakya, İskenderun, and Kırıkhan in Hatay province during the years 2018-2019. The Fülleborn flotation and Benedek sedimentation methods were applied to these samples. Additionally, feces were examined for the presence of *Cryptosporidium* spp. oocysts using Kinyoun acid-fast staining and nested PCR methods. **Results:** Microscopic examination revealed that 14 animals (58.33%) were infected with one or more parasite species. The prevalence rates of gastrointestinal parasites were as follows: *Eimeria* spp. 45.83%, *Metastrongylus* spp. 20.83%, *Ascaris suum* 20.83%, *Strongyloides* spp. 12.5%, and *Oesophagostomum* spp. 8.33%. Furthermore, no *Cryptosporidium* spp. was detected in any of the fecal samples. The infection rate was higher in male boars (64.70%) compared to females (42.85%). According to the results of Fisher's Exact Test, there was a statistically significant difference in parasite presence among the sampled districts, while no significant difference was found between parasite presence and sex. **Conclusions:** In conclusion, this study indicates that wild boar populations can serve as potential reservoirs of parasites for both animals and humans.

Keywords: *Ascaris suum, Cryptosporidium, Eimeria, Strongyloides*, Boar

1. Introduction

As most of the emerging zoonotic diseases originate from wildlife, studies on wildlife constitute a crucial component of infectious disease surveillance [1, 2]. Therefore, disease monitoring research, laboratory diagnostics, and molecular analyses in wild animal species are fundamental for understanding the epidemiology of zoonotic diseases and implementing health measures for humans and animals [3]. This is essential because the transmission of diseases between wild animals, domestic animals, and humans poses an increasing threat to human and animal health [4]. In this context, one of the significant wild animals for both human and animal health is the wild boar (*Sus scrofa*). The wild boar is a wild animal species native to Europe, Asia, and North Africa [5,6]. This species, which has one of the broadest geographical distributions of all terrestrial wild animal species [7], has seen a significant increase in population in Europe in recent years [8]. Taxonomically, the wild boar is classified in the order Artiodactyla (even-toed ungulates), suborder Nonruminantia, and family Suidae [9]. According to the International Union for Conservation of Nature (IUCN), it is listed as "Least Concern" (LC) on the Red List of Threatened Species [10]. Additionally, due to its

ability to cause significant damage to agricultural and natural habitats, threaten native species, and act as a source of infection for domestic animals and humans, it is categorized as one of the world's top 100 invasive species [11]. These animals can serve as reservoir hosts for various infectious agents, posing a disease source for wild animals, domestic animals, and humans. Consequently, they can play a role in the transmission of various viral, bacterial, and parasitic zoonotic diseases [12, 13, 14, 15]. Some parasitic zoonoses and domestic animal disease agents that may originate from wild boars include *A. suum*, *Babesia bigemina*, *Balantidium coli*, *Blastocystis* spp., *Cryptosporidium* spp., *Echinococcus multilocularis*, *Entamoeba histolytica*, *Enterocytozoon* spp., *Fascioloides magna*, *Giardia* spp., *Hepatozoon* spp., *Taenia solium*, *Theileria* spp., *Trichinella* spp., *Toxoplasma gondii*, *Sarcoptes scabiei*, and *Sarcocystis suihominis* [16, 17].

Cryptosporidium spp. is the fifth most common foodborne pathogen, with over eight million cases reported annually [18]. The infectious dose for cryptosporidiosis is extremely low, with just 10 oocysts sufficient to cause infection. Given the environmental resistance of *Cryptosporidium* spp. oocysts, the parasite has a high potential to spread to new hosts [19]. *Cryptosporidium* spp. can be transmitted either through the consumption of contaminated water and food or through direct contact with contaminated humans and animals. Pigs have been identified as potential environmental reservoirs, contaminating water and food sources not subjected to any disinfection process prior to human consumption [18, 20]. Among the reported *Cryptosporidium* species to date, *C. suis* and *C. scrofarum* are frequently detected in wild pigs and have also been found in humans, indicating the zoonotic potential of this parasite, which should not be overlooked [18].

Wild boars, whose hunting is permitted during specific periods, can be found almost everywhere in Türkiye, except for a few provinces in Central and Eastern Anatolia characterized by vast steppes and plains [21, 22]. Despite their presence across nearly all ecosystems in Türkiye, there are limited studies on the prevalence of gastrointestinal parasites in wild boars [23, 24, 25, 26]. Moreover, no studies have been found regarding the parasites of wild boars in Hatay province. This study aims to determine the prevalence of gastrointestinal parasites in wild boars hunted in Hatay province.

2. Materials and Methods

2.1 Study Area

Located at the southernmost point of Türkiye, bordering Syria, Hatay province is surrounded by the Orontes River, the Amik Plain, the Amanos Mountains, the Samandağ coast, and the Gulf of İskenderun. Due to its combination of terrestrial and marine ecosystems, it is one of the most unique areas in Türkiye in terms of biodiversity and ecological significance. The region, rich in endemic and other wild animal species [27], is also situated on one of the world's most critical migratory bird routes [28]. The most commonly encountered wild animal in Hatay province is the wild boar [29]. In this study, wild boars were obtained from the districts of Antakya, İskenderun, and Kırıkhan in Hatay province (Fig. 1).

2.2 Collection and Examination of Fecal Samples

The material for this study consisted of fecal samples collected from 24 adult (>12 months) wild boars (seven females, 17 males) hunted in the mountainous regions of the Antakya, İskenderun, and Kırıkhan districts of Hatay province between 2018 and 2019. Specifically, the samples were collected from 10 boars in Antakya, four in İskenderun, and 10 in Kırıkhan. These wild boars were legally hunted by hunters during the permitted hunting period. Within 24 hours following the hunt, approximately 10-20 grams of fresh fecal samples were taken from the rectum of each animal using disposable gloves. The samples were then placed in sterile, leak-proof plastic containers labeled with protocol numbers and transported to the laboratory under a cold chain, where they were examined for parasites within 24-48 hours. Each fecal sample was analyzed for the presence of nematode larvae, cestode eggs, and coccidian protozoan oocysts using the Fülleborn flotation method, and for trematode eggs using the Benedek sedimentation technique. The samples were examined under a light microscope [30]. The identified parasitic forms (eggs/cysts/oocysts/larvae) were diagnosed with the aid of relevant literature [30] and photographed. Additionally, the presence of *Cryptosporidium* spp.

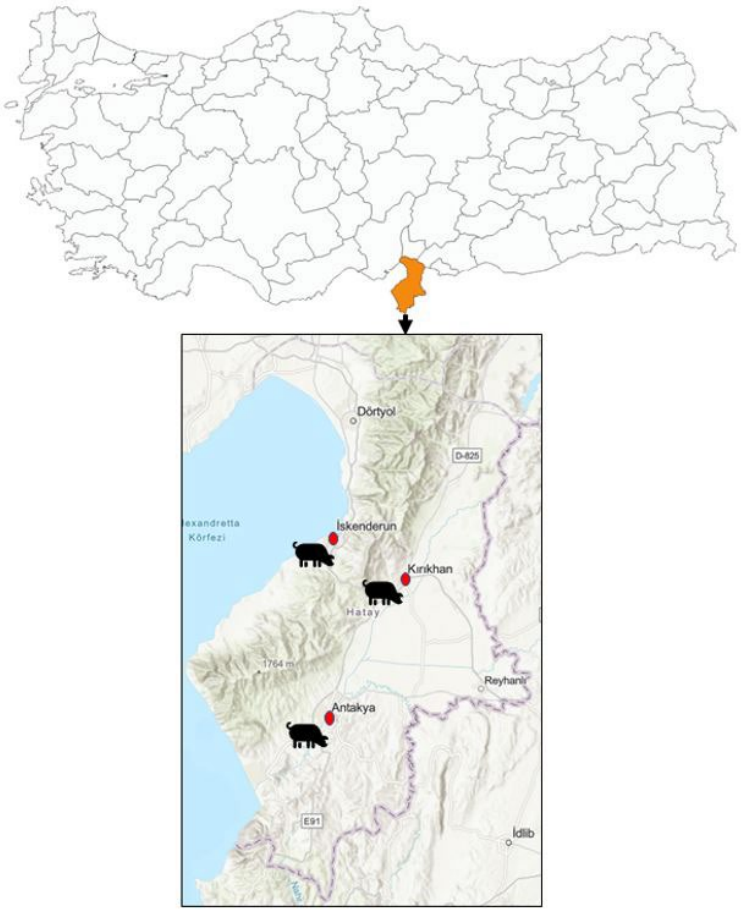

Figure 1. Hatay province, the area where the study was conducted

oocysts in the fecal samples was investigated using Kinyoun acid-fast staining [31] and nested PCR methods [32].

2.3 Molecular Investigation of *Cryptosporidium* spp.

2.3.1 DNA Extraction

For DNA isolation from the fecal samples, the GeneMATRIX® Stool DNA Purification Kit was used following the kit's procedure. The isolated DNA samples were stored at -20°C until the PCR reactions were conducted.

2.3.2 PCR Assay

The primers used for the nested PCR reaction were defined by Xiao *et al*. [32]. In the first PCR reaction, primers 5′-TTCTAGAGCTAATACATGCG-3′ and 5′-CCCATTTCCTTCGAAACAGGA-3′ were used to amplify a 1325 bp DNA fragment of the SSU rRNA gene region of *Cryptosporidium* species. In the second PCR stage, primers 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and 5′-AAGGAGTAAGGAACAACCTCCA-3′ were used to amplify an 824-864 bp gene fragment. For both reactions, the mixture was prepared using 4 µL of 5x FIREPol® Master Mix (12.5 mM MgCl2), 0.5 µL each of forward and reverse primers, 5 µL of DNA, and 10 µL of nuclease-free water. In the second PCR reaction, instead of 5 µL of DNA, 5 µL of the first PCR reaction product was used. The first PCR reaction consisted of an initial denaturation at 95°C for 5 minutes, followed by 25 cycles of 95°C for 30 seconds, 58°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 5 minutes. The second PCR reaction was performed under the same conditions, but with 35 cycles. Genomic DNA obtained from the feces of a calf infected with *Cryptosporidium* spp. was used as a positive control, while distilled water was used as a negative control. The PCR products were run on

a 1% agarose gel at 120 V for 45 minutes. The gel was then visualized under UV light.

2.4 Statistical Analysis

Statistical analyses were performed using the SPSS 23.0 software package (Version 23.0 Armonk, NY: IBM Corp.). The Chi-Square test was utilized to investigate the significance of differences in the frequency of parasitic infections concerning the genders of the wild boars and the districts where sampling was conducted. Differences were considered significant at the $p<0.05$ level.

3. Results

Microscopic examination revealed that out of 24 wild boars (seven females, 17 males), 14 (58.33%) were found to be infected with one or more parasite species. A total of five parasites were identified, including one coccidian oocyst and four nematode eggs (Fig. 2). The prevalence of parasites detected in three districts of Hatay province is shown in Table 1. The highest prevalence among parasites was observed in *Eimeria* spp. (45.83%), followed by *Metastrongylus* spp. and *A. suum* with equal prevalences (20.83%), *Strongyloides* spp. (12.5%), and *Oesophagostomum* spp. (8.33%). Additionally, *Cryptosporidium* spp. was not detected in any of the fecal samples (Fig. 3) subjected to Kinyoun acid-fast staining and nested PCR methods (Table 1). The number of wild boars infected with multiple parasites was higher than those infected with a single parasite. Accordingly, 28.57% (4/14) of wild boars were infected with a single parasite, 57.14% (8/14) with two parasites, and 14.28% (2/14) with three parasites (Table 2). The rate of parasite infection was higher in male boars (64.70%) compared to females (42.85%). Parasites were found in wild boars in all districts. The highest percentage of infected boars was recorded in the Kırıkhan district (100%), followed by İskenderun (50%) and Antakya (20%) (Table 3). According to the Fisher's Exact Test, a statistically significant difference was observed among the districts sampled in terms of parasite presence, while no significant difference was found between parasite presence and sex.

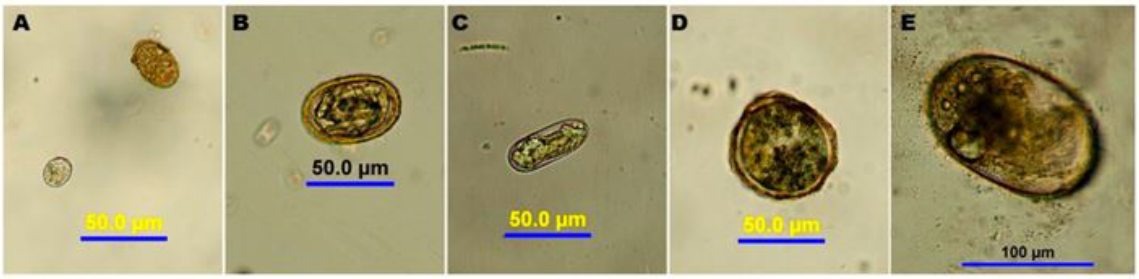

Figure 2. Parasites detected in the study. The photomicrographs of the images were taken under 400× magnifcation. **A.** *Eimeria* spp. oocyst **B.** *Metastrongylus* spp. egg, **C.** *Strongyloides* spp. egg, **D.** *Ascaris suum* egg, **E.** *Oesophagostomum* spp. egg

Table 1. Parasites Detected in Wild Boars in Hatay Province and Their Prevalence

Parasites	Number of infected wild boars in districts			Prevalence (%)
	Antakya	İskenderun	Kırıkhan	
Eimeria spp.	-	1	10	45.83
Metastrongylus spp.	2	1	2	20.83
Ascaris suum	2	-	3	20.83
Strongyloides spp.	-	-	3	12.5
Oesophagostomum spp.	-	1	1	8.33
Cryptosporidium spp.	-	-	-	-

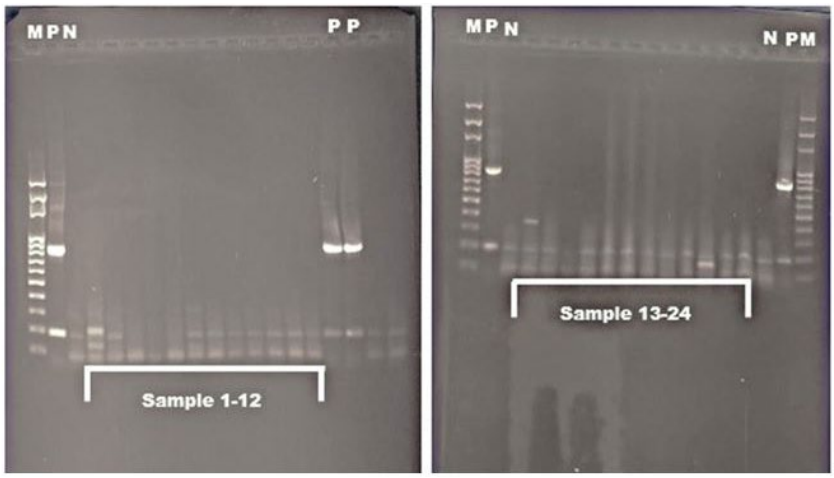

Figure 3. The gel image of the PCR product of the *Cryptosporidium* spp. M: Marker, P: Positive control, N: Negative control

Table 2. Parasites Detected in Wild Boars in Hatay Province and Their Prevalence

Parasites detected in per wild boar	Number of infected wild boars
Eimeria spp.	3
Eimeria spp. + *Metastrongylus* spp.	1
Eimeria spp. + *Strongyloides* spp.	1
Eimeria spp. + *Ascaris suum*	3
Eimeria spp. + *Oesophagostomum* spp.	1
Eimeria spp. + *Strongyloides* spp. + *Oesophagostomum* spp.	1
Eimeria spp. + *Strongyloides* spp. + *Metastrongylus* spp.	1
Metastrongylus spp.	1
Ascaris suum + *Metastrongylus* spp.	2
	Total: 14

Table 3. Prevalence of Parasitic Infections in Wild Boars Based on Gender and Sampling Districts

Variable	Examined	Infected	%	P-value
Sex				$p > 0.05$
Female	7	3	42.85	
Male	17	11	64.70	
Total	24	14	58.83	
District				$p < 0.05$
Antakya	10	2	20	
İskenderun	4	2	50	
Kırıkhan	10	10	100	

4. Discussion

Wild boar is a wildlife animal widely hunted for food and sport purposes worldwide [33]. However, hunting activities carried out without proper hygiene measures during the processing of meat and offal pose a potential risk of transmitting various pathogens, including parasites, from these animals to humans and other domestic animals. In this regard, research is being conducted in many countries around the world to determine the risks of wild boar populations transmitting pathogenic parasites. In addition, DNA metabarcoding has been used in recent years as a valuable analysis method to identify gastrointestinal parasite species in wild animals, thereby helping to prevent the transmission of disease-causing zoonotic parasite species to humans and domestic animals

[34]. Studies have reported that wild boars harbor various parasites of significant importance to public health and veterinary medicine [35]. Therefore, there is a need for studies in Türkiye, as in the rest of the world, to reveal data on the parasitic fauna of wild boars. In Türkiye, which is a largely Muslim country, zoonotic parasites such as *Trichinella* are seen in sporadic cases due to the strict prohibition of consuming pork and wild boar meat according to Islamic regulations [36]. However, since Hatay is a province with various beliefs, a segment of the population can consume pork.

Although ectoparasites such as Ixodid ticks [16, 37] and protozoans like *Babesia* spp. [37] and *T. gondii* [38] have been detected in wild boars in Türkiye, the majority of reported parasites belong to the group of helminths. These include *T. spiralis* [39, 40, 41, 42], *A. suum* [43], *Echinococcus multilocularis* [44], *Echinococcus granulosus* [45], and *M. pudendotectus* [46]. However, most of these studies that focused on a single parasite are in the form of case reports. Consequently, research on the presence or prevalence of multiple helminth parasites in wild boars in Türkiye is limited [23, 24, 25, 26]. In studies conducted by Merdivenci [23, 24] on wild boars, eight species were reported, including *M. apri*, *Taenia hydatigena*, *Gongylonema pulchrum*, *Trichuris suis*, *Fasciola hepatica*, *A. suum*, *O. dentatum*, and *Haemonchus contortus*. Senlik *et al.* [25] identified 12 species in wild boars from Bursa province, including *M. apri*, *Metastrongylus salmi*, *Metastrongylus pudendotectus*, *Dicrocoelium dendriticum*, *Globocephalus urosubulatus*, *Macracanthorhynchus hirudinaceus*, *Gongylonema pulchrum*, *Physocephalus sexalatus*, *Trichuris suis*, *Ascarops strongylina*, *Hyostrongylus rubidus*, and *T. hydatigena*, during necropsy. Another study conducted on 30 wild boars in the Burdur region reported the presence of *T. suis* and *Dicrocoelium dendriticum* helminth species [26]. In the present study sampling wild boars from Antakya, İskenderun, and Kırıkhan districts of Hatay province, a total of 5 parasites were found in fecal samples of 24 wild boars, including *Eimeria* spp. among protozoans and *Metastrongylus* spp., *A. suum*, *Strongyloides* spp., and *Oesophagostomum* spp. among helminths. Additionally, *Cryptosporidium* spp. was not detected in any sample.

Eimeria spp. can cause significant health problems in young or weaned piglets. In severe infections, piglets may experience bloody diarrhea, loss of appetite, weight loss, lethargy, and even death [47]. *Eimeria* oocysts are resistant to environmental conditions and can persist in the environment, thus maintaining their ability to cause infection under suitable climatic conditions. Therefore, *Eimeria* is a commonly reported protozoan in wild boars worldwide. However, its presence has not been reported in wild boars in Türkiye in studies conducted until now. In this study, *Eimeria*, which was found to be the most prevalent parasite at rates of 12.5% as a single infection and 33.33% in mixed infections, with an overall prevalence of 45.83%, is reported in wild boars in Türkiye for the first time to our knowledge. There are studies reporting *Eimeria* spp. as the most prevalent parasite in wild boars worldwide, as in this study [35, 47, 48]. The prevalence of this parasite has been found to be 92.3% in Denmark [48], 75.6% in Mexico [35], 58.8% and 39.3% in two studies conducted in Poland [47, 49], 33.33% in Italy [50], 7.5% in Bulgaria [51], and 3% in Russia [52]. Climate differences, the age of the studied wild boars, and the size of the areas they inhabit are considered as reasons for these differences in prevalence.

Metastrongylus species are lungworms found in the respiratory tract of pigs. Acquired immunity develops in adults against *Metastrongylus* parasites, which are particularly common in young pigs. In wild boars, *Metastrongylus* spp. can cause destruction of interstitial tissues, lung obstruction, shortness of breath, progressive weight loss, and even fatal bronchopneumonia [14]. Additionally, some *Metastrongylus* species with zoonotic potential (*M. elongatus, M. salmi*) can rarely cause metastrongylosis in humans. Severe lung distress with bilateral pleural effusion, bloody, heavy phlegm cough, fatigue, and progressive shortness of breath are clinical symptoms observed in humans [53]. Due to their use of various worm species as intermediate hosts in their life cycles, these nematodes have a wide geographical distribution and are among the most common parasites of wild boars [52]. Indeed, studies conducted in different countries, including Türkiye, have reported the prevalence of this nematode in wild boars ranging from 13.6% to 85% [12, 25, 51, 52, 54, 55]. In these studies, it has been the most commonly detected parasite among nematodes. In this study, *Metastrongylus* spp., detected with equal prevalence as *A. suum* (20.83%), has been one of the two most prevalent nematodes, consistent with the aforementioned literature.

Ascaris suum is one of the common intestinal parasites of both domestic and wild pigs, with a worldwide distribution. Infections caused by this nematode can negatively affect the health and weight gain of wild boars. Wild boars play a role in transmitting this parasite to both domestic pigs and humans by serving as reservoirs. The eggs of this ascarid species, which are highly resistant to harsh environmental conditions such as freezing and extreme heat, can survive in the soil for up to 10 years. In this regard, the presence of wild boars in certain areas significantly increases the likelihood of spreading *A. suum* infections [56]. The prevalence of this species in wild boars has been reported to range from 3% to 88% in studies conducted in some countries [48, 51, 52, 53, 57, 58, 59]. *Ascaris suum*, which shows variability in frequency of occurrence in different geographic locations and population densities [60], was detected at a rate of 20.83% in this study. The presence of this parasite in a wild boar was reported in a case study in Türkiye [43], while its prevalence in domestic pigs was reported to be 4.1% [61]. To our knowledge, the prevalence of *A. suum* parasite in wild boars has been demonstrated for the first time in this study in Türkiye.

Strongyloides spp. and *Oesophagostomum* spp. are the least detected helminths in this study, with the rates of 12.5% and 8.33%, respectively. These nematodes have been reported in wild boars in various studies worldwide [35, 51, 62]. In Türkiye, *O. dentatum* has been reported by Merdivenci [23, 24]. To our knowledge, the presence of *Strongyloides* spp. in wild boars has been demonstrated for the first time in this study in Türkiye.

Cryptosporidium spp. is one of the most important protozoans with zoonotic potential that acts as a host in wild boars [18]. The prevalence of this protozoan in wild boars has been reported as 1.4% in Portugal [17], 13.3% in Central Europe [20], 8.2% [63] and 21.7% [18] in two studies in Spain, and 25% in Sweden (20). However, *Cryptosporidium* was not detected in any of the 24 wild boars in this study, which differs from the aforementioned studies. This difference could be due to the relatively low number of pigs examined in this study compared to the literature. Another reason could be that all the boars examined in this study were adults, which could be attributed to the factor of age. Indeed, studies have been reported that age is one of the most important factors related to prevalence, and the prevalence of *Cryptosporidium* is higher in piglets and youngsters than in adults [18]. Although *Cryptosporidium* was reported at a rate of 8.8% (21/238) in a study conducted on pig farms in Türkiye [61], its presence has not been reported in wild boars. This was the first effort to detect *Cryptosporidium* in the wild boar fecal sample in this region by both morphological and molecular methods, however the results were negative. The use of these complementary methods aimed to maximize the reliability and accuracy of the findings. While morphological examination facilitates the visualization and identification of oocysts based on their structural features, molecular methods provide enhanced sensitivity and specificity, particularly for detecting low-level infections or identifying species/genotypes. Despite this comprehensive approach, no *Cryptosporidium* was detected in the samples analyzed in this study.

In the current study, a significant difference was found in terms of the presence of parasites among the sampled districts according to Fisher's Exact Test, while no significant difference was found between the presence of parasites and gender. In the Kırıkhan district, parasites were detected in all wild boars, with the highest prevalence rate (100%), followed by İskenderun (50%) and Antakya (20%), respectively. This situation in the Kırıkhan district can be explained by the more favorable environment for the survival and development of parasites. Additionally, there are studies indicating both statistically significant differences in parasite prevalence or presence between male and female wild boars [35, 52], as well as studies reporting no statistically significant difference, similar to this study [58, 59, 63].

The small sample size and the lack of molecular investigation of parasites other than *Cryptosporidium* are the limiting factors of this study. The reason for the small sample size of this study is that wild boars are difficult to catch but can only be obtained after hunting. It has been reported that to obtain positivity in the PCR method, 20 *Cryptosporidium* oocysts are required per gram of stool, while in microscopic methods, 100,000 to 500,000 *Cryptosporidium* oocysts are needed per gram of stool [64], Therefore, when

there are a small number of oocysts in the stool, the likelihood of obtaining false-negative results using microscopic methods is high. To prevent this, microscopic and molecular diagnostic methods have been used together only for *Cryptosporidium*. In future studies, if possible, increasing the sample size and examining parasites with zoonotic potential both microscopically and molecularly should not be ignored.

5. Conclusions

In conclusion, this study has demonstrated that wild boar populations serve as potential reservoirs of parasites for both animals and humans. Because the prevalence of nematodes with zoonotic potential (20.83%), *Metastrongylus* spp. and *A. suum* in this study is at a level that should not be underestimated. In addition, the pathogenic species of *Eimeria* protozoa (45.83%), the most common parasite found in wild boars in this study, may pose a serious health threat to both farm pigs and livestock. When wild boars venture from their natural habitats into public areas, they contaminate the environment with their feces, inevitably facilitating contact between the zoonotic pathogenic parasites they carry and humans. To prevent the spread of these zoonotic parasites, it is crucial for veterinarians to raise awareness among the public, particularly hunters, about the importance of this issue. Furthermore, conducting studies to determine the parasitic fauna of wild boars in different regions of Türkiye in the future would be beneficial.

Availability of Data and Materials

All of the data generated and analyzed during this study are included in this published manuscript.

Author Contributions

Conceptualization, A.Z., O.C., and İ.E.; Investigation, O.C. and İ.E.; Methodology, O.C. and İ.E.; Formal analysis, O.C.; Writing - Original draft preparation, A.Z.; Visualization, O.C.; Writing - Review and editing, A.Z., O.C., and İ.E.; Collection, recording and arranging of the samples, S.A.Ş.

Ethics Approval

This study was approved by Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee (Decision number: 2024/03-04).

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Conflict of Interest

No conflict of interest was declared by the authors.

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(Original Research)

Parasites Detected in Aquarium Fish in Konya Province of Türkiye

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Abstract

Background: Parasitic infections in aquarium fish, similar to those in other animal species, can lead to significant economic losses for the industry and hobby breeders. This study aimed to determine the prevalence of parasitic infections in aquarium fish in Konya province by assessing various fish species collected from different aquariums. **Methods:** A total of 104 aquarium fish representing nine species from eight taxonomic families were examined. These included 39 goldfish (*Carassius auratus*) from the Cyprinidae family, 42 guppies (*Poecilia reticulata*) and two mollies (*Poecilia sphenops*) from the Poeciliidae family, nine bushymouth catfish (*Ancistrus dolichopterus*) from the Loricariidae family, seven Ornate tetras (*Hyphessobrycon bentosi*) from the Characidae family, one Kenyi cichlid (*Maylandia lombardoi*) from the Cichlidae family, one cory catfish (*Corydoras sp.*) from the Callichthyidae family, two Siamese fighting fish (*Betta splendens*) from the Osphronemidae family, and one shark catfish (*Pangasius* sp.). Each fish underwent a macroscopic examination, followed by microscopic analysis of skin, fin, and gill scrapings using the native examination method. Protozoa, metazoa, and arthropods were identified based on relevant literature. **Results:** Parasitic infections were detected in 30.8% (32 out of 104) of the aquarium fish. The identified parasites included *Gyrodactylus sp.*, *Dactylogyrus sp.*, *Tetrahymena sp.*, *Chilodonella sp.*, *Trichodina sp.*, *Ichtyobodo necator*, *Ichthyophthirius multifilis*, *Argulus sp.*, and an aquatic mite. In some cases, mixed infections with more than one parasitic species were observed. **Conclusions:** The findings demonstrate that a variety of parasitic infections are present in aquarium fish sold in Konya's pet shops, posing risks to both the fish industry and private breeders. The detection of multiple parasites and the presence of mixed infections suggest that regular parasitic screenings in pet shops are essential. These preventive measures could reduce the transmission of infections among aquarium fish and mitigate economic losses for those involved in the fish trade.

Keywords: *Cyprinidae*, *Poeciliidae,* Arthropod, Helminth, Protozoa, Türkiye

1. Introduction

Engaging in a hobby is one of the best ways for people to relieve stress, and worldwide surveys on hobbies show that interest in aquariums ranks second only to photography [1]. Although aquarium keeping is generally considered a hobby, it is also an important sector within aquaculture. Aquarium fish farming holds a commercially significant position in aquaculture in both developed and developing countries. In many economically challenged tropical countries, local people catch aquarium fish from natural habitats or raise them for export, generating income for their families and contributing to the national economy [1]. Today, aquarium fish farming has become a multimillion-dollar industry worldwide. In Türkiye, it has been practiced for 30 years and has grown increasingly popular in recent years [2].

Some microorganisms, including parasites, constitute the normal flora of the skin, fins, gills, and digestive tract of fish. The presence of various stress

factors predisposes fish to invasion by these opportunistic pathogens, leading to increased morbidity and mortality [3]. Parasitic infections and infestations are frequently encountered in all commercially important food and aquarium fish farms globally. These parasites can cause significant losses when favorable conditions, such as crowded ponds and rising water temperatures, occur [4]. Studies report that mortality due to infections in fish is approximately 10-20%, with parasites accounting for a quarter of these losses [2].

Helminthic parasites possess various attachment organs such as hooks, pincers, and suction organs on certain parts of their bodies. These parasites attach to the skin or gill filaments of their hosts, causing mechanical damage at the attachment sites, which can become entry points for secondary infections [5]. Parasites negatively impact host behavior and feed intake. When feeding is disrupted, fish divert their limited energy from reproduction toward resisting parasites, which indirectly suppresses their reproduction [6]. Many parasite species can settle on the gills or among the gill filaments of aquarium fish. In mild infections, discoloration or mottling of the gill filaments may occur. Severe infections can result in marked symptoms, including epithelial hyperplasia of the filaments, lamellar fusion, and excessive mucus secretion, all of which contribute to respiratory difficulties [5, 7].

Despite the rising interest in aquarium fish farming in Türkiye, studies on parasitic diseases in aquarium fish remain limited. To implement effective measures against parasitic diseases, identifying the parasitic agents that cause these diseases and conducting epidemiological studies are essential. This study aimed to identify the parasites responsible for diseases in aquarium fish in Konya and to contribute to the limited research and literature on this subject.

2. Materials and Methods

The study material consisted of 104 deceased aquarium fish collected from various aquariums and pet shops in the Konya province of Türkiye. The sampling area and information on the aquarium fish sampled in the study are presented in Figure 1. The

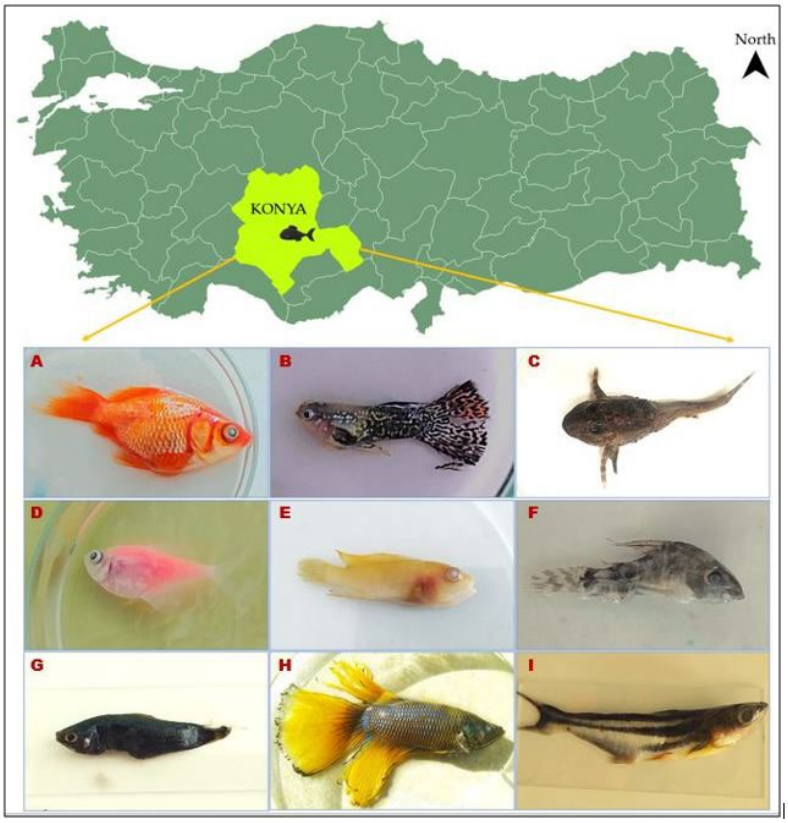

Figure 1. Fish samples collected from Konya province of Türkiye, **A.** *Carassius auratus,* **B.** *Poecilia reticulata,* **C.** *Ancistrus dolichopterus,* **D.** *Hyphessobrycon bentosi,* **E.** *Maylandia lombardoi,* **F.** *Corydoras* sp., **G.** *Poecilia sphenops,* **H.** *Betta splendens*, **I.** *Pangasius* sp.

fish were collected from 13 different pet shops with the permission of the pet shop owners. Goldfish (*Carassius auratus*, n=39) from Cyprinidae family, guppies (*Poecilia reticulata,* n=42) from *Poeciliidae* family, bushymouth catfish (*Ancistrus dolichopterus,* n=9) from *Loricariidae* family, Ornate tetra (*Hyphessobrycon bentosi*, n=7) from *Characidae* family, Kenyi cichlid (*Maylandia lombardoi*, n=1) from *Cichlidae* family, cory catfish (*Corydoras* sp., n=1) from *Callichthyidae* family, molly fish (*Poecilia sphenops*, n=2) from *Poeciliidae* family, Siamese fighting fish (*Betta splendens*, n=2) from *Osphronemidae* family, and shark catfish (*Pangasius* sp., n=1) from *Pangasiidae* family were sampled in the study (Figure 1). These fishes, which had died due to various causes, were taken from the pet shops. Fish samples were randomly selected from different tanks in pet shops and aquariums, ensuring representation from various sources. The information about the species of fish and the information required during the collection of the samples were recorded. After sample collection, each fish was transported to the laboratory under a cold chain to preserve parasite integrity. The aquarium fish were delivered to the Parasitology Laboratory at the Faculty of Veterinary Medicine, Selcuk University. Upon arrival, they were placed in separate petri dishes with water from their original tank. Their entire body surface and gills were examined macroscopically. Afterward, scrapings from the skin, gills, and internal organs were examined for parasitism using a binocular light microscope (Leica DM 1000, Wetzlar, Germany) and stereo microscope (Nikon SMZ745T, Tokyo, Japan). Protozoa, helminths, and arthropods detected during microscopic examination were identified at the genus or species level using the relevant literature [8, 9].

3. Results

Of the 104 deceased fish examined, 32 (30.8%) were found to be infected with one or more parasites (Table 1). Parasites were found in all fish species except for the shark catfish (*Pangasius* sp.). Notably, only protozoan agents were detected in guppy fish (16.6%), which constituted the majority of the study sample. No helminths or arthropods were identified in this species. Each infected fish harbored at least one and up to three different parasite species. Among the infected fishes, 24 (75%) were infected by one type of parasite, six (18.8%) had dual infection, and two (6.25%) had triple co-infection. All parasites were detected in the skin and gills of fish species.

The majority of the parasites detected in our study were protozoa. *Chilodonella* sp. had the highest prevalence, observed in 59.4% of infected fish, followed by *Tetrahymena* sp. (31.3%). This study reports the first identification of *Argulus* sp. in Konya province. Typically causing infestations in summer and autumn, *Argulus* sp. was detected in goldfish sampled during the autumn season. Additionally, a water mite from the Halacaridae family which is commonly found in rivers was identified in a molly fish (*Poecilia sphenops*) in the present study. Photographs of the detected parasitic agents, and detailed information on parasitic

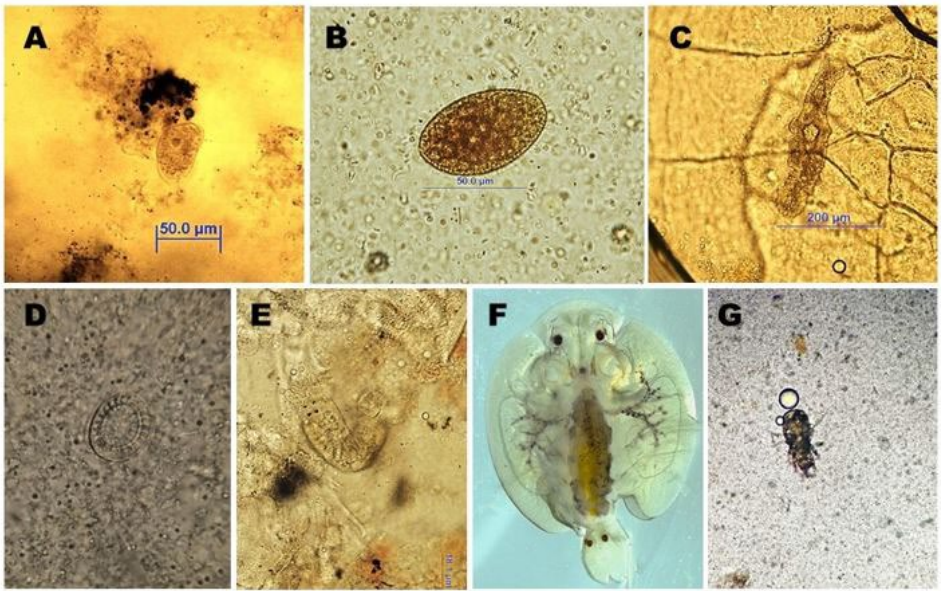

Figure 2. Parasites identified in the examined aquarium fishes, **A.** *Chilodonella* sp., **B.** *Tetrahymena* sp., **C.** *Gyrodactylus* sp., **D.** *Trichodina* sp., **E.** *Dactylogyrus* sp., **F.** *Argulus* sp., **G.** Aquatic mite

infection rates and numbers according to species are provided in Figure 2, Table 1, and Table 2.

conditions provide an ideal environment for parasites to thrive [10, 11]. Parasitic diseases

Table 1. General information regarding the studied aquarium fish samples from Konya province of Türkiye

Common name	Species	Family	N	N_i (%)
Goldfish	*Carassius auratus*	*Cyprinidae*	39	15 (38.46%)
Guppy	*Poecilia reticulata*	*Poeciliidae*	42	7 (16.6%)
Molly fish	*Poecilia sphenops*	*Poeciliidae*	2	1 (50%)
Ornate tetra	*Hyphessobrycon bentosi*	*Characidae*	7	3 (42.85%)
Bushymouth catfish	*Ancistrus dolichopterus*	*Loricariidae*	9	3 (33.3%)
Cory catfish	*Corydoras sp.*	*Callichthyidae*	1	1 (100%)
Kenyi cichlid	*Maylandia lombardoi*	*Cichlidae*	1	1 (100%)
Siamese fighting fish	*Betta splendens*	*Osphronemidae*	2	1 (50%)
Shark catfish	*Pangasius spp.*	*Pangasiidae*	1	0 (0%)
Total			104	32 (30.8%)

N: number of host sampled, N_i: number of infected fish (%)

Table 2. Parasitic agents detected in aquarium fish species from Konya province of Türkiye

Parasitic agents	Aquarium Fishes									Prevalence among infected fish	Total
	Goldfish	Guppy	Bushymouth catfish	Ornate tetra	Kenyi cichlid	Cory catfish	Molly fish	Siamese fighting fish	Shark catfish		
Gyrodactylus sp.	2	-	-	-	-	-	-	-		6.3%	1.9%
Dactylogyrus sp.	8	-	-	-	-	-	-	-	-	25%	7.7%
Tetrahymena sp.	2	1	3	3	1	-	-	-	-	31.3%	9.6%
Chilodonella sp.	7	8	1	-	1	1	-	1	-	59.4%	18.3%
Argulus sp.	2	-	-	-	-	-	-	-	-	6.3%	1.9%
Trichodina sp.	1	-	-	-	-	-	-	-	-	3.1%	0.9%
Aquatic mites (Halacaroidea)	-	-	-	-	-	-	1	-	-	3.1%	0.9%
Ichtyobodo necator	-	-	1	-	1	-	-	-	-	6.3%	1.9%
Ichthyophthirius multifiliis	-	-	-	-	-	1	-	-	-	3.1%	0.9%

4. Discussion

The global aquarium fish industry has grown into a multimillion-dollar market, with increasing popularity in Türkiye [2]. Aquarium fish, often wild-caught from tropical and subtropical regions, are typically fed with live food such as water fleas and are kept in confined aquarium spaces. These

which can rapidly spread and cause high mortality in aquarium fish, thus require particular attention. In recent years, research on parasites infecting aquarium fish has been conducted worldwide, revealing varying rates of parasitic infection. In studies conducted in Türkiye, the prevalence of parasitic infections in aquarium fish has ranged from 43.3% to 90.9% [12, 13]. In this

study, at least one parasite species was detected in 30.8% of the aquarium fish examined. This rate is notably lower than those reported in Türkiye. These discrepancies may be attributed to various factors, including differences in maintenance conditions of aquariums and ornamental ponds, fish feeding practices, and housing incompatible fish species together. The synergistic effect of these factors increases stress and thus promotes susceptibility of fishes to infections. Other influencing factors include the number and diversity of fish examined, the use of antiparasitic treatments by aquarium owners, cleaning routines, delays prior the removal of deceased fish from the aquarium, and the diagnostic preparation methods used.

The study found protozoa in a significant proportion of the aquarium fish examined. Here, *Chilodonella* sp. and *Tetrahymena* sp. were detected in 59.4% and 31.3% of infected fish, respectively. In a similar study by Gökpınar et al. [12], a high prevalence of *Chilodonella* sp. (61%) and *Trichodina* sp. (26.3%) was reported with a much lower prevalence of *Tetrahymena* sp. (1.6%) in aquarium fish in Kırıkkale province. Although some parasite prevalences were comparable, notable differences emerged, particularly for *Tetrahymena* sp. This variation in infection rates may be influenced by factors such as differences in aquarium conditions, species and numbers of fish examined, timing of examination, and any treatments applied within aquariums. *Chilodonella* sp. primarily colonize the gills, with infected fish displaying symptoms such as anorexia, skin depigmentation, ulceration, scale loss, excessive mucus production, and gill lesions [14]. Severe infections may lead to the weakening and eventual death of the fish [8]. In previous studies conducted in Türkiye, *Chilodonella* sp. prevalence has ranged from 26% to 51% [12, 15, 16], indicating a relatively higher prevalence in this study. In a comprehensive review of marine, freshwater, brackish, and aquarium fish in Türkiye, Öktener et al. [17] found that *Trichodina* sp. was the most common parasite, followed closely by *Chilodonella* sp. Given the widespread presence and pathogenic impact of *Chilodonella* sp., further research is warranted to understand this parasite's biology and control. Additionally, more awareness among breeders is needed regarding its effects. Interestingly, *Chilodonella* sp. was not detected in Ornate tetra, molly, and shark catfishes in this study. This absence may be attributable to the limited sample sizes for these species or potentially to inherent resistance.

Tetrahymena sp. can infect various organs, including muscles, abdominal cavity, intestines, liver, eye sockets, and spinal cord in ornamental fish and cause organ destruction, which can ultimately lead to death [18]. The pathogenic potential of these protozoa underscores the need for vigilant monitoring and improved management practices in aquarium environments. *Tetrahymena* species predominantly infect guppies, which has led to the disease being commonly referred to as guppy disease. However, it has also been reported in other fish species, such as cichlids and tetras [19, 20]. In Konya province, *Tetrahymena* sp. has been identified in the gills and skin of seven discus fish [21], and a separate study from the same region found it in 11% of examined tetra fish [22]. In our study, the prevalence reached 31.3%, a notably high rate. This elevated prevalence may be attributed to the large proportion of guppies (40.4%) among the examined fish, a species particularly susceptible to this pathogen. Given the significant impact of this high infection rate in guppies, further studies focusing on both the host species and *Tetrahymena* are warranted.

Ichthyophthirius multifiliis, which causes white spot disease, is a well-documented issue for aquarists, with its characteristic clinical signs being the presence of white spots on the skin, fins, eyes, oral cavity, and gills of affected fish [24]. Gökpınar *et al*. [12] reported an infection rate of 0.8% in a local fish population, while previous studies in both Türkiye and other countries have observed varying infection rates between 2.8% and 8% [10, 12, 16]. Our findings, with a prevalence of 3.1%, align closely with these values, suggesting a stable presence of *I. multifiliis* within this range. The protozoan *Ichthyobodo*, another critical parasite in aquaculture, targets the skin and gills of fish, leading to severe health issues and potentially substantial losses. Its prevalence has been documented in studies from Türkiye and Sri Lanka, with reported rates ranging from 1.7% to 27.6% [12, 16, 30]. Gökpınar et al. [12] noted a prevalence of 8.4% in the Kırıkkale province, while our study found a slightly lower rate of 6.3%, which is consistent with previous research [12, 16, 30]. *Ichthyobodo* sp. is often overlooked due to its morphological similarities with other protozoa, underscoring the need for careful laboratory examination to ensure accurate diagnosis.

Trichodina sp. was observed in 3.1% of parasitized aquarium fish, aligns with global prevalence rates reported in previous studies, ranging from 3% to 26.6% [12, 15, 16]. *Trichodina* species are well-known protozoan parasites found in both freshwater and marine fish, typically on the body surface, gills, and oral cavity, but they can also occasionally inhabit the intestines, kidneys, and urinary bladder of their hosts [27]. High infestations, especially on skin and gills, have been associated with mortality, as documented by Koyuncu and Cengizler [28] in goldfish (*Carassius auratus*). Parasitological and bacteriological examinations indicated that a significant burden of *Trichodina* sp. contributed to fish deaths. The prevalence of *Trichodina* sp. varies widely among aquarium fish populations, which may be due to differences in fish husbandry practices, water quality, host susceptibility, and environmental conditions. Gökpınar et al. (2023) reported a prevalence of 26.3% in aquarium fish, highlighting the potential risk posed by this parasite in managed aquatic systems. Given *Trichodina*'s pathogenic potential, it is essential for aquarists to recognize the risks associated with these protozoans and to adopt preventive measures. Awareness and further studies on *Trichodina* sp. are necessary to better understand its epidemiology, host interactions, and control strategies, particularly in aquarium settings where outbreaks can lead to significant fish mortality and welfare concerns.

Dactylogyrus species, typically recognized as gill parasites of fish, are monogenean trematodes that may also inhabit the skin under certain conditions [31]. The infection symptoms in discus fish highlight the significant clinical impact of *Dactylogyrus*, including pallor and excessive mucus production in the gills, lethargic swimming near the water surface, abnormal swimming behaviors, loss of balance, reduced activity, and, in severe cases, high mortality rates [21]. Our findings align with the prevalence reported by Gökpınar et al. [12], who observed a 21.9% prevalence rate for *Dactylogyrus* sp., whereas the current study reports a slightly higher prevalence of 25%. One plausible reason for this elevated prevalence could be the aquarium water temperature, as the reproductive rate of monogenean parasites, including *Dactylogyrus*, increases with warmer temperatures. Consequently, *Dactylogyrus* infections are likely more frequent during warmer periods, such as the summer months. This seasonal effect, driven by temperature-dependent reproductive dynamics, may have contributed to the high prevalence observed in our study. In related research, Dewi and Fadhilla [32] investigated the prevalence of monogenean parasites in apparently healthy guppies and goldfish, finding the highest prevalence rates among *Gyrodactylus* sp. and *Dactylogyrus* sp. infections in guppies, at approximately 14% and 8%, respectively. In contrast, our study reports *Gyrodactylus* sp. and *Dactylogyrus* sp. infection rates of 6.3% and 25%, respectively, with infections detected exclusively in goldfish. Notably, no monogenean parasites were found in the guppies sampled, which constituted nearly half of the total fish population studied. This discrepancy in species-specific parasite prevalence may suggest differential susceptibility or environmental tolerance to monogenean infections between goldfish and guppies.

In this study, we identified *Argulus* sp., commonly known as fish lice, as an additional parasitic agent affecting the fish examined. *Argulus* sp. infestations are known to cause a range of symptoms in infected fish, including anemia, scale loss, lethargy, irregular swimming, reduced appetite, surface hanging, and localized hemorrhages where the parasite attaches. Although *Argulus* sp. can grow up to 1 cm in length, its detection is often challenging due to its ability to blend with the fish's mucous membranes. However, they can be distinguished by their characteristic two black-pigmented eyes. Fish lice are also capable of temporarily leaving their host to reproduce and subsequently infesting new hosts [8]. These parasites typically thrive in warmer months, with infestation peaks observed during summer and fall. Consistent with this trend, *Argulus* sp. was identified in fish sampled during the fall in our study. The lice were found attached to the skin, gill chambers, and mouth, where they induced local inflammation attributed to mechanical damage and the effects of digestive enzymes. Various studies have documented *Argulus* species in both freshwater and marine fish across different regions [33], with particular prevalence in ornamental species such as goldfish and koi [24]. Of note, Koyuncu [34] reported *Argulus japonicus* for the first time in koi farmed in Mersin, Türkiye. Similarly, Gökpınar et al. [12] identified *Argulus* infestations in 5% of 502 aquarium fish, while in our study, the infestation

rate was found to be lower, at 1.9% of the 104 fish sampled. The relatively low prevalence of *Argulus* sp. in our findings could be due to the parasite's visibility, which allows aquarium owners to remove them manually. Notably, in our study, *Argulus* sp. was exclusively detected in goldfish, marking this as the first documented occurrence of *Argulus* sp. in Konya province. *Argulus* species are generally associated with freshwater environments, as observed in other studies [35-37].

Water mites (Hydrachnidia), comprising over 5,000 species, are widely distributed in aquatic environments. Commonly represented families include Oribatida, Astigmata, and Halacaroidea [38]. Despite their prevalence, the small size of water mites often leads to their being overlooked [39]. For example, Proctor [39] found no oribatid or halacarid mites in stream samples from tropical Queensland, indicating potential habitat-based variability. In our study, we observed the presence of Halacaroidea mites on *Poecilia sphenops* skin in an aquarium environment, contrasting with findings in natural habitats. This suggests that aquarium conditions may support mite proliferation as effectively as natural waters. The presence of Halacaroidea could stem from environmental or management-related factors, such as insufficient aquarium maintenance, which may foster infestation. Additionally, our observations imply that in captive environments, mites may exhibit low host specificity, potentially infecting multiple species.

5. Conclusions

The number of studies on protozoa, helminths, and arthropods that adversely affect aquarium fish, causing anorexia, growth retardation, reproductive disorders, and mortality, remains limited in Türkiye. This study identified a 30.8% prevalence of parasitic infections in aquarium fish collected from pet shops in Konya province, highlighting the need for increased surveillance and control efforts. Coinfections were observed, emphasizing the complexity of parasite-host interactions and the necessity of further research on species-specific susceptibility. Additionally, new reports of certain parasites contribute to filling knowledge gaps in the field. Given the high prevalence, aquarists and pet shop owners should prioritize quarantine, quality acquisitions, and rigorous maintenance to prevent parasite introduction. Monitoring water quality and implementing rotating treatments are crucial to minimizing infection risk and preventing drug resistance in aquatic mites. These findings underscore the importance of continued vigilance and preventive strategies to mitigate the impact of parasitic infections in aquarium fish.

Availability of Data and Materials

All data are available in this study

Author Contributions

Conceptualization, S.V., F.G., and O.C.; Methodology, S.V., F.G., and O.C. Sample collection, S.V.; Data curation, S.V., F.G., and O.C.; Writing – original draft, S.V., F.G., and O.C.; Writing – review and editing, S.V., F.G., and O.C.

Ethics Approval and Consent to Participate

All experimental procedures followed the ethical guidelines of the Veterinary Faculty of Selcuk University, Experimental Animals Production and Research Center Ethics Committee (SUVDAMEK: 2024/135)

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Conflict of Interest

The authors declare no conflict of interest.

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(Original Research)

MHC-II DRB Gene Polymorphism and its Association to Gastrointestinal Parasite Burden of Crossbred Anglo-Nubian Goats from a Single Animal Farm in Sultan Naga Dimaporo, Lanao del Norte, Philippines

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Abstract

Background: The Major Histocompatibility Complex (*MHC*), which codes for proteins essential to immune response, is frequently cited as a candidate gene associated with gastrointestinal parasite (GIP) resistance in small ruminants. This study aimed to investigate the polymorphism of the *MHC-DRB* gene in a crossbred Anglo-Nubian goat population and assess its association with GIP burden. **Methods:** Fecal analysis was conducted to estimate worm burden based on egg per gram (EPG) count. Sequence-based genotyping was used to analyze polymorphisms within the 285 bp fragment of the *MHC-DRB* gene exon 2. **Results:** The results confirmed high polymorphism of the *MHC-DRB* gene in goats, identifying 23 SNPs, 20 of which were non-synonymous mutations leading to 14 amino acid changes. Additionally, three Linkage Disequilibrium (LD) blocks and 21 closely linked SNP pairs ($r^2 > 0.9$) were identified. **Conclusion:** Association analysis revealed that individual SNPs, LD blocks, and SNP pairs were not significantly associated ($p > 0.05$) with worm burden. Thus, with the established significance of MHC in immune response mechanism it is recommended to conduct further investigation with larger sample sizes including different goat breeds. Additionally, it is recommended to explore other *MHC* loci and to associate the polymorphisms identified with other immune-related traits.

Keywords: *MHC-DRB* fragment, Non-synonymous mutations, Ruminants, Worm burden

1. Introduction

Gastrointestinal parasitism (GIP) significantly contributes to losses in goat farming worldwide [1-5]. Although anthelmintic agents are commonly used to control GIP, their indiscriminate use has led to the development of resistant parasites and the accumulation of chemical residues in the environment and animal products [4-9]. A sustainable alternative to control GIP in animal farming is selecting animals with favorable genetic markers for disease and parasite resistance [4-6, 8, 10-12].

The Major Histocompatibility Complex (*MHC*) codes for proteins that are primarily expressed on

the surface of immune cells and responsible for immune regulation [13-16]. In goats, the caprine MHC molecules are encoded on chromosome 23 by a diverse gene family [14,19,21]. The *MHC* consists of three main classes: I, II, and III [17-20], of these three, it is the *MHC-II* that received great attention especially in association studies [20]. The *MHC-II* codes for peptide-binding sites that is functionally important for the organisms' immune response [17,21]; it takes part in the antigen presentation to CD4+ T cells which help B cells to produce appropriate immunoglobulins to trigger a corresponding immune response [13,17,18,21-23]. The *MHC-II* is composed of two subtypes: DQ and DR which are mostly polymorphic among organisms and play a role in the development of MHC-specific immune response [13,14,16,19,20, 24]. Specifically, in exon 2, the *MHC-II DRB* gene codes for the first beta domain which is in close contact with foreign antigens [13,17]. This locus of *MHC* gene displays a great degree of polymorphism which is associated to the ability of the *MHC-II* molecules to recognize a wide variety of different antigen derived peptides [13-18,20].

The *MHC-DRB* locus is frequently cited in association studies of GIP resistance in sheep [23, 25-28] and in goats [4,5,19,29]. In association studies regarding endoparasite resistance the commonly used phenotypic parameter for GIP host resistance is determining the egg per gram (EPG) count of GIP per animal through fecal analysis or fecal egg counting (FEC) [2,5,10,11,23,30,31]. In different sheep breeds, different genotypes of the *MHC-DRB* were associated with resistance or susceptibility to GIP. In Ghezel sheep, the genotype 'A1A1' was observed with a lower fecal egg count [28]; in Deccani sheep, the genotype 'J' was frequently observed with a high FEC count [25] and in Southern Indian sheep breed 'bb' genotype was found to be associated with a higher EPG [26]. Notably, several polymorphism studies on the partial sequence (285bp fragment) of the *DRB* locus of *MHC* gene in exon 2 have been conducted on several goat populations [13,15,17, 20-22,32,33] using the primers that previously described [34], however association studies using this locus were limited.

In connection to the information presented, in the Philippines where goat industry is predominantly composed of backyard raisers [35], GIP infestation is among the top three concerns for animal farms [36] and although goat farming is considered as a sunrise industry in the country [36, 37], studies to control GIP infestation in goats through sustainable methods such as the use of possible genetic markers are scarce. Thus, this study was conducted as a preliminary investigation on the polymorphism of the 285 bp fragment of the *MHC-DRB* exon 2 gene and its association with the gastrointestinal parasite burden of crossbred Anglo-Nubian goats reared on an animal farm located in Sultan Naga Dimaporo, Lanao del Norte, Philippines. It also aims to contribute to improving marker-assisted breeding practices in the country and the data on possible markers for selection candidates in goat breeding, which will ultimately improve livestock quality and survivability.

2. Materials and Methods

2.1 Animals

Crossbred Anglo-Nubian X Native goats reared at the *SaGoat Kita* Farm, located at Mindanao State University – Lanao del Norte Agricultural College in Sultan Naga Dimaporo, Lanao del Norte, Philippines, were utilized as the sample population for this study. The animal house, established in 2001, initially had a buck-to-doe ratio of 3:40. During sample collection, the population consist of 117 goats, of which 34 were males and the remaining were females. A total of thirty goats were included in the study, excluding those younger than four months [5, 28], as this age threshold ensures the presence of a mature immune response [38]. Does were also excluded from the study due to the possibility of pregnancy, thus avoiding potential stress to the animals during sampling period. The goats were raised in a cut-and-carry system with *ad libitum* access to water. The dry season in the region occurs from January to June, while the rainy season spans from late June to early January. The goats were dewormed alternately with Fenbendazole and Ivermectin every 60 days. Additionally, supplements were provided, including Vitamin B complex for goats under one year of age and Vitamin ADE for those over one year of age.

2.2 Fecal Sampling and Fecal Analysis

Sample collection was performed by collecting an estimated five grams of feces directly from the rectum of the animals using sterile gloves [28].

Samples were placed separately inside labeled clean, sealable plastics, transported to the laboratory, and refrigerated at 4°C. The collection was conducted 30 days post-deworming and performed thrice at one-week intervals [28, 39]. Fecal analysis was performed immediately within 48 h after the collection [40].

The McMaster flotation technique was used for fecal analysis to determine the EPG count for each animal [41]. Fecal samples from each animal were homogenized individually to ensure thorough mixing. Two grams of fecal matter were then weighed and combined with 28 mL of a concentrated NaCl solution, which was prepared by dissolving 180 grams of NaCl in 500 mL of distilled water. The mixture was filtered through a mesh sieve, and the resulting suspension was pipetted into the chambers of a McMaster slide (Eggzamin® McMaster Microscope Slides), with each chamber filled separately. The slide was left to stand for five minutes to allow the parasite eggs to float then examined under a compound microscope at 10x magnification to count parasite eggs. To prevent crystal formation, the counting was completed within 60 minutes. Only eggs larger than 60–80 microns [41,42] were included in the count and reported as EPG using the formula: (Chamber 1 + Chamber 2) × 50. A single EPG value was used for each animal by calculating the average EPG from the three collections.

2.3 Amplification of *MHC-DRB* Gene

Hair follicles were collected in the rump area of the animals and were stored in a resealable plastic. Genomic DNA was extracted using a QIAGEN DNEASY kit with some minor modifications. The 285 bp fragment in Exon 2 of the *MHC-DRB* sequence was amplified using the primers previously described: F: 5' - TATCCCGTCTCTGCAGCACATTTC-3'; R: 5'-TCGCCGCTGCACACTGAAACTCTC-3' [5,34,43]. A 30 uL PCR mixture was used consisting of 1 X buffer, 1.5 uM of each primers, 0.2 uM dNTPs, 0.5 U/uL Taq Polymerase and 4 uL genomic DNA. The thermocycler (35x) was set at 95°C for 15 min for initial denaturation, 95°C for 30 sec for denaturation, 67°C for 30 sec for annealing, 72°C for 1 min for extension, and a final extension of 72°C for 5 min. Amplicons were confirmed and assessed via AGE and were sent to Biofact Co., Ltd, South Korea, for PCR purification and sequencing.

2.4 Sequence Analysis

Multiple sequence alignments with the reference sequence (NC_030830.1) [44] from NCBI were performed using MEGA11 [45]. Genotyping and SNP confirmation were then performed using the chromatograms [27] of each sequence through Geneious Prime [46] at the default setting (minimum variant coverage of 1 and at a minimum variant frequency of 0.15).

2.5 Data Analysis

The final EPG value for each goat was distributed among the worm burden categories: Low/mild worm burden (EPG < 500), moderate worm burden (EPG 501-1500), heavy/high worm burden (EPG 1501-3000), and fatal worm burden (>3000) [36]. The mean EPG for each category was computed by adding all EPG values within the category and then dividing by the total number of animals within that category.

On the other hand, identified SNPs were examined for their diversity indices, including allele and genotypic frequencies, observed heterozygosity (Ho), expected heterozygosity (He), deviation from Hardy-Weinberg equilibrium (HWE), and polymorphism information content (PIC). SNP blocks and linkage disequilibrium (LD) coefficient (r^2) of the SNPs were also determined. These analyses were performed using R studio [47], employing the following packages: 'genetics' [48], 'BioManager' [49], 'ggplot2' [50], and 'reshape2' [51]. The associations of the individual SNPs, LD blocks, and linked SNP pairs to the EPG and worm burden categories were determined using the Chi-square test. All association tests were conducted using Jamovi Software [51, 53]. All statistical analyses were performed with 95% confidence interval.

3. Results

3.1 Distribution of Worm Burden of crossbred Anglo-Nubian goats

Descriptive statistics for each worm burden category are presented in Table 1. Goats with low EPG counts (<500) are generally considered GIP-resistant, whereas those with EPG counts greater

Table 1. Descriptive statistics for the worm burden categories of the thirty crossbred Anglo-Nubian goats

Categories	N*	Mean EPG**
Low/mild (EPG of <500)	4	359
Moderate (EPG of 501-1500)	16	900
Heavy/high (EPG of 1501-3000)	4	1929
Fatal (>3,000)	6	3936

*number of animals; **mean egg per gram per category.

than 500 are classified as GIP-susceptible (Khobra *et al.*, 2012; Pratap *et al.*, 2024). In this study, only four animals fell into the GIP-resistant category, while the rest were classified as GIP-susceptible. Sixteen goats had moderate worm burdens, while four had high EPG counts and six were classified with fatal EPG counts. The animals that displayed moderate worm burdens had a mean EPG value of 900, which is approaching the upper limit of the moderate category, indicating that animals within this group were more likely to have higher EPG counts. Supplementary Table 1 presents the individual EPG count of animals.

3.2 Detection of Polymorphism and Diversity Indices of *MHC-DRB* gene

Genotyping analysis of the DNA sequence of the *MHC-DRB* gene revealed 23 SNPs. Based on the findings, heterozygosity was observed to be low (He>Ho) in the samples. HWE analysis revealed 15 SNP loci that have significantly deviated ($p<0.05$) from the HWE, and only eight SNP loci revealed no significant deviation ($p\geq0.05$). Alignment of the translated sequence of this fragment of the MHC gene containing the 23 SNPs revealed 14 amino acid changes. The complete summary of the SNPs and the diversity indices is presented in Supplementary Table 2.

On the other hand, Table 2 presents four SNPs out of twenty-three which include two non-biallelic loci (DRB9489A>C/T and DRB9546G>T/A) whose allele and genotype frequencies were highly varied, resulting with the PIC values of 0.460 and 0.504, indicating that these loci are moderate informative. Twelve biallelic loci were also observed to obtain moderately informative PIC values (0.250<PIC<0.460) [19,32].

For a clear illustration, Fig. 1 exhibits the *MHC-DRB* gene of the goats found on the exon 2

Table 2. Diversity indices of SNP in *MHC-DRB* gene of crossbred Anglo-Nubian goats.

SNP No*	SNP ID**	Type of Mutation	Amino Acid Change	Allele	Allele Frequency	Genotype	Genotype Frequency	Ho	He	HWE (*p* value)	PIC
12	DRB9489A>C/T	N[12,13]	I>L/F	A	0.63	AA	0.53	0.300	0.526	0.076	0.460****
				C	0.25	AT	0.07				
				T	0.12	AC	0.13				
						CC	0.13				
						CT	0.10				
						TT	0.03				
13	DRB9491T>C			T	0.05	TT	0.03	0.033	0.095	0.002***	0.090
				C	0.95	TC	0.03				
						CC	0.93				
22	DRB9546G>T/A	N[22,23]	G>F/I/V	G	0.55	GG	0.47	0.300	0.578	0.000***	0.504****
				T	0.32	GT	0.10				
				A	0.13	TT	0.23				
						TA	0.07				
						AA	0.07				
						GA	0.07				
23	DRB9547G>T			G	0.52	GG	0.40	0.233	0.499	0.014***	0.375****
				T	0.48	GT	0.23				
						TT	0.37				

* SNP location within the 23 SNP array, ** SNP ID ware based on NCBI reference no NC_030830.1, ***significantly deviated from Hardy-Weinberg Equilibrium (HWE) ($p<0.05$), **** moderately informative polymorphism information content (PIC), Non-synonymous mutation (N), Observed heterozygosity (Ho), Expected heterozygosity (He), Phenylalanine (F), Glycine (G), Isoleucine (I),

in chromosome 23. Out of 23 SNPs detected in this study, four SNPs were previously reported by other researchers [16, 19, 43].

Furthermore, the twenty-one biallelic SNPs of the *MHC-DRB* gene were subjected to linkage disequilibrium analysis. LD plays a crucial role in mapping and identifying r^2>0.9000 (Fig. 2), indicating close to complete linked genetic variants [56]. The closest pair, with 1 bp apart, exhibited a complete linkage (r^2=1) (DRB(*BsaHI*)9459 and DRB(*BasHI*)9460), and the farthest in these selected SNP is with only 157 bp (DRB9367T>A and DRB(*PstI*)9524) (r^2=0.9022).

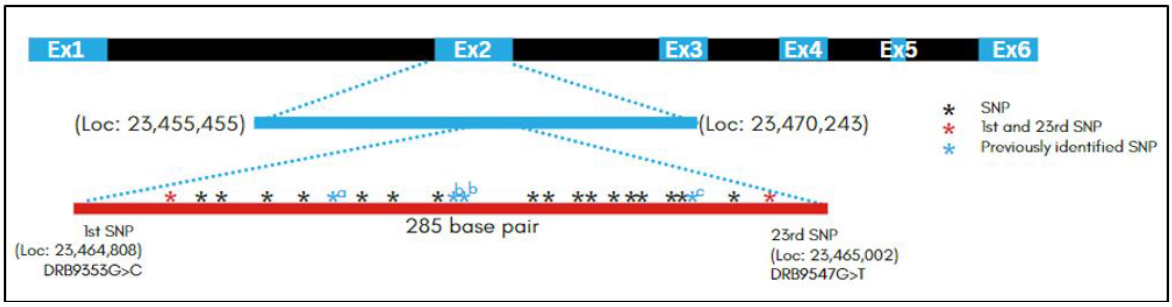

Figure 1. Illustration of the 285bp fragment of *MHC-DRB* gene showing: *locations of the single nucleotide polymorphisms within the fragment, a [19], b [16, 19], c [43].

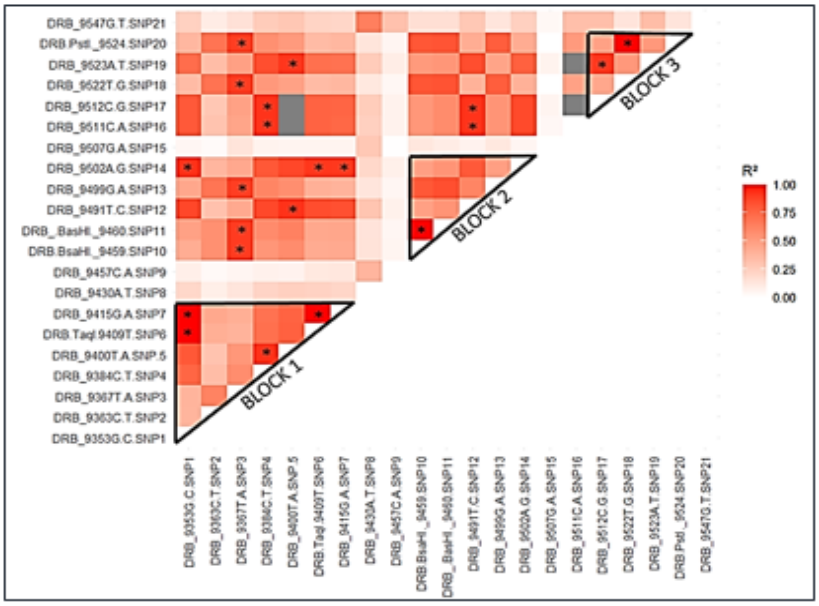

Figure 2. Heatmap-generated blocks based on the Linkage disequilibrium (r^2) values of 21 balletic SNPs in MHC-DRB gene in crossbred Anglo-Nubian goats. Dark red (high r^2 value) indicate that SNPs are most likely to be inherited together. *SNP pairs with r2> 0.9000.

haplotype blocks. It is frequently utilized to quantify the association between two genetic loci [29] and is utilized as a fundamental tool for investigating economically important traits, degree of diversity among animal breeds; r^2 represent correlation between two bi-allelic loci [29, 54, 55]. LD analysis revealed three haplotype blocks and 21 linked SNP pairs with

3.3 Association of *MHC-DRB* Gene Polymorphism with Animal's Worm Burden Category based on EPG

Goats were distributed based on EPG categories which are: Low/mild worm burden (EPG of <500), Moderate worm burden (EPG of 501-1500), Heavy worm burden (EPG of 1501-

3000), and (>3000) [36]. The 23 SNPs were found to be non-significant ($p \geq 0.05$) associated with worm burden even with the locus having the highest PIC value (DRB9546G>T/A) which might be due to the limited number of samples used in the study. The complete summary of the association of the 23 SNPs with the worm burden is presented in Supplementary Table 3.

Furthermore, Table 3 shows the association of the SNPs with high PIC values (DRB9489A>C/T and DRB9546G>T/A) and a locus (DRB9491T>C) which obtained a *p*-value of 0.03. However, the distribution of individual EPG values among the genotypes of locus DRB9491T>C was inconclusive. Specifically, 16 animals with the CC genotype exhibited moderate worm burdens, while animals with the TT and TC genotypes displayed varying worm burdens. This variability among the genotypes in terms of worm burden does not provide a clear, consistent pattern linking genetic variations specific genetic variations at the investigated loci. The diverse range of EPG values within each genotype group weakens any potential correlation between genotype and worm burden category.

The discrepancy in EPG distribution raises concerns about the statistical power of the analysis, particularly considering the small sample size used in the study. The limited sample size may have reduced the ability to detect meaningful associations, resulting in inconclusive findings. Therefore, increasing the sample size in future studies is crucial for enhancing the robustness of the analysis and more accurately assessing genetic factors influencing worm burden susceptibility.

Furthermore, LD blocks and pairs were analyzed separately to assess their association with worm burden in the animals. Consistent with the individual SNP analyses, the association analysis revealed no significant correlations between the three LD blocks (Table 4) or the 21 SNP pairs (Table 5) and EPG values ($p > 0.05$). However, one SNP pair (pair No. 10) yielded a p-value of 0.03, which is statistically significant at the 0.05 threshold. Despite this, it is important to note that one of the SNPs in this pair (DRB9491T>C) had already shown a significant p-value in the individual SNP analysis of worm burden, which was deemed inconclusive due to inconsistent EPG distribution across genotypes, with no distinct

Table 3. Association of the SNPs in the *MHC-DRB* gene of crossbred Anglo-Nubian goats with worm burden categories.

SNP ID	Genotype	Number of goats in Worm Burden Category				x^2	*p*-value
		Low	Moderate	High	Fatal		
DRB9489A>C/T	AA	3	7	2	4	13.7	0.548
	AT	0	2	0	0		
	AC	1	2	0	1		
	CC	0	2	1	1		
	CT	0	3	0	0		
	TT	0	0	1	0		
DRB9491T>C	TT	1	0	0	0	13.9	0.030*
	TC	1	0	0	0		
	CC	2	16	4	6		
DRB9546G>T/A	GG	1	7	2	4	14.6	0.481
	GT	2	1	0	0		
	TT	1	4	1	1		
	TA	0	2	0	0		
	AA	0	1	1	0		
	GA	0	1	0	1		

*significance based on *p*-value is dismissed as the distribution of genotypes per worm burden is inconclusive.

Table 4. Association of the Heatmap-generated LD blocks of the SNPs in the *MHC-DRB* gene of crossbred Anglo-Nubian goats with worm burden categories using chi-square test.

Block No.	SNPs	x^2	*p*-value
Heat Map Block 1	DRB_9353G>C DRB_9363C>T DRB_9367T>A DRB_9384C>T DRB_9400T>A DRB(*TaqI*)9409* DRB_9415G>A	28.1	0.707
Heat Map Block 2	DRB(*BsaHI*)9459* DRB(*BasHI*) 9460* DRB_9491T>C DRB_ 9499G>A DRB_9502A>G	23.6	0.788
Heat Map Block 3	DRB_9512C>G DRB_9522T>G DRB_9523A>T DRB(*PstI*)9524*	8.86	0.963

*SNPs that were previously detected in other studies.

pattern linking genotypes to worm burden categories.

4. Discussion

The *MHC II –DRB* gene in exon 2 of chromosome 23 investigated in this study code for proteins that are found on the surface of B cells and antigen-presenting cells such as macrophages, dendritic, and Langerhans cells; these molecules take part in the antigen presentation to CD4+ T cells which help B cells to produce appropriate immunoglobulins against infection [13,17,18,21-23]. This locus exhibits the most polymorphism in the *MHC* gene [4,5,13-17,19-22,29,33,43] as confirmed by the 23 SNPs identified in this study.

Table 5. Association of the linked SNP pairs based on LD of crossbred Anglo-Nubian goats with worm burden categories using chi-square test.

No.	SNP Pairs		r^2*	x^2	*p*-value
1	DRB_9353G>C	DRB(*TaqI*)9409	1.0000	7.62	0.267
2	DRB(*BsaHI*)9459	DRB(*BasHI*)9460	1.0000	5.28	0.809
3	DRB_9353G>C	DRB_9415G>A	1.0000	6.25	0.395
4	DRB(*TaqI*)9409	DRB_9415G>A	1.0000	9.22	0.417
5	DRB_9522T>G	DRB(*PstI*)9524	1.0000	5.53	0.786
6	DRB_9384C>T	DRB_9400T>A	0.9333	1.88	0.931
7	DRB_9353G>C	DRB_9502A>G	0.9329	10.3	0.326
8	DRB_9400T>A	DRB_9491T>C	0.9310	14.7	0.099
9	DRB_9384C>T	DRB_9511C>A	0.9286	4.76	0.855
10	DRB_9491T>C	DRB_9511C>A	0.9286	18.4	0.03**
11	DRB_9384C>T	DRB_9512C>G	0.9286	4.04	0.909
12	DRB_9491T>C	DRB_9512C>G	0.9286	15.6	0.076
13	DRB_9400T>A	DRB_9523A>T	0.9200	8.29	0.505
14	DRB_9512C>G	DRB_9523A>T	0.9200	9.29	0.678
15	DRB(*TaqI*)9409	DRB_9502A>G	0.9192	14.9	0.245
16	DRB_9415G>A	DRB_9502A>G	0.9158	14.1	0.297
17	DRB_9367T>A	DRB(*BsaHI*)9459	0.9073	7.44	0.944
18	DRB_9367T>A	DRB(*BasHI*)9460	0.9073	7.44	0.944
19	DRB_9367T>A	DRB_9522T>G	0.9042	13.2	0.591
20	DRB_9367T>A	DRB(*PstI*)9524	0.9022	13.5	0.76
21	DRB_9367T>A	DRB_9499G>A	0.9000	19.5	0.363

*Only pairs with r^2> 0.9000, **significance is dismissed.

Notably, four SNPs detected in the current study were also identified in previous studies [16,19,43]. These include the DRB(*TaqI*)9409 restriction site recognized by *TaqI* at 163bp/122bp, coding for the allele B/T restriction pattern or the undigested fragment allele A/t pattern [19]; DRB(*BsaHI*)9459 and DRB(*BsaHI*)9460 at 174bp/112bp, which code for allele B [16,19]; and DRB(*PstI*)_9524C>G at the site of 241bp/44bp, also coding for allele B [43]. In these studies, DNA amplification utilized similar forward and reverse primers for the *MHC-DRB* locus [34]. Subsequently, it was determined that the goat population under investigation were undergoing inbreeding, as supported by the higher He values compared to Ho and deviation from HWE [57,58] of the SNPs.

Although SNPs were determined to be moderately informative for association study, as indicated by the PIC values [19,32,59], association results revealed otherwise. The high degree of polymorphism in this gene is generally associated with the ability of the MHC-DRB molecule to recognize a wide variety of antigen-derived peptides [13-18,20]. The 14 amino acid substitutions caused by the non-synonymous SNPs found in these loci of the *MHC-DRB* gene may alter the functionality of the MHC-DRB molecule [29,60] by changing the three-dimensional conformation of the protein and affecting its ability to interact with antigenic peptides [60,61].

However, the results of this study suggest that these changes in amino acids did not significantly impact the worm burden in goats, which aligns with findings from other studies that similarly find no significant association between *MHC-DRB* polymorphisms and parasitic infections [5,19]. The lack of association may be due to various factors, including the limitations of the current study, such as a small sample size and inbreeding within the goat population. These factors highlight the need for larger and a diverse sample sizes in future studies, as well as the importance of considering population genetics and breeding practices. Such improvements could provide clearer insights into whether the *MHC-DRB* gene fragment plays a role in GIP resistance or susceptibility.

As mentioned by various researchers, *MHC* genes have great potential as a marker for selection programs in livestock breeding [24,30, 38,62]. However, studies using the fragment sequenced in the current study, primarily focus on polymorphism analysis [13,15,17,20-22,32,33] and those that explored its role in parasite infection did not find association [5,19]. In contrast, two studies sequenced two different loci of *MHC-DRB* gene using two different sets of primers. One study found allele C, based on *HaeIII* restriction enzyme pattern, to be highly expressed with high EPG counts of goats [4]. Another study associated haplotypes CCC and GCT of *MHC-DRB* with GIP infected goats [29].

Thus, given the established function of the *MHC-DRB* gene and its role in interacting with various antigens, alterations in this gene in the form of polymorphisms and changes in amino acids are expected to influence an animal's immune response. However, these changes may not be associated with GIP resistance or susceptibility. Instead, the *MHC-DRB* locus examined in this study may be linked to other economically significant traits, or the association with GIP may involve a different locus within the *MHC-DRB* gene.

5. Conclusion

Research on the correlation between gene polymorphism and GIP resistance in goats is scarce in the Philippines. With this study, the *MHC-DRB* of the crossbred Anglo-Nubian goats was confirmed to be highly polymorphic, of which the majority were non-synonymous mutations resulting in amino acid changes. Three LD blocks and eleven closely linked genetic variants were identified. However, the association analysis revealed that the individual SNP, LD blocks, and linked SNPs are not associated with the worm burden of the goats. Nevertheless, the highly polymorphic fragment investigated in this study may be utilized as a potential marker for other association studies. With these, further investigation of the SNPs, LD blocks, and linked variants identified in this study, using a larger sample size, additional goat breeds, and goats from different farms, is recommended. Such studies should focus on associating these possible markers with immune-related and other economically important traits, which would significantly contribute to the development of genetic-based strategies for animal farming, particularly in the Philippines. Additionally, the authors also recommend exploring polymorphisms in other *MHC* loci.

Availability of Data and Materials

All data are available in this study.

Author Contributions

Conceptualization, A.N.N.S., J.M.D.D., S.R.M.T., N.H.N.S., E.T.A., K.S.K., and C.S.O.M.; Methodology, A.N.N.S., J.M.D.D., S.R.M.T., N.H.N.S., E.T.A., K.S.K., and C.S.O.M.; Software, A.N.N.S., and J.M.D.D.; Validation, A.N.N.S., J.M.D.D., S.R.M.T., N.H.N.S., E.T.A., K.S.K., and C.S.O.M.; Formal Analysis, A.N.N.S., J.M.D.D., K.S.K., and C.S.O.M.; Investigation, A.N.N.S., J.M.D.D., S.R.M.T., N.H.N.S., E.T.A., K.S.K., and C.S.O.M.; Resources, A.N.N.S., J.M.D.D., E.T.A., K.S.K., and C.S.O.M.; Data Curation, A.N.N.S., J.M.D.D., K.S.K., and C.S.O.M.; Writing, A.N.N.S., J.M.D.D., S.R.M.T., N.H.N.S., E.T.A., K.S.K., and C.S.O.M.; Visualization, A.N.N.S., J.M.D.D., and C.S.O.M.; and Supervision, A.N.N.S., J.M.D.D., S.R.M.T., N.H.N.S., E.T.A., K.S.K., and C.S.O.M.

Ethics Approval and Consent to Participate

The study was conducted with the approval of the Research Integrity and Compliance Office, Institutional Animal Care and Use Committee of the Mindanao State University Iligan Institute of Technology with the IACUC Protocol Approval No.: 2024A02.

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Conflict of Interest

The authors declare no conflict of interest.

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(Original Research)

Inhibitory Effects of Processed Bignay [*Antidesma bunius* (L.) Spreng.] Fruit Pulp Against Carbohydrate - Digesting Enzymes Related to Type 2 Diabetes

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Abstract

Background: *Antidesma bunius*, locally called bignay in the Philippines, is an indigenous fruit traditionally used as an antidiabetic agent. To promote its utilization, various processes can be applied to extend its shelf life and produce functional ingredients. **Methods:** In this study, freeze dried, oven dried, spray dried, and concentrated bignay were evaluated and compared with acarbose for their *in vitro* inhibitory activities against α-amylase, α-glucosidase, and sucrase as well as its potential to inhibit glucose movement across a membrane using spectrophotometric methods. **Results:** The inhibitory activities of processed bignay against α-amylase, α-glucosidase, and sucrase were lower than acarbose and showed concentration dependence. Among the processed bignay, the freeze dried showed the maximum inhibitory activity towards α-amylase (53.47 ± 0.34%), α-glucosidase (40.59 ± 1.01%), and sucrase (44.22 ± 1.82%) at 500 µg/mL. Moreover, the freeze dried bignay had the highest glucose movement inhibition across a dialysis membrane at 500 µg/mL and at different time intervals. **Conclusions:** The inhibition of the key enzymes for carbohydrate hydrolysis and glucose movement across a membrane were best exhibited by the freeze dried bignay, followed by oven dried bignay, concentrated bignay, and least by the spray dried bignay.

Keywords: *Antidesma bunius*, Enzyme inhibition, Glucose movement inhibition, Type 2 diabetes mellitus

1. Introduction

Type 2 diabetes mellitus (DM) is considered as one of the fastest growing chronic metabolic disorders caused by the lack of insulin production, insulin action, or both resulting in an increased blood glucose level or hyperglycemia [1]. Postprandial hyperglycemia should be controlled during the early stages of type 2 DM as it results in other health complications such as heart and blood vessel disease, kidney disease, retinopathy, and peripheral neuropathy [2]. Although the pathogenesis of type 2 DM is already well studied, its overall treatment still remains a challenge. As a result, there is a continuous effort to manage type 2 DM leading to recent advances both in drug and non-drug treatments [3].

Managing type 2 DM has two main considerations, first is the prevention of carbohydrate breakdown into glucose and second is the regulation of glucose diffusion through the intestinal membrane to the blood stream [4]. The

three major enzymes in carbohydrate hydrolysis are the α-amylase, α-glucosidase, and sucrase [5-6]. The α-amylase hydrolyzes carbohydrates to smaller oligosaccharides which are further broken into glucose by the α-glucosidase for absorption in the brush border membrane of the small intestine [7]. In addition, sucrase is also present in the small intestines' brush border membrane and hydrolyzes sucrose, releasing fructose and glucose [8]. Inhibiting these three enzymes can prevent postprandial hyperglycemia by reducing the blood glucose level [9]. Acarbose, miglitol, and voglibose are the most common prescription drugs in the market that can inhibit these carbohydrate-digesting enzymes, but these are reported to be costly and have several negative gastrointestinal effects such as recurrent stomach pain, flatulence, and diarrhea [1,6,10]. Thus, plants and their natural products have been explored as alternatives to regulate diabetes due to their pharmacological properties, minimal toxicity, and lesser side effects [1]. Different plant polyphenolic compounds have been reported to have antidiabetic properties [11] by inhibiting the different digestive enzymes involved in lipid and carbohydrate hydrolysis resulting in blood glucose control [12]. However, only around 10% of these secondary metabolites have been characterized and investigated for their antidiabetic activity.

Antidesma bunius, locally called as bignay in the Philippines, has been traditionally used as medicine for a widespread diseases such as indigestion, cough, hypertension, and diabetes [13]. Several studies report that its leaves, bark, and fruits have α-amylase and α-glucosidase inhibitory activities due to the presence of different phenolic compounds with antioxidant activities [9,14-16]. However, these reported studies were done on fresh samples and none yet on dried or processed bignay. Since the fruits of bignay is seasonal, it is usually stored by conventional freezing which may cause negative biochemical changes such as loss of antioxidant properties [17]. As an alternative, different drying and concentrating processes can be applied to extend its shelf-life and convert it to functional ingredients. Carbonera *et al.* (2023) [18] reported that freeze drying and oven drying bignay fruits can improve the polyphenolic content and antioxidant activity, all of which can contribute to its antidiabetic potential. Thus, it is important to analyze for the possible inhibitory effects of bignay fruit pulp against the carbohydrate-digesting enzymes linked to type 2 diabetes as well as its ability to inhibit glucose movement across a membrane to determine if the antidiabetic potential still remains after processing.

The main purpose of this study was to determine the inhibitory potential of bignay fruits which underwent freeze drying, oven drying, spray drying, and juice concentrating against enzymes related to carbohydrate digestion such as α-amylase, α-glucosidase, and sucrase. In addition, the ability of the processed bignay to prevent glucose movement across a membrane was also investigated. The combination of both these effects will support and establish the intent to convert bignay fruit into a functional ingredient with antidiabetic potential.

2. Materials and Methods

2.1 Chemicals and raw materials

The enzymes α-amylase, α-glucosidase, and sucrase (Sigma-Aldrich, USA) and standard drug acarbose (Thermo Fisher Scientific, USA) used for the inhibitory assays were all analytical grade. In addition, the other chemicals used were also analytical grade from Sigma-Aldrich (USA), Fluka™ (Switzerland), Ajax Finechem (New Zealand), Loba Chemie (India), J.T. Baker (China), and RCI Labscan (Thailand).

Bignay fruits [*Antidesma bunius* (L.) Spreng cv. 'common'] were collected from the Institute of Food Science and Technology, University of the Philippines Los Baños (UPLB) in the month of August 2020. The harvested fruits were sorted and only the dark purple to almost black mature fruits were used. Approximately 20 kg of the mature fruits were washed and fed through a de-pulper machine (Kiya Seisakusho, Ltd., Toyo Japan) to obtain the pulp and separate the seeds and peel. The collected pulp slurry was portioned at 2 kg each in polyethylene bags and kept in the freezer (Fujidenzo, Philippines) at -20 °C.

2.2 Processing of bignay

Freeze drying, oven drying, spray drying, and juice concentration were the processing methods used for this study. Freeze drying and oven drying were selected based on prior studies reporting their ability to enhance phenolic content

and antioxidant activity, which are linked to enzyme inhibition potential. Spray drying and juice concentrating were also included as these are commonly used in industry for scalability, despite known challenges such as thermal degradation of bioactive compounds

The portioned bignay pulp slurries were dried using different methods following the parameters based on previous studies [18-21] with minor modifications to achieve a moisture content of not more than 10%. For the freeze dried bignay (FDB), the slurry was placed in the freeze dryer (Gecar Machine Solutions Inc., Philippines) for 31 h with a heater setting of 35 °C with a chilling temperature of -30 °C [18-19]. For the oven dried bignay (ODB), a pack of the thawed slurry was placed in an industrial convection oven (Memmert GmbH + Co. KG, Germany) for 31 h set at 50 °C [20]. For the spray dried bignay (SDB), the slurry was prepared according to Carbonera *et al.* (2023) [18] and then loaded to the spray dryer (MachineLab Technology Inc., Philippines) with an inlet temperature of 180 °C and outlet temperature of 103 °C [21]. For the bignay concentrate (BC), the procedure of Carbonera *et al.* (2023) [18] was also followed to obtain a concentrate with a °Brix value within 25-60 which is the recommended total soluble solids for fruit concentrates [22].

The FDB, ODB, and SDB were placed in sealable dark colored containers then inside a desiccator at room temperature. On the other hand, the BC was placed in clean and sterile PET bottles then kept in refrigerated temperature (0–4 °C).

2.3 Extract preparation

Sample extracts of FDB, ODB, and SDB were obtained using the methods of Sayah *et al.* (2017) [23] with minor revisions. For each dried sample, 50 g was weighed, combined with 500 mL 80% methanol, and mixed using a mechanical shaker for 24 hr at room temperature. The extracts were filtered and the collected filtrate was concentrated using a rotary evaporator at 40 °C. On the other hand, the BC was filtered and did not undergo any extraction step. Appropriate dilution for each sample extract were prepared prior to each analysis.

2.4 α-amylase inhibition assay

The α-amylase inhibition assay was carried out following Shettar *et al.* (2017) [24]. Different concentrations of each sample and the standard drug acarbose at 100, 200, 300, 400, and 500 µg /mL were prepared. A volume of 0.5 mL for each sample was added with 0.5 mL α-amylase solution (0.5 mg/mL) in 0.02 M sodium phosphate buffer (pH 6.9). The mixture was allowed to stand for 10 min at room temperature and added with 0.5 mL 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9). The solution was mixed and allowed to stand for another 10 min at room temperature then added with 1 mL of dinitrosalicylic acid (DNS) to stop the reaction. The solution was placed in a 100 °C water bath for 5 min, cooled to 30 °C, added with 10 mL of deionized water, then mixed. The absorbance for each solution was read at 540 nM. A blank (buffer only) and control (buffer with amylase solution) were also prepared and absorbances were determined using the same wavelength. The α-amylase inhibitory activity was computed as:

$$\%\ \alpha - amylase\ inhibition = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

2.5 α-glucosidase inhibition assay

The α-glucosidase inhibition assay was done following Ma and Liu (2020) [25] and Bhatia *et al.* (2019) [1] with modifications using different concentrations of each sample and acarbose at 100, 200, 300, 400, and 500 µg/mL. The sample solutions, acarbose, α-glucosidase (0.1 U/mL), and *p*-NPG (1 mM) were prepared with 0.1 M phosphate buffer (pH 6.8). For each sample, 1 mL was added with 0.2 mL α-glucosidase solution and 2.5 mL 0.1 M phosphate buffer (pH 6.8) then mixed. The solution incubated at 37 °C for 20 min. Then, 0.4 mL 1mM *p*-NPG was added and the solution was incubated for another 30 min at 37 °C. The reaction was stopped with the addition of 1 mL 0.1 N Na_2CO_3. Then the absorbance determination was done at 405 nM. The absorbances of the blank (buffer only) and control (buffer instead of extract) solutions were also

obtained. The α-glucosidase inhibitory activity was calculated as:

$$\%\ \alpha - glucosidase\ inhibition = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

2.6 Sucrase inhibition assay

The sucrase inhibition assay was conducted following the procedure of Antora *et al.* (2018) [6] using different concentrations of each sample and acarbose at 100, 200, 300, 400, and 500 µg /mL. The samples, acarbose, sucrase (4.8 U), and sucrose (60 mM) were prepared using phosphate buffer saline (pH 7.4). In a test tube, 0.25 mL of each sample was added with 0.25 mL sucrase solution, mixed, and allowed to stand for 10 min at 37 °C. The mixture was added with sucrose solution, incubated for 30 min at 37°C, added with 1 mL DNS, and boiled for 5 min. It was then added with 1 mL distilled water, mixed, and the absorbance was read at 540 nM. The absorbances of the blank (buffer only) and control (buffer instead of extract) solutions were also read. The % inhibition was determined using the equation:

$$\%\ sucrase\ inhibition = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

2.7 Inhibitory concentration 50% (IC_{50}) value calculation

The concentration of each sample extract which can inhibit 50% of the enzyme activity or IC_{50} was computed including the standard drug acarbose for comparison. For each of the sample extract, a standard dose response curve was plotted using the different concentrations and the IC_{50} was calculated using the software Graph Pad Prism version 8.1.

2.8 Glucose diffusion inhibition assay

This inhibition assay proceeded following the methods of Roy and Mahalingam [4] with minor modifications using different concentrations of each sample and acarbose at 100, 200, 300, 400, and 500 µg/mL. Strips of 12000 MW dialysis membrane (Sigma Aldrich, USA) were prepared following the protocol provided by the manufacturer. The prepared dialysis membrane was soaked in distilled water and kept in the refrigerator at 4 °C prior to the analysis.

In a test tube, 2 mL of each sample and the acarbose solution were added with 2 mL of 0.15 M sodium chloride with 0.22 mM glucose solution, mixed, and then transferred to the prepared dialysis membrane with one end sealed. The other end of the dialysis membrane strips was also sealed and placed in 100 mL beakers containing 40 mL 0.15 M sodium chloride solution, and 10 mL distilled water. A magnetic stirrer was added at the bottom of the beaker and then placed in a stirring plate set at 200 rpm. Sample aliquot of 1 mL was taken from each beaker every 30 min for 3 hr. The collected sample aliquots were tested for glucose concentration using phenol sulfuric acid method using the protocols of Tamboli *et al.* in 2020 [26]. The relative inhibition of glucose

$$\%\ Inhibition\ of\ glucose\ movement = \frac{(glucose\ conc._{initial} - glucose\ conc._{final})}{glucose\ conc._{initial}} \times 100$$

movement (%) was computed as:

2.9 Statistical analysis

The assays were done in three trials for each concentration of all the samples as well as the acarbose and the calculated data were presented as mean ± standard deviation. The results were tested for homogeneity with Levene's test and evaluated by one-way analysis of variance (ANOVA) using the Minitab® version 18.1 (Minitab, Inc., USA). Tukey's Honestly Significant Difference (HSD) test ($p \leq 0.05$) then followed to determine the significant differences among the means.

3. Results

3.1 α-amylase, α-glucosidase, and sucrase inhibitory activities

The α-amylase inhibitory activity of the different processed bignay in comparison with the standard drug acarbose is presented in Figure 1. Results showed that all processed bignay inhibited α-amylase activity in a concentration-dependent manner (100–500 µg/mL) wherein a higher concentration resulted in higher inhibitory activity. For each concentration, the α-amylase

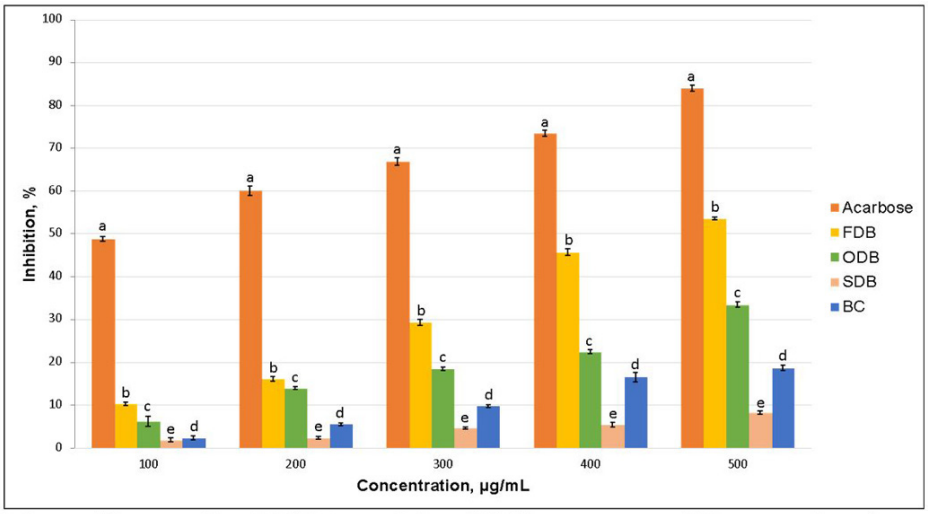

Figure 1. α-amylase inhibitory activity of different processed bignay and acarbose at different concentrations. Processed bignay samples: freeze dried bignay (FDB), oven dried bignay (ODB), spray dried bignay (SDB), and bignay concentrate (BC). Means keyed with different letters within each concentration indicate significant differences at $p < 0.05$.

inhibitory activity of all processed bignay were significantly different from each other and were all lower than the standard drug acarbose. Among the processed bignay at the 500 µg /mL concentration, FDB has the highest α-amylase inhibitory activity (53.47 ± 0.34%) followed by the ODB (33.42 ± 0.62%), BC (18.61 ± 1.04%), and lastly the SDB (8.19 ± 0.48%).

In addition, the α-glucosidase inhibitory activity of the processed bignay and the standard drug acarbose at different concentrations are summarized in Figure 2. Results also showed that all processed bignay inhibited α-glucosidase activity in a concentration-dependent manner (100–500 µg/mL) and at a higher concentration, the higher the inhibition. The inhibitory activity of α-glucosidase for all processed bignay showed significant differences with each other at each concentration and were also lower than acarbose. At 500 µg/mL, FDB also has the highest inhibitory activity (40.59 ± 1.01%) among the processed bignay against α-glucosidase followed by the ODB (28.63 ± 0.81%), the BC (18.95 ± 1.45%), while the SDB has the lowest inhibitory activity (13.98 ± 1.01%).

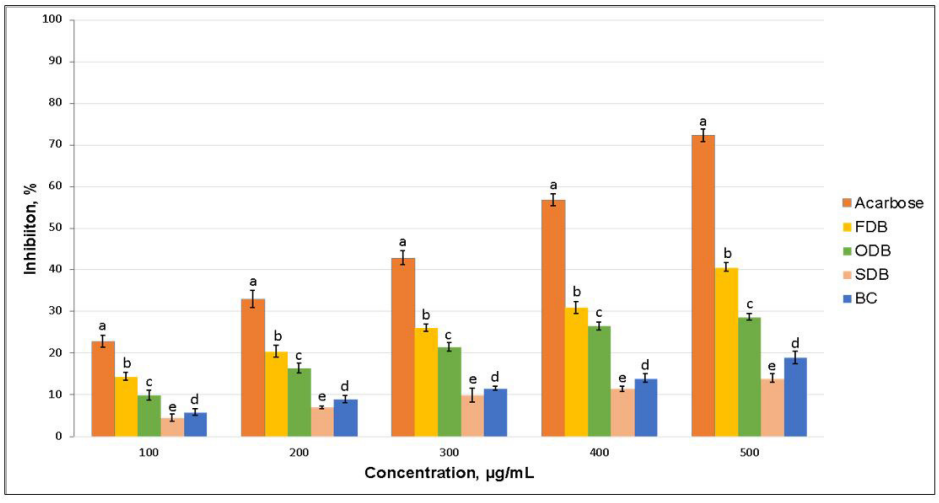

Figure 2. α-glucosidase inhibitory activity of different processed bignay and acarbose at different concentrations. Processed bignay samples: freeze dried bignay (FDB), oven dried bignay (ODB), spray dried bignay (SDB), and bignay concentrate (BC). Means keyed with different letters within each concentration indicate significant differences at $p < 0.05$.

Likewise, the processed bignay have also shown concentration-dependent (100–500 µg/mL) inhibitory effect on sucrase (Figure 3). The inhibitory effect of all processed bignay at the different concentrations were also lower than acarbose. Comparing the processed bignay at the highest concentration, FDB showed the highest inhibitory effect on sucrase (44.22 ± 1.82%), followed by the ODB (31.99 ± 1.42%), the BC (20.70 ± 1.01%), and lastly by the SDB (13.98 ± 0.84%).

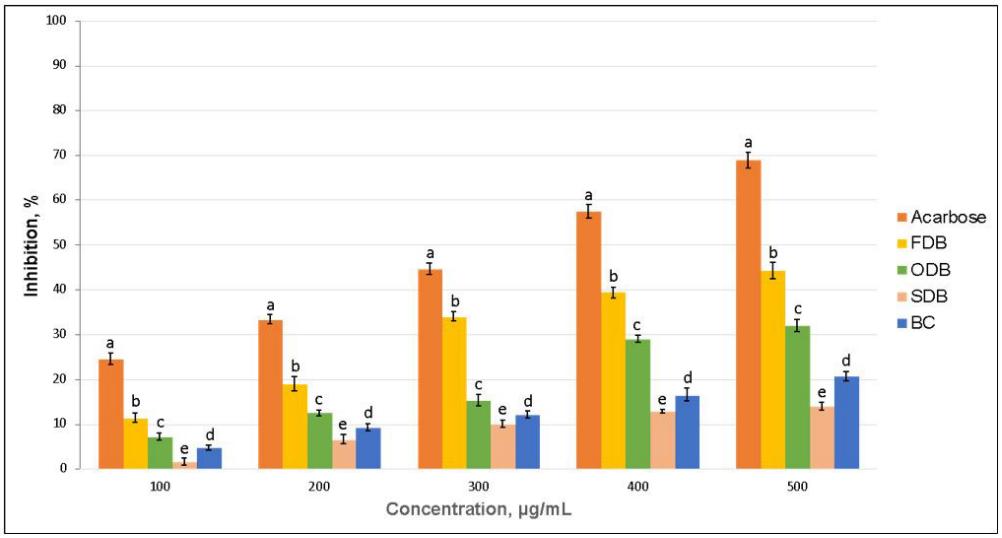

Figure 3. Sucrase inhibitory activity of different processed bignay and acarbose at different concentrations. Processed bignay samples: freeze dried bignay (FDB), oven dried bignay (ODB), spray dried bignay (SDB), and bignay concentrate (BC). Means keyed with different letters within each concentration indicate significant differences at $p < 0.05$.

The IC_{50} values of the different processed bignay and acarbose showed significant differences within the three tested carbohydrate-digesting enzymes as presented in Table 1. The IC_{50} values of acarbose for α-amylase (100.68 ± 7.97 µg/mL), α-glucosidase (336.61 ± 1.90 µg/mL), and sucrase (337.06 ± 3.44 µg/mL) indicated strong inhibition as compared to all processed bignay. Among the processed bignay, FDB is the most potent inhibitor against α-amylase, α-glucosidase, and sucrase having IC_{50} values of 464.30 ± 1.90 µg/mL, 675.93 ± 35.71 µg/mL, and 538.09 ±18.91 µg/mL, respectively, while the lowest was the SDB for all three tested enzymes. The substantial differences in the IC_{50} values of acarbose and the processed bignay is mainly because acarbose is a

Table 1. The IC_{50} values for α-amylase, α-glucosidase, and sucrase of processed bignay and acarbose.

Sample	IC_{50} values (µg/mL)		
	α-amylase	α-glucosidase	Sucrase
Acarbose	100.68 ± 7.97^{e}	336.61 ± 1.90^{e}	337.06 ± 3.44^{e}
FDB	464.30 ± 1.90^{d}	675.93 ± 35.71^{d}	538.09 ±18.91^{d}
ODB	794.12 ± 15.72^{c}	919.54 ± 10.05^{c}	767.54 ± 33.92^{c}
SDB	3196.02 ± 367.86^{a}	2062.21 ± 311.54^{a}	1637.56 ± 178.68^{a}
BC	1206.72 ± 33.15^{b}	1542.77 ± 181.59^{b}	1255.03 ± 70.05^{b}

Value Means ± SD (n=3); IC_{50}: Half-maximal inhibitory concentration
FDB = Freeze-dried bignay; ODB = Oven dried bignay at 50 °C; SDB = Spray dried bignay; BC = Bignay concentrate.
Means keyed with different letters within each column indicate significant differences at $p < 0.05$.

pure standard drug while the extracts from the processed bignay were crude and not purified.

3.2 Glucose diffusion inhibitory activity

The relative inhibition of glucose movement across a membrane of the different processed bignay and acarbose at 500 µg/mL and different time intervals is summarized in Figure 4. It can be observed that there is a constant decrease in the percent inhibition of glucose movement over time for all processed bignay as well as the acarbose. In addition, the relative inhibition of glucose movement of the FDB and ODB were not significantly different with each other from 30 to 90 min while the rest of the processed bignay were significantly different from each other at each time interval and were all lower than the standard drug acarbose. Among the processed bignay, FDB and ODB showed substantial amounts of inhibition while the SDB showed poor inhibition.

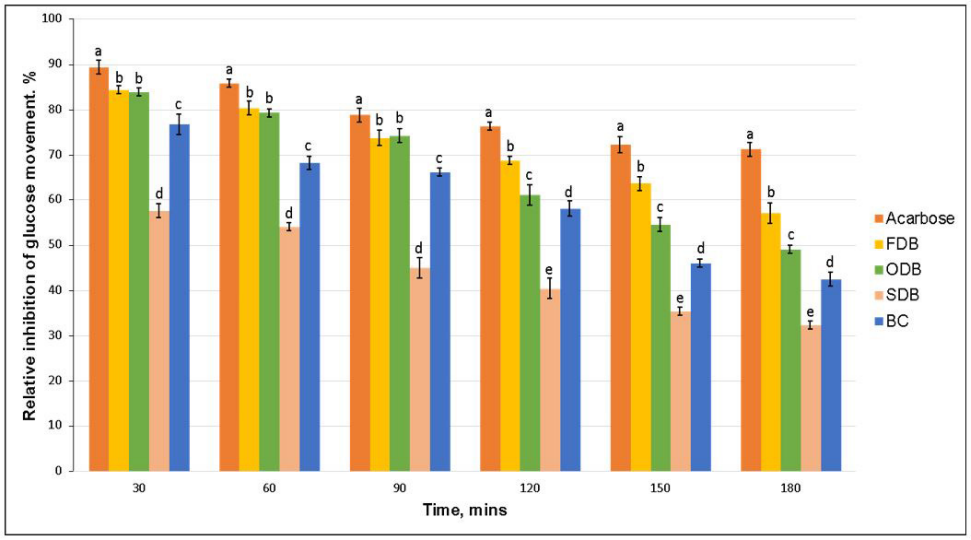

Figure 4. Glucose movement inhibition across a membrane of different processed bignay and acarbose at 500 µg/mL at different time intervals. Processed bignay samples: freeze dried bignay (FDB), oven dried bignay (ODB), spray dried bignay (SDB), and bignay concentrate (BC). Means keyed with different letters within each time indicate significant differences at $p < 0.05$.

4. Discussion

The inhibitory property of the processed bignay, specifically the FDB, against the enzymes responsible for carbohydrate digestion could be related to the phenolic compounds present. Several studies report that different phenolic compounds show inhibitory activities against α-amylase, α-glucosidase, and sucrase [6,23, 27-28]. Specifically, flavonoids, flavonols, and anthocyanins show inhibitory effect to α-amylase and α-glucosidase [1, 29] while eugenols, rosmarinic acids, luteolin, apigenin, glucosides, saponins were found to have sucrase inhibitory property [30-31].

Fresh bignay fruits are reported to have high total phenolics, total flavonoids, and total anthocyanins [19, 32] which have inhibitory effects towards α-amylase, sucrase [9] as well as α-glucosidase [10]. For processed bignay fruits, Carbonera et al. in 2023 [18] reported that freeze drying as well as oven drying significantly improved the total phenolic, total flavonoid, and total anthocyanin contents while spray drying and juice concentration have detrimental effects. In addition, the major phenolic compounds in dehydrated bignay are reported to be epicatechin, catechin, and gallic acid [18] and these have inhibitory effects towards α-amylase [32]. This study extends previous related studies by demonstrating that processing methods such as freeze drying can improve the antidiabetic activity of bignay fruit. But since phenolic compounds are heat sensitive due to their thermolabile molecular structure [33], the high temperature of spray drying (103–180 °C) and juice concentrating (80 °C) may have caused thermal degradation on the phenolic compounds, which in turn affected their inhibitory effects against α-amylase, α-glucosidase, and sucrase. Similar studies also reported that heat treatment at 100°C and higher

can decrease the activity of polyphenolic compounds to inhibit α-amylase [34] and α-glucosidase [35].

Glucose movement across the small intestines' brush border membrane plays an important role in the regulation of carbohydrate uptake where generally glucose is transferred by the sodium-dependent glucose transporter (SGLT1) and the facilitated-transporter glucose transporter (GLUT2) [36]. The processed bignay can decrease glucose movement because of the presence of polyphenols. The different phenolic compounds can suppress the absorption of glucose into the intestinal cells by inhibiting SGLT1 and GLUT2 [11, 37]. This is also in agreement with other studies on plants with high phenolic content and antioxidant activity such as *Caralluma europaea* [38], *Cleome viscora* [39], and *Phoenix roebelenii* leaves [4] which also demonstrated significant inhibitory effect on the movement of glucose through a dialysis membrane to an external solution at different time intervals. Furthermore, a study on the effect of spray drying on the blueberry juice polyphenolic compounds showed a 76-78% loss in the total phenolics and 57% loss on the anthocyanins [40]. Thus, the poor inhibition activity exhibited by the SDB is due to the high temperature of the spray drying process which resulted in the thermal degradation of the phenolic compounds in bignay.

While the *in vitro* assays in this study provide valuable insights into the inhibitory effects of processed bignay fruit on carbohydrate-digesting enzymes and glucose movement, they do not account for the complexities of physiological systems. Thus, further studies that include in vivo models to evaluate bioavailability, metabolism, and potential side effects in diabetic individuals should be considered.

5. Conclusions

Processed bignay displays potential antidiabetic activity by preventing the breakdown of complex carbohydrates through inhibition of the key carbohydrate-digesting enzymes as well as reducing the rate of glucose movement across a membrane. The inhibitory effect of the processed bignay is more towards α-amylase, followed by sucrase, and lastly towards α-glucosidase – all of which are concentration-dependent. Among the processed bignay, the FDB had the highest inhibitory activity against the three tested enzymes and on the inhibition of glucose movement. Therefore, it is recommended to use this drying process in converting bignay fruit into a functional ingredient. Since this study included only *in vitro* assays, further validation through in vivo animal models or clinical studies is recommended to confirm and validate the observed effects.

Availability of Data and Materials

All data are available in this study

Author Contributions

Conceptualization, Resources, Funding acquisition, and Writing – Review & Editing, A.F.A.C, L.M.A, M.A.C.E, S.M.D., R.C.M.L.A., and K.A.T.C.I.; Methodology, A.F.A.C. and K.A.T.C.I.; Investigation and Writing – Original Draft, A.F.A.C.; Supervision and Project administration, L.M.A, M.A.C.E, S.M.D., R.C.M.L.A., and K.A.T.C.I.

Ethics Approval and Consent to Participate

Not applicable

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Not applicable

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Conflict of Interest

The authors of this study declare no conflict of interest.

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(Short Communication)

Peste des Petits Ruminants (PPR) Outbreaks in Wildlife Populations in IRAN, 2001- 2024

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Abstract

Background: Peste des Petits ruminants (PPR) is a highly contagious viral disease affecting small ruminants, with outbreaks reported in over 65 countries. This study aimed to assess PPR outbreaks in wildlife populations in Iran. **Methods**: Data on PPR outbreaks from 2001 to October 2024 were compiled from Farsi literature and the Google search engine for confirmed reports. **Results**: A total of 36 outbreaks were recorded among Bovidae species, including wild goats (*Capra aegagrus*), Mouflon (*Ovis gmelini*), and Urial (*Ovis vignei*), with over 77% occurring in protected areas. Most outbreaks (73%) occurred in summer and autumn. No outbreaks have been reported since July 2023, likely due to mass livestock vaccination as part of the national PPR eradication program. Inadequate vaccination coverage, illegal livestock trading, and non-compliance with quarantine measures have contributed to recurrent outbreaks in wildlife. Traditional livestock practices and grazing in protected areas have facilitated spillover into wild populations. **Conclusions**: The 36 reported outbreaks across 16 provinces, spread across diverse geographical regions, highlight the urgent need for comprehensive strategies, including consistent vaccination programs for domestic herds, stricter border control measures, and enhanced law enforcement in protected areas to prevent further outbreaks in wildlife populations.

Keywords

Peste des Petits Ruminants, Outbreak, Eradication, Wildlife, Livestock

1. Introduction

Peste des Petits ruminants (PPR) is a highly infectious disease of small ruminants, caused by a virus from the *Morbillivirus* genus in the *Paramyxoviridae* family. PPR occurs across Africa (excluding the southern countries), the Middle East, Turkey, West and South Asia, and China [1]. Morbidity and mortality rates are typically high, and the disease can trigger epidemics with up to 100% mortality in susceptible sheep and goat populations [2].

The host ranges for PPR virus and Rinderpest virus are likely identical, encompassing members of the order *Artiodactyla* [3]. Despite its ability to infect various wild animal species, there is currently no evidence to suggest that PPR is maintained within wild populations, an important factor for eradication efforts. Several characteristics of PPR favor its eradication: it has a short infectious period, no carrier status, and transmission occurs mainly through direct contact instead of being vector-borne [1]. Additionally, the virus's survival traits are believed to be similar to those of the Rinderpest virus, the only animal disease officially eradicated, as declared in 2011 [4]. The PPR virus does not persist for extended

periods in outdoor environments, as it is sensitive to heat, ultraviolet light, and dehydration. It remains stable at 4°C within a pH range of 7.2–7.9, with a half-life of 3.7 days [5]. Due to these characteristics, PPR has been designated as a target for the Global Eradication Programme (PPR GEP), launched by the Food and Agriculture Organization of the United Nations (FAO) and the World Organisation for Animal Health (WOAH), with the goal of eradicating the disease by 2030 [1,4,6].

Placed at the crossroads of the Palearctic, Saharo-Arabian, and eastern zoogeographic domains, and with its substantial environmental diversity, Iran is a habitat of high complexity and richness of fauna and flora [7]. Listed known terrestrial mammals of Iran, including 192 species, dispersed in 90 genera, 34 families, and seven orders. Ten species among them belong to the order *Artiodactyla* (six species in the family *Bovidae*, three species in *Cervidae*, and one in *Suidae*) [7].

Since the first reported outbreak among domestic herds in 1995, PPR has been endemic in Iran and spread to all provinces within the following decade [8]. Catastrophic deaths and severe clinical signs have been reported, which can be attributed to poor hygiene and a lack of proper vaccination coverage in domestic flocks over the past decades. In addition to domestic flocks, the disease has been reported among wild populations across a wide geographical range of the country, from the north to the south, since 2001 [9]. Due to numerous challenges in studying wildlife diseases, particularly PPR, in Iran, few studies have been conducted on the occurrence of PPR in wildlife species. Furthermore, since there is no comprehensive list of PPR outbreaks in wildlife populations in Iran in previous literature, we have attempted to compile as detailed a list of outbreaks (including some suspected cases) as possible. We have also sought to retrospectively analyze the different aspects of the disease transmission and spillover from domestic herds to wildlife.

2. Results and Discussion

In total, 36 outbreaks of PPR have been recorded in the wildlife of Iran. The first outbreak was reported in May/June 2001, and the most recent outbreak occurred in August 2023 (Table 1). Since then, no additional outbreaks have been reported by provincial environmental organizations' websites, collaborating laboratories, or the press. Outbreaks have occurred in 16 provinces (Fig. 1). With one exception, all outbreaks have involved wild goat and sheep populations. Taleghan Protected Area, with four outbreaks, had the highest number of reported cases. In terms of provincial divisions, Fars Province had the highest number of outbreaks, totaling seven. The outbreaks have occurred in both protected and free areas across a variety of regions with different altitudes, mean monthly temperatures, and habitats (ranging from semi-desert to forest). In four outbreaks, the number of collected carcasses was not reported. In the remaining 32 outbreaks, at least 4,804 carcasses were collected. The approximate number of collected carcasses in the first two outbreaks was much higher than in other incidents. A total of 25% of all reported outbreaks (nine out of 36) occurred in 2021, followed by five outbreaks in 2020 and four in 2015. Of all reported outbreaks, 38.9% (14 out of 36) occurred in National Parks, which have the highest level of protection compared to other categories. The seasonal occurrence of outbreaks was evaluated in 33 cases (one outbreak lacked time information, and two outbreaks lasting over seven to eight months were excluded). Outbreaks were reported in all seasons, with the highest prevalence in autumn and summer (both 36.4%) and the lowest in winter (6%) (Fig. 2).

In the past two decades, PPR has been detected in over 65 countries across Africa, Asia, and the Middle East [4]. In 2017, an outbreak in Mongolia led to an 80% reduction in the population of Saiga antelope (*Saiga tatarica mongolica*) [4]. Additional outbreaks have also been reported in China, affecting ibex (*Capra ibex sibirica*), argali sheep (*Ovis ammon*), and goitered gazelle (*Gazella subgutturosa*) populations. Serological studies in Africa have indicated repeated PPR infections in a variety of wildlife species, including African buffalo (*Syncerus caffer caffer*), blue wildebeest (*Connochaetes taurinus*), impala (*Aepyceros melampus*), common tsessebe (*Damaliscus lunatus*), Grant's gazelle (*Nanger granti*), Dorcas gazelle (*Gazella dorcas*), common duiker (*Sylvicapra grimmia*), kob (*Kobus kob*), waterbuck (*Kobus ellipsiprymnus*), and western hartebeest (*Alcelaphus buselaphus*) [10]. Despite American white-tailed deer (Odocoileus virginianus) being experimentally fully susceptible to PPR virus [11],

Table 1. PPR outbreaks in wildlife populations in Iran, 2001-2024.

	Region	Year	Month	Species	Collected Carcasses	Source of Data
1	Kavir National park	2001	May/Jun	Wild goats (*Capra aegagrus*) - Goitered gazelles (*Gazella subgutturosa*)	1500	[9]
2	Sarigol National park	2011	Aug/Sept	Urial (*Ovis vignei*)	550-700	[9]
3	Bamou National park	2014	Sept–Nov	Wild goats (*Capra aegagrus*)	50	[9]
4	Haftad-qolleh Arak	2015	Apr/May	Wild goats (*Capra aegagrus*) Mouflon (*Ovis gmelini*)	458	[9], IEW
5	Kharmaneh sar Tarom	2015	Aug/Sep	Wild goats (*Capra aegagrus*)	126	[9], IEW
6	Alamout protected area	2015	Sept–Nov	Wild goats (*Capra aegagrus*)	30	[9]
7	Taleghan protected area	2015	Nov/Dec	Wild goats (*Capra aegagrus*)	204	[9], IEW
8	Sorkheh Hesar National park	2016	April	Mouflon (*Ovis gmelini*)	10	IEW
9	Khojir National park	2016	Apr/May	Wild goats (*Capra aegagrus*)	85	[9], IEW
10	Baba Aman park	2016	September	Wild goats (*Capra aegagrus*)- Urial (*Ovis vignei)*	50	IEW
11	Seidova National park (Semnan)	2017	September	Wild goats (*Capra aegagrus*)- Mouflon (*Ovis gmelini*)	80	[9], Workshop2021, IEW
12	Salook National park	2017	November	Urial (*Ovis vignei*)	40	[9], Workshop2021, IEW
13	Khabr National park	2018	Jun	Wild goats (*Capra aegagrus*)- Wild sheep (*Ovis gmelini* × *O. vignei*)	56	[9], Workshop2021, IEW
14	Taleghan protected area	2018	October	Wild goats (*Capra aegagrus*)- Wild sheep (*Ovis gmelini* × *O. vignei*)	28	Alborz.doe
15	Siyahdaran forest park (Talesh)	2019	May	Red deer (*Cervus elaphus*)	6	Gilan.doe
16	Hormud protected area ((Larestan)	2019	August	Wild goats (*Capra aegagrus*)- Mouflon (*Ovis gmelini*)	123	Workshop2021, Fars,doe
17	Khorasan Razavi (Gonabad & Begestan)	2019	September	Urial (*Ovis vignei*)	200	Workshop2021, Sko.doe
18	Southern Khorasan (Ferdows & Sarayan)	2020	February	Urial (*Ovis vignei*)	70	Sko.doe

19	Kolah Ghazi National park	2020	Feb-Oct	Wild goats (*Capra aegagrus*)-	460	Workshop2021, Isfahan.doe, IEW
20	Toot-e Siyah hunting prohibited area	2020	September	Wild goats (*Capra aegagrus*)	55	Workshop2021, Fars.doe
21	Damavand hunting prohibited area	2020	September	Wild goats (*Capra aegagrus*)- Wild sheep (*Ovis gmelini* × *O. vignei*)	24	Workshop2021, Tehran,doe
22	Tang-e Sayad National park	2020	…….	Wild goats (*Capra aegagrus*)	29	[8]
23	Bahram-e Gur protected area	2021	Jan-June	Wild goats (*Capra aegagrus*)	100	Fars.doe, Mehrnews
24	Darz and Sayeban (Larestan)	2021	April	Wild goats (*Capra aegagrus*)	6	Irna
25	Marvar Yazd hunting prohibited region	2021	June	…	…	Yazd.doe, Jamejam
26	Chaharmahal and Bakhtiari Province	2021	July	Wild goats (*Capra aegagrus*)- Mouflon (*Ovis gmelini*)	40	Irna, Jamejam
27	Ghamishlou National Park	2021	July	Wild goats (*Capra aegagrus*)- Mouflon (*Ovis gmelini*)	…	Irna, Jamejam
28	Tandooreh National Park	2021	August	Wild goats (*Capra aegagrus*)	24	Irna, Jamejam, Rko.doe
29	Mazandaran (Different area)	2021	Sept-Dec	Wild goats (*Capra aegagrus*)	172	Hakimemehr
30	Salook National park	2021	October	Wild goats (*Capra aegagrus*)	16	Hakimemehr
31	Taleghan protected area	2021-22	Oct-Feb	Wild goats (*Capra aegagrus*)- Wild sheep (*Ovis gmelini* × *O. vignei*)	100	Alborz.doe
32	Do Hezar & Se Hezar, Mazandaran	2022	April	Wild goats (*Capra aegagrus*)	…	Ghatreh
33	Hava hunting prohibited area (Lamerd)	2022	May	Wild goats (*Capra aegagrus*)- Mouflon (*Ovis gmelini*)	5	Unpublished Data from Fars.doe
34	Taleghan protected area	2022	August	Wild goats (*Capra aegagrus*)	104	Alborz.doe
35	Angoran protected area (Zanjan)	2023	July	Wild goats (*Capra aegagrus*)	…	Irna
36	Bamou National park	2023	July	Wild goats (*Capra aegagrus*)	3	Farsnews

Figure 1. Estimated geographic distribution of PPR outbreaks in wildlife populations in Iran since 2001. (Outbreaks were numbered in the same order as listed in the table.)

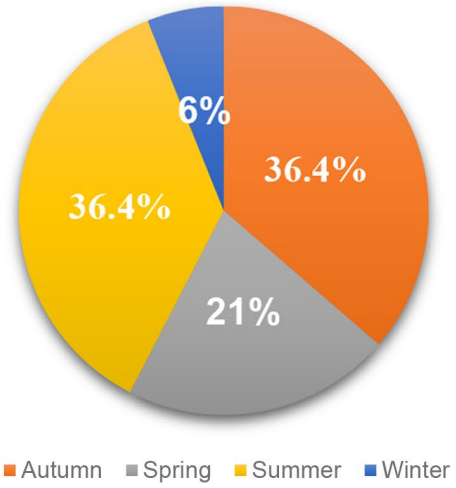

Figure 2. Percentages of PPR Outbreaks in each Season [Autumn (12 outbreaks) - Spring (7 outbreaks) - Summer (12 outbreaks) - Winter (two outbreaks)] in wildlife in Iran 2001- 2024.

Red deer appeared to be insusceptible [12]. However, an outbreak of PPR in Siyahdaran Forest Park (Talesh) that resulted in the death of six red deer challenges this assumption.

In Iran, the first documented outbreak of PPR was reported in 1995 in the Ilam province. In the same year, eight other provinces engaged, and a total of 39 affected domestic sheep and goat flocks were detected [13]. The first outbreak among wild species occurred in 2001 in Kavir National Park, where approximately 1,500 carcasses of wild goats (*Capra aegagrus*) and Persian gazelles (*Gazella subgutturosa*) were collected. It was estimated that 25% to 40% of the wild goat population was lost due to PPR. From 1995 to 2004, the disease

spread to 28 provinces in Iran [13]. However, no outbreaks were reported in wildlife populations until 2011. Given that the disease had spread to domestic livestock in nearly all provinces during that period, and that over 5,000 outbreaks among domestic goat and sheep herds in Iran were reported to the OIE between 2005 and 2011, accounting for 32% of all reported outbreaks in that timeframe [14], it is unlikely that wildlife populations were unaffected. The third outbreak occurred in Bamou National Park in 2014, three years later. Since then, at least one outbreak occurred every seven months from September 2014 to July 2023. The absence of any reported outbreaks afterward can be attributed to organized and consistent mass vaccination of livestock throughout the country, aiming to cover at least 85% of the population for three consecutive years as the control stage (Phase II) of the national eradication program, as outlined in the Global Eradication Program [6,1]. Unreported data from the Veterinary Organization indicate that the number of focal points for PPR outbreaks in livestock has declined from 850 in 2018 to 25 in 2024.

The role of wildlife in the spread of PPR is not completely understood, and there is no evidence to confirm that PPR circulates in wild animals independently of domestic animals [3]. Nevertheless, it is widely recognized that the PPR virus circulates among domestic ruminants, acting as a potential reservoir for transmission to wild animal species. The contribution of small domestic ruminants to the epidemiology of PPR in wild ruminants is well-documented [15]. The virus cannot survive for long in the environment [5] and is not self-sustaining in wild small ruminants [3]. On the other hand, nomadic and semi-nomadic forms of pastoralism, are still widely practiced in most of Iran [4]. According to the Food and Agriculture Organization Statistics (FAOSTAT) in 2020, the population of domestic sheep and goats in Iran was about 63,250,731 [4]. In Iran, due to animal-browsing licenses issued decades ago, as well as a lack of quality pastures, avoiding shared pastures between domestic herds and wild small ruminants can be a source of conflict. It is likely that, in cases where pasture resources are exchanged between domestic and wild animals, the spillover of the PPR virus is facilitated between the two populations [15]. Therefore, controlling PPR in livestock populations is essential to prevent its spread to wildlife. More than 77% of all outbreaks have occurred in different types of protected areas (National Parks, Protected Areas, Hunting Prohibited Areas). At least 15 outbreaks in 13 National Parks have been reported. Although legislation prohibits livestock grazing in National Parks, the Environmental Organization lacks the necessary authority and resources to effectively enforce these regulations and prevent livestock flocks from entering the parks.

A previous study on PPR seroprevalence in small ruminants in Garmsar City [16], showed that the odds ratio of PPR infection in autumn is higher than in other seasons. In contrast, some studies reported that the PPR infection rate was higher in spring than in other seasons [17,18]. In this study, the disease has been recorded in all months of the year. More than 27 outbreaks (75%) out of 36 were reported in summer and autumn (or part of the outbreak occurred during these seasons). One reason could be the higher likelihood of indirect contact with domestic flocks in summer due to limited water and food resources. Another possible reason could be that favorable environmental conditions in autumn enhance the virus's viability.

PPR is not only endemic in Iran but also in many of Iran's neighboring countries, and it has been demonstrated that the PPR virus strains detected in those countries share the same lineage as the strains found in Iran [19]. Weaknesses in the border quarantine system and animal trafficking from neighboring countries are other challenges in controlling the disease in Iran [20]. PPR has been documented in Pakistan since 1991, and despite the use of a live attenuated vaccine in small ruminants, it has remained endemic [21]. The first detection of PPR in Turkey occurred in 1999 in Eastern Anatolia, with outbreaks also reported among wildlife populations [4]. In Iraq, the first documented case was in 1998, and in recent years, occurrences have been reported from various regions in both the north and south of the country [22]. Additionally, PPR caused more than 750 deaths among wild goats (*Capra aegagrus*) in the Iraqi Kurdistan region [23]. In Afghanistan, PPR was detected in 1995 and has since been endemic throughout the country [24]. Furthermore, except for Qatar, PPR outbreaks have been reported in all other countries with maritime borders with Iran in the Persian Gulf, including Kuwait, Oman, Saudi Arabia, the United Arab Emirates, and Bahrain (PPR has

never officially been reported in Qatar, but several studies have confirmed the presence of the virus) [23]. Livestock trafficking and inadequate monitoring of cross-border livestock trade may play a significant role in the circulation of the PPR virus and its persistence among domestic flocks in the Middle East. In addition to regional livestock trafficking, illegal livestock trade represents a significant challenge within the borders of several countries in this region.

3. Conclusions

The thirty six (36) recorded outbreaks in 16 provinces indicate that while mass vaccination of livestock is likely the most crucial measure for eradicating PPR, merely controlling epidemics and outbreaks will not suffice. Outbreaks were reported throughout the year, with the highest prevalence in autumn and summer. It is essential to consider traditional husbandry practices, interprovincial illegal animal trade, and livestock trafficking, alongside the inadequate coverage of mass vaccination, as factors contributing to the establishment of stable infection reservoirs in domestic sheep and goat populations. Amending laws related to animal-browsing licenses and gradually transitioning from traditional to modernized livestock husbandry systems, especially in Iran, where over 60% of pastures are of low quality and poor, are essential steps not only for eradicating PPR but also for preventing the spillover of other transmissible diseases between livestock and wildlife.

Availability of Data and Materials

All data are available in this study

Author Contributions

Conceptualization, E.S.; Methodology, E.S. and H.K.; Investigation, F.K.; Writing – Original Draft, E.S. and F.K.; Writing – Review & Editing, E.S. and H.K.

Ethics Approval and Consent to Participate

This study did not require ethical approval as it did not involve any procedures with animals or human participants that are subject to ethical review.

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(Original Research)

Evaluating Palatability of Lipopolysaccharide Supplement in Cats With and Without Flavoured Treats

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Abstract

Background: Administering oral medication to cats poses significant challenges, particularly due to their acute sensitivity to odours, which can hinder acceptance of drug-infused food. **Methods**: This study investigated the palatability of lipopolysaccharide (LPS) supplement, with or without, flavoured semi-liquid treats for cats, in five domestic shorthair cats using a two-bowl preference test conducted over seven days. Key behavioural metrics included first approach, first consumption, total approach, total intake, and intake ratios. Additionally, a sensory evaluation was conducted with human participants (n=18) to assess the aroma acceptability of treats formulated with either salmon or sprat. Statistical analyses included Chi-Square tests for approach and consumption frequency, one-way ANOVA for total consumption, and descriptive statistics for sensory outcomes (p<0.05). **Results**: A consistent preference for salmon-based treat, with or without LPS, reflected in higher consumption frequencies and intake ratios (0.76 and 0.74, respectively), compared to sprat-based and LPS-only options. Human sensory ratings also favored the aroma of salmon-based over sprat-based treat. **Conclusion**s: These findings underscore the critical role of palatable formulations—particularly salmon—in improving voluntary ingestion of medicated supplements and enhancing compliance in feline care.

Keywords

Aroma, Lipopolysaccharide, Medicine wastage, Cat treats, Palatability, Two-bowl method

1. Introduction

Cats are widely recognized for their remarkable adaptability to compact environments, autonomous behavior, and minimal care demands. They offer emotional comfort, companionship, and affection, which in turn nurtures a sense of responsibility in their owners. Numerous cat owners report forming bonds with their pets that resemble familial or close friendships, underscoring the profound emotional ties that may arise between felines and their human counterparts [1]. Despite these benefits, cats are susceptible to a range of health conditions that often necessitate either therapeutic or palliative interventions. Administering such treatments poses significant difficulties for caregivers. Compliance with veterinary-prescribed medication regimens remains a largely underexplored area in feline health management. Owners frequently encounter challenges when medicating uncooperative cats, which may result in physical harm, incomplete dosing, and broader concerns such as the emergence of antimicrobial resistance [2]. A variety of techniques are employed to deliver oral medications, including the

"poke down" method—placing the drug at the base of the tongue, followed by closing the mouth and gently massaging the throat to prompt swallowing. Another frequently used strategy involves concealing medication within food; however, this is not always effective, particularly when drugs must be administered on an empty stomach or possess a bitterness that cannot be easily camouflaged [3]. Moreover, many cats exhibit aversion to physical restraint, adding further complexity to the medication process [4].

In optimal scenarios, medications should be consumed voluntarily, particularly when gentle handling is required due to the animal's medical condition [5]. For voluntary ingestion to be successful, the formulation must be palatable. Palatability encompasses the sensory and chemical characteristics of a substance that influence an animal's pre-ingestive behavior, and it plays a vital role in determining acceptance of food or medication products [6]. This concept involves multiple sensory factors, including visual appearance, smell, mouthfeel, and taste, all of which stimulate gustatory and olfactory receptors that contribute to the animal's flavor perception [7,8]. Felines exhibit a unique palatability profile, primarily shaped by their taste receptor physiology. Despite possessing only around 470 taste buds—a number lower than that found in humans and various other species—cats demonstrate a marked sensitivity to bitter flavors, a trait that often underlies their reluctance to accept medicinal compounds [9]. Moreover, olfactory cues are a key factor in food acceptance, as the nasal cavity's scent receptors, together with the vomeronasal organ (Jacobson's organ), provide heightened scent sensitivity in both felines and canines [10,11].

Further complicating the situation, a significant number of cat owners report receiving insufficient instructions from veterinarians regarding proper medication administration techniques. As noted by [2], approximately half of surveyed cat owners stated they either "sometimes" or "never" received guidance on how to administer prescribed treatments. Additionally, one-third of respondents were not advised on whether medications could be delivered with food, and only a minority received information about the appropriateness of crushing tablets or opening capsules. Providing clear and comprehensive instructions is vital for effective communication and for ensuring optimal therapeutic outcomes in companion animal care. Considering the existing challenges—such as issues with palatability, the need for physical restraint, and communication gaps—it is crucial to investigate alternative strategies. The present study aims to evaluate the use of flavored semi-liquid treats, specifically those incorporating salmon or sprat, as a potential means of enhancing medication palatability, streamlining the administration process, and reducing the likelihood of injury to both cats and their caregivers.

2. Materials and Methods

2.1 Research location, study period, and animals

The study involved five neutered, adult domestic shorthair cats, consisting of two males and three females, with a median age of two years. The cats' body weights ranged from 3 to 4 kg, with a median weight of 3.5 kg. A detailed history of cats is provided in Table 1. The cats, which were exclusively housed indoors together, were owned by a single individual who voluntarily consented to their inclusion in the study. These cats were housed in an individual cages (in the same room) at the Animal Research Facility within the Faculty

Table 1. Summary of physical and health parameters of study cats.

Cat ID	Sex	Age (years)	Weight (kg)	Health Status	Neuter Status	BCS (1-9 scale)
Cat 1	Male	2	3.3	Healthy; vaccinated, dewormed, defleaed	Neutered	5
Cat 2	Female	1.5	3.4	Healthy; vaccinated, dewormed, defleaed	Neutered	5
Cat 3	Male	2.5	3.7	Healthy; vaccinated, dewormed, defleaed	Neutered	5
Cat 4	Female	2	3.5	Healthy; vaccinated, dewormed, defleaed	Neutered	5
Cat 5	Female	2	3.6	Healthy; vaccinated, dewormed, defleaed	Neutered	5

of Veterinary Medicine at Universiti Putra Malaysia from September 4 to September 14, 2023. To ensure the health and welfare of the cats throughout the experimental period, routine physical examinations were carried out by a licensed veterinarian. These examinations took place at both the commencement of the study and at regular intervals during the research, ensuring that the well-being of the animals was continuously monitored.

2.2 Acclimatization and palatability test

An acclimatization period of one week was provided, during which the cats were given unrestricted access to their habitual diet—seafood-flavoured dry kibble of the same commercial brand routinely provided by the owner. The kibble was administered twice daily, at 6 a.m. and 6 p.m., throughout this period. Upon completion of the acclimatization phase and for the remainder of the study, the quantity of kibble offered was adjusted based on the calculated maintenance energy requirements (MER) expressed in kilocalories per day (kcal/day), as determined using the approach described in [12]:

$$\text{MER (kcal/day)} = 70^{*} (\text{Body weight}_{kg})^{0.75}$$

To evaluate the palatability of the LPS supplement and treats, a two-bowl test was conducted following the protocol described by [13], with modifications. Briefly, cats were offered two identical bowls (round, plastic, double-feeding dish) simultaneously, each containing either the 1) LPS only, 2) flavoured semi-liquid treat for cats—specifically Sprat or Salmon, or 3) combination of LPS and treat over a one-week period. The LPS was administered in powder form (white, 140 mg per tablet; dosage: two tablets per 5 kg body weight), while the treats were provided in a semi-liquid form (4 g per sachet; one sachet per cat). To ensure consistency, all test feedings were conducted at 8 a.m. and 8 p.m., precisely two hours following the provision of standard kibble. Both the LPS and the treats were obtained from a commercial supplier, the identity of which remains undisclosed. Where necessary, two tablets of LPS were crushed and blended with the treats to facilitate administration.

Over the course of seven days, each cat was presented with various combinations of treats and LPS. The schedule was as follows: Day 1 (A and B), Day 2 (A and C), Day 3 (C and D), Day 4 (B and C), Day 5 (B and D), Day 6 (A and D), and Day 7 (A or B with E). In this context, (A) represented Sprat treat; SpT, (B) Salmon treat; SaT, (C) SpT + LPS, (D) SaT + LPS, and (E) LPS alone. During the test, the treats and LPS (either individually or combined) were placed in labelled bowls (1 and 2). Consumption was observed qualitatively for a period of 30 minutes (finished or unfinished). To minimize any potential bias, the bowl placement was alternated during the evening trial.

2.3 Human volunteer olfactory assessment

A total of 18 volunteers were presented with random samples of Salmon- or Sprat-based treats and instructed to evaluate the odor using a rating scale from 1 to 4, where 1 indicated the least pleasant scent and 4 indicated the most pleasant. To minimize potential bias and neutralize the olfactory effects of the previous sample, volunteers were provided with coffee beans to sniff between evaluations, allowing them to reset their sense of smell before proceeding with the next sample.

2.4 Statistical analysis

Data analysis was conducted using Statistical Software for Social Sciences (SPSS) for Windows, Version 19.0 (SPSS Inc., Chicago, USA). The Chi-Square test was utilized to assess the variables "First Approached" and "First Consumed," while the "Total Consumption" variable was examined using one-way analysis of variance (ANOVA). Descriptive statistics were applied to the data collected from the human sensory evaluation, and statistical significance was established at a threshold of $p<0.05$.

3. Results

The data from the "First to Approached" (Fig. 1.) measure clearly indicate that cats showed a significant preference for Salmon-based formulations, both with and without LPS, compared to Sprat-based options and LPS alone

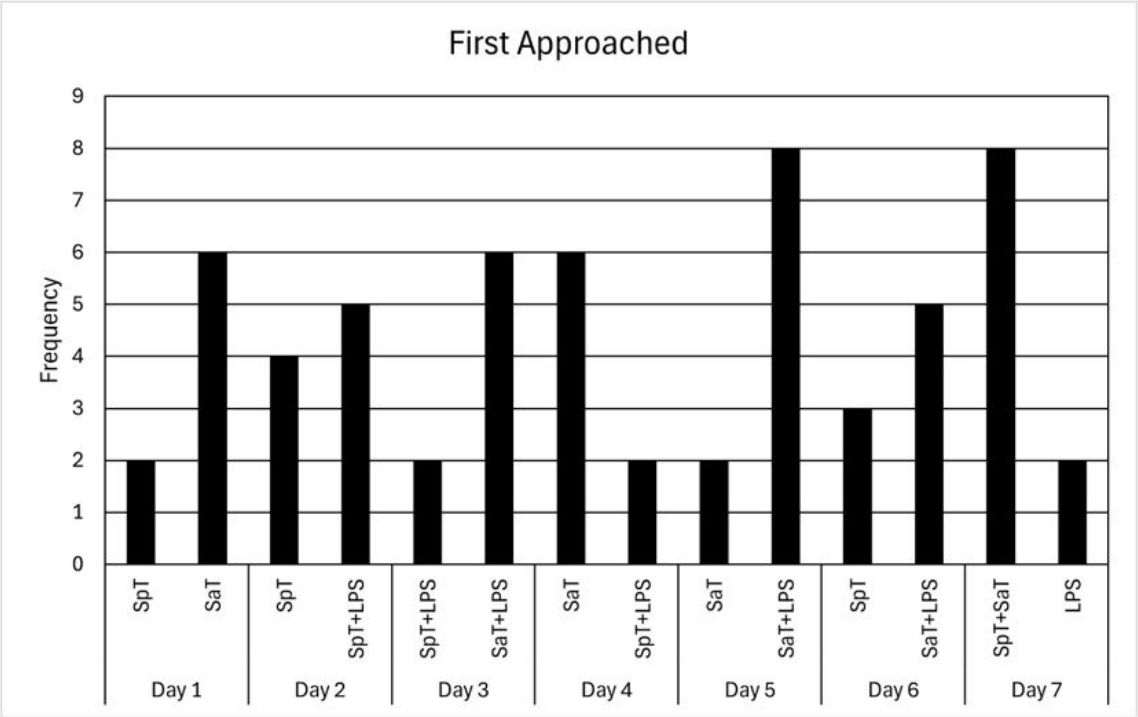

Fig. 1. Cat preferences for first approached in a two-bowl test over seven days.

(p<0.05). The highest levels of initial engagement were observed with SaT and SaT + LPS, while the Sprat-based options exhibited more inconsistent responses, and LPS alone was the least attractive. These findings underscore the superior palatability of salmon-based treats in capturing the cats' attention. This has practical implications for the development of pet products, suggesting that salmon-based treats, particularly when paired with functional ingredients like LPS, may enhance initial interest and promote voluntary intake. The statistical significance of the results (p<0.05) further confirms the strong preference for salmon, which could be an important consideration for creating more effective products aimed at improving feline health and medication adherence.

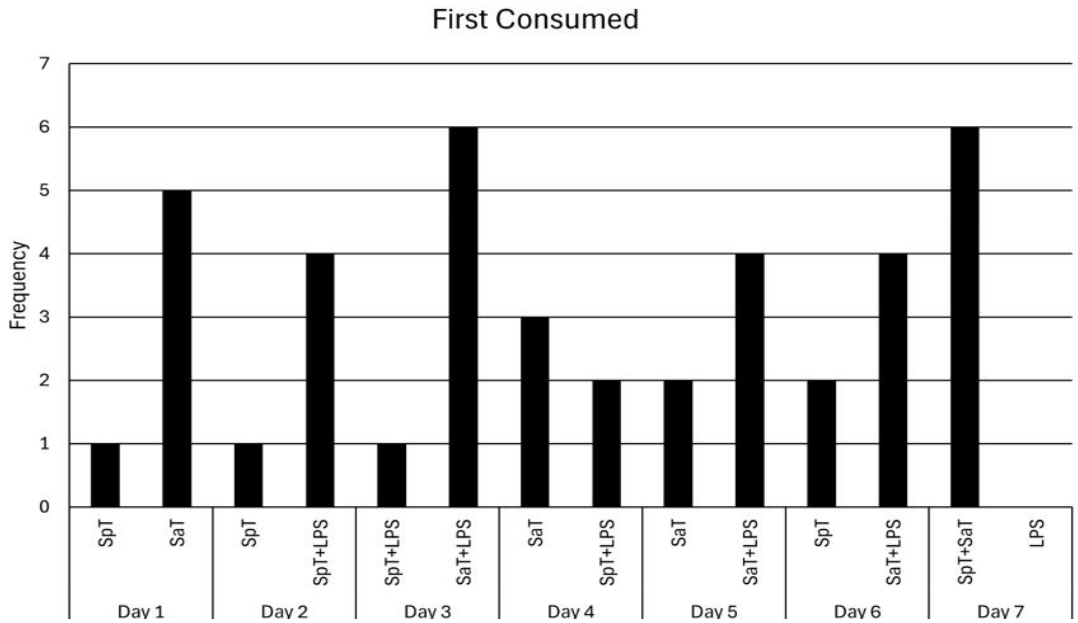

Figure 2. Cat preferences for first consumed in a two-bowl test over seven days.

The "First to Consumed" data (Fig. 2.) showed that salmon-based formulations, particularly SaT and Sat + LPS, were consistently consumed first, indicating high palatability. In contrast, Sprat-based options were less accepted, and LPS alone was never consumed. These differences were statistically significant ($p<0.05$), highlighting the preference for salmon-based treats.

The "Total Approached" data (Fig. 3.) revealed that SaT and SaT + LPS were approached more frequently (17 and 19 times, respectively), indicating a strong preference for Salmon. In contrast, SpT and SpT + LPS were approached less often (14 times each), and the LPS-only treatment was the least approached (2 times), suggesting low palatability. These results, with $p<0.05$ considered statistically significant, emphasize the importance of palatability in promoting approach behavior. The strong attraction to Salmon-based treatments suggests that enhancing sensory appeal, particularly through Salmon, could improve the effectiveness of pet products, especially in medication adherence.

The "Total Consumed" data (Fig. 4.), with $p<0.05$ considered statistically significant, shows that Salmon-based treatments (SaT: 13 and SaT + LPS: 14) were consumed the most, highlighting the strong palatability of Salmon. In contrast, Sprat-based treatments (SpT and SpT + LPS) were consumed only seven times each, suggesting lower palatability and acceptance. The LPS-only treatment was not consumed at all, further reinforcing its lack of appeal. These results emphasize the practical implication that

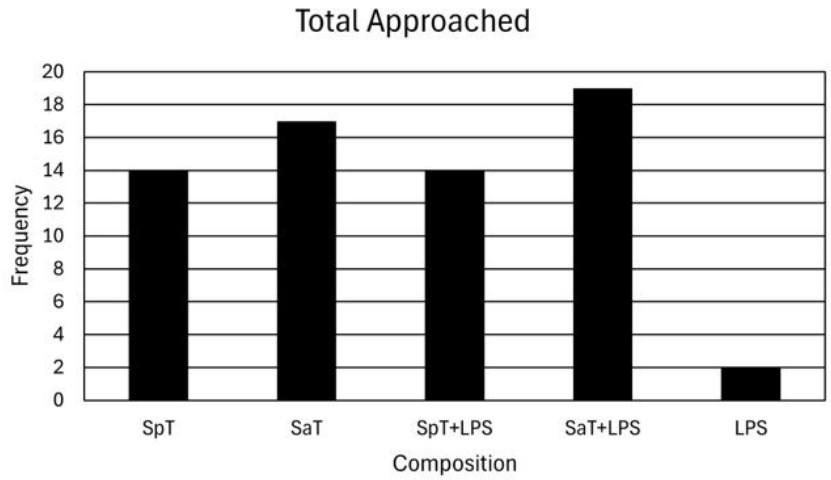

Figure 3. Total approached counts for treats and LPS across the study period.

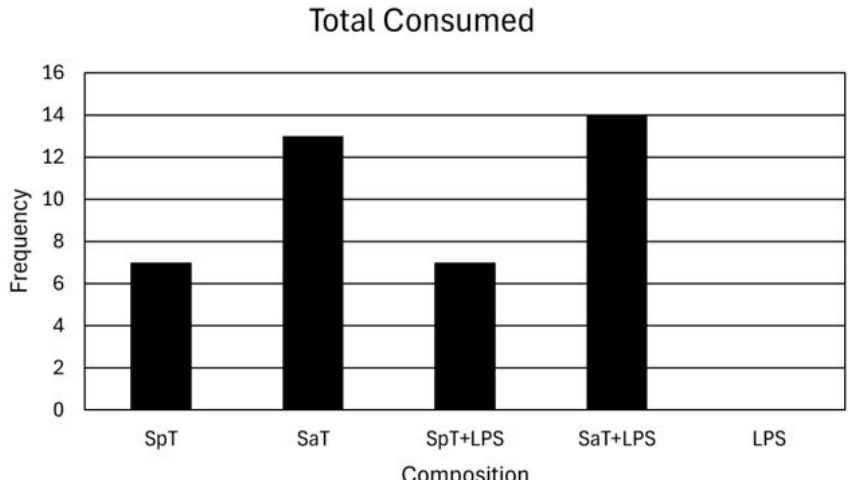

Figure 4. Total consumed counts for treats and LPS across the study period.

combining a highly palatable treat like Salmon with supplements (such as LPS) can significantly increase consumption.

The "Intake Ratio" data (Fig. 5) reveal notable differences in consumption rates among the treatments. SaT had the highest intake ratio (0.76), indicating its strong palatability, with SaT + LPS showing a slight decrease (0.74) due to the addition of LPS. These differences were statistically significant (p<0.05), suggesting that while the addition of LPS reduced the intake slightly, the combination still maintained high palatability. In contrast, SpT and SpT + LPS had lower intake ratios (0.50), highlighting the lower appeal of Sprat. The LPS-only treatment had an intake ratio of 0.00, emphasizing its lack of attractiveness.

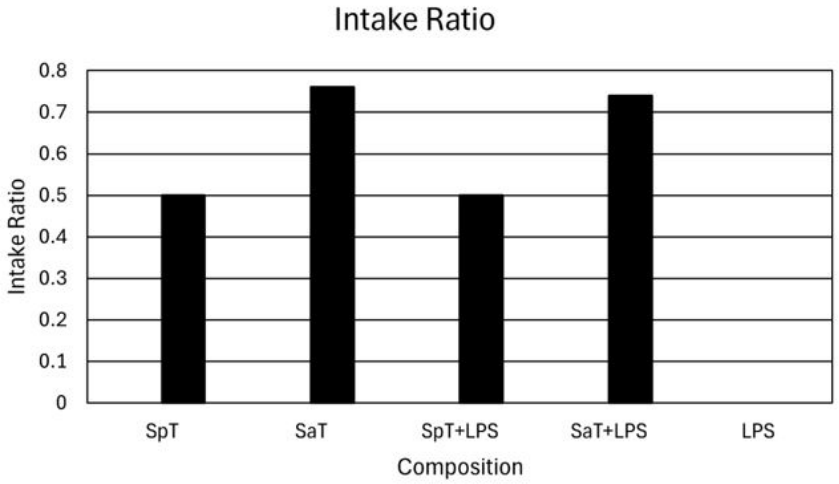

Figure 5. Intake ratio of treats and LPS among cats.

A total of 18 participants participated in the sensory survey. Table 2 summarizes their perceptions of the aromas of two types of treats: Sprat and Salmon. The aroma of each treat was rated on a 1 to 4 scale, where 1 indicated the least pleasant and 4 the most pleasant. The results demonstrated significant differences in the aroma perceptions of the two treats. Overall, most participants found the aroma of SaT to be more pleasant than that of SpT.

4. Discussion

In this study, the two-bowl test was adapted to evaluate the palatability of lipopolysaccharide (LPS) supplement, with or without, flavoured semi-liquid treats for cats. The assessment incorporated various parameters, as defined by [14], including measures of "First Approached," "First Consumed," "Total Approached," "Total Consumed," and "Intake Ratio." Additionally, a sensory evaluation was conducted to assess the acceptability of the treat aromas by human participants.

The results demonstrated that cats consistently showed a marked preference for SaT + LPS, as opposed to plain SaT or SpT. On the other hand, LPS administered alone was notably aversive to the cats. These findings suggest that SaT may enhance the palatability of LPS when combined.

Table 2. Human olfactory assessment of treats' aroma.

Flavors of treat	Score			
	Least Pleasant (1)	Less Pleasant (2)	More Pleasant (3)	Most Pleasant (4)
Sprat (SpT)	13	10	8	5
Salmon (SaT)	8	5	10	13

Moreover, the cats displayed a stronger preference for SaT over SpT, aligning with prior research that suggests cats generally favour fish, especially salmon, over other flavours commonly found in commercial cat foods, such as liver, chicken, beef, or even rats [15]. Several factors influence feeding behaviour and preferences in cats, including flavour, aroma, texture, temperature, appearance, and size [9; 11].

Aroma appeared to be the dominant factor influencing the palatability of treats for cats, as indicated by the "First Approached" and "Total Approached" results. Most cats were attracted to SaT, whether with or without LPS. Similarly, the human sensory assessment revealed that SaT's aroma was generally rated as more pleasant than that of SpT. While these findings are significant, palatability involves both aroma and flavour [16], with flavour not being assessed in this study by human participants. Unlike humans, cats possess highly developed olfactory abilities [17] and rely on both aroma and taste to select their diets. Cats' olfactory systems are especially responsive to high-protein foods and fatty acids, which are abundant in salmon. As noted by [11], cats utilize their olfactory senses to detect and process various environmental chemicals, enabling them to distinguish both familiar and novel odours. Furthermore, the volatile compounds released by salmon during cooking likely contribute to its appeal to cats [18]. This heightened olfactory sensitivity may explain the cats' selective attraction to aromas.

Additionally, the protein content in treats may also play a role in palatability. SaT contains a higher protein content (10.5%) and lower carbohydrate content (1.4%) compared to SpT (protein: 7.9%, carbohydrate: 1.6%). Cats generally prefer high-protein and high-fat foods while avoiding those high in carbohydrates [19]. Research by [20] indicated that protein content significantly influences the amount of food consumed by cats, suggesting that they prioritize protein over carbohydrates, which is consistent with the current study's findings. Cats' low carbohydrate intake is associated with various sensory and metabolic adaptations, including the absence of sweet taste receptors, which prevents them from perceiving sweet flavours [19]. This is attributed to the pseudogenization of the T1R2 receptor. In contrast, cats have more sensitive umami receptors than humans, allowing them to detect a broader range of amino acids. As obligate carnivores, cats do not encounter sweet compounds in their diet, but their ability to detect proteins and amino acids is crucial for their nutritional needs [21]. Previous studies have demonstrated that cats favour foods with pronounced "umami" or savoury flavours, typically associated with high concentrations of amino acids [14, 20, 22]. Additionally, cats can recognize diets that meet their specific nutritional requirements, particularly their higher protein and amino acid needs, with taurine supplementation often included to ensure adequate intake [23].

Another factor influencing palatability is a cat's prior exposure to food, linked to neophobia and neophilia. Cats tend to show greater acceptance and preference (neophilia) for foods they are familiar with, while unfamiliar foods may induce hesitation or initial rejection (neophobia) [24]. Studies of feline food preferences support this notion, showing that when presented with multiple food options, cats often choose based on sensory properties such as aroma, flavour, and texture, as well as their past dietary experiences [25]. Maternal influences also shape food preferences in cats [18]. Kittens raised on a single food type are more likely to develop neophobia when exposed to new foods, whereas those exposed to a variety of meats early in life are less likely to exhibit this behaviour [26]. Domestic cats frequently show a preference for novel foods over their regular diet, although their exploratory behaviour does not always apply to all new foods, even those that meet their nutritional needs [27].

Overall, these findings have direct implications for product development in the pet food industry. The demonstrated preference for salmon-based treat suggests that incorporating highly palatable, high-protein ingredients such as salmon can enhance the acceptance of functional treats or supplements, particularly those containing bioactive compounds like LPS. This may guide the formulation of more effective, voluntarily consumed medication-delivery systems for companion animals.

5. Conclusions

The use of treats in this study revealed a clear feline preference for salmon over sprat, with the addition of LPS further enhancing overall

palatability. These findings align with previous research indicating that cats favor fish-based flavours, particularly salmon, and suggest that functional additives like LPS can be more readily accepted when delivered in a palatable matrix. To gain a more comprehensive understanding of feline dietary preferences, future research should explore additional variables such as food temperature, texture, and presentation. Importantly, these results highlight the potential clinical applications of treats. By improving the palatability of medications, treats offer a promising strategy to enhance treatment compliance in cats, especially for chronic or long-term therapies. This approach could simplify medication administration for pet owners and contribute to more effective veterinary care.

Availability of Data and Materials

All data are available in this study.

Author Contributions

Conceptualization, M.A; Funding acquisition, M.A.; Project administration, M.A., N.I., G.T.S., and M.F.W.C.; Supervision, M.A., N.I., G.T.S., and M.F.W.C.; Resources, M.A.; Methodology, M.A., N.I., G.T.S., and M.F.W.C.; Investigation, N.A.A.A.R. and A.R.R.; Data curation, N.A.A.A.R., A.R.R., and N.I.; Formal analysis, N.A.A.A.R., M.A., and N.I.; Software, N.I.; Visualization, N.I.; Validation, M.A., N.I., G.T.S., and M.F.W.C.; Writing – Original Draft, N.A.A.A.R., and N.I.,; Writing – Review & Editing, N.I., and M.A.

Ethics Approval and Consent to Participate

This study was approved under the permission and guidelines of the Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (UPM) with reference number UPM/IACUC/AUP-U031/2023.

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Conflict of Interest

The authors declare no conflict of interest.

Abbreviations

LPS, lipopolysaccharide; MER, maintenance energy requirements; SpT, sprat-flavoured treat; SaT, salmon-flavoured treat; BCS, body condition score.

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(Short Communication)

Effects of Proportion of Brahman Genetics on the Reproductive Performance of Female Crossbreds in Western Highlands of Vietnam

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Abstract

Background: Brahman crossbreds exhibit heavy weight, good health, nice coat-color and docility, making them advantageous for beef production. Appropriate proportion of Brahman genetics in female herds is crucial for sustainable development in Western Highlands of Vietnam. This study aims to evaluate the effects of different proportions of Brahman genetics on reproductive performance of crossbred females. **Methods:** Data were collected from 1,285 breeder cows in Dak-Lak and Gia-Lai provinces between 2017 and 2021. The traits measured included age at first service (AFS), weight at first service (WFS), age at first calving (AFC), and days open (DO). Data was analyzed using the GLM and linear regression. The statistical models included province, dam breed group, birth year of heifers and parities as fixed effects. **Results:** AFS, WFS, AFC and DO were higher in female crossbreds with higher proportion of Brahman genetics. Incorporating Brahman genetics to female herds improved WFS, but not AFS, AFC and DO. For every 1% increase of Brahman genetics, AFS, WFS, AFC and DO increase by 1.39 days, 1.55 kg, 1.42 days and 0.813 days, respectively. **Conclusions:** When breeder cows were upgraded with Brahman genetics, body weight at first service was improved. However, the other reproductive traits were negatively affected.

Keywords

Beef cattle, Body weight, Brahman cattle, Reproductive performance

1. Introduction

Breeding strategy, herd management, and the establishment of breeding herd are key determinants of economic efficiency in beef cattle production [1,2]. The use of breeder cows with high genetic merit for production traits and reproduction traits can serve as foundation breeding stocks [1,3,4-11]. Further, to produce calves for feedlot fattening, cows with Brahman blood, Local Yellow cattle (LYC), and Red Sindhi cattle (RSC) are often crossed with introduced breeds [19-26].

Brahman cattle is widely recognized for their adaptability to hot and humid climates, resistance to internal and external parasites, and excellent reproductive performance in tropical and subtropical environments [12-17]. In Vietnam, the Brahman breed is particularly valued in rural and remote areas for its appealing appearance, including its red coat, skin color, high wither, and large dewlap, aligning with local cattle farmers' preferences. This breed also exhibits a large frame, heavier weight compared to the LYC or RSC, with superior carcass performance, and efficient feed conversion. Additionally, Brahman crossbreds in Vietnam demonstrate strong disease resistance, heat tolerance, tick resistance, and docility [18]. On the other hand, LYC and RSC are common choices as base genetics of crossbred cows to leverage maternal effects and improve reproductive traits with economic importance such as age at first service (AFS), weight at first service

(WFS), age at first calving (AFC), and days open (DO).

Determining the reproductive performance of female breeders with varying proportions of Brahman genetics is therefore warranted. Establishing the best performing female genetics in a base herd is crucial for its efficient utilization. The findings of this research may provide a long-term solution for local offices and central government authorities involved in improving beef cattle productivity in the Western Highlands of Vietnam.

2. Materials and Methods

2.1 Animals

Breeder females on each farm were individually identified based on physical characteristics such as coat and skin color, horn type, frame size, ear tag number, or tattoos, with additional identification provided by cattlemen. The WFS was estimated using standardized tape measurements. Records of AFS, AFC, and DO were provided by owners, local technicians, or inseminators.

Local Yellow Cattle (LYC) and Red Sindhi Cattle (RSC) were identified based on physical appearance and verified through its owners, local technicians, or inseminators. As shown in Table 1, crossbred females were classified according to the proportion of Brahman genetics, which is reflected first, followed by either a proportion of LYC or RSC (example, 25Br-75LYC). "LYC" or "RSC" are breed groups without Brahman blood (0%). A total of 1,285 females from 113 farms in Dak-Lak and Gia-Lai provinces were included in the study. Data were collected between 2017 and 2021 and classified into 10 breed groups based on Brahman genetic proportions and base female breeds.

2.2 Management practices

Cattle grazed on pastures during the daytime and supplemented with concentrate while housed in barns. They were released daily between 08.00 and 09.00 AM and returned to barns between 18.00 and 19.00 PM. Concentrate was provided twice daily using a uniform formulation across all farms (Table 2). Pregnant female and lactating cow were fed 0.5 kg/day and 0.7 kg/day. While housed in barns, cattle were offered forage, hay, and rice straw *ad libitum*.

2.3 Data analyses

Fixed-effects models were constructed to evaluate the impact of varying Brahman genetic proportions while controlling for non-genetic factors. Three models were utilized: Model 1 analyzed age at first service (AFS), weight at first service (WFS), and age at first calving (AFC) in heifers; Model 2 analyzed days open (DO) in cows; and Model 3 assessed the effect of a 1% increase in

Table 1. Data distribution based on breed groups and provinces.

Breed groups by Brahman genetics	Breed groups by dam resources	Dak-Lak province	Gia-Lai province	Total
25% Brahman	25Br-75LYC (25BrLYC)	19	29	48
	25Br-75RSC (25BrRSC)	14	22	36
50% Brahman	50Br-50LYC (50BrLYC)	35	40	75
	50Br-50RSC (50BrRC)	125	155	280
75% Brahman	75Br-25LYC (75BrLYC)	8	20	28
	75Br-25RSC (75BrRSC)	17	97	114
87.5% Brahman	87.5Br-12.5LYC (875BrLYC)	8	14	22
	87.5Br-12.5RSC (875BrRSC)	7	18	25
0% Brahman	LYC	179	128	307
	RSC	198	152	350
	Overall	**610**	**675**	**1,285**

Table 2. Ingredients, nutritional values, and regimes for concentration.

A	Ingredients	(%) in ration
1	Rice bran	45
2	Yellow maize powder	45
3	Bone meal	9
4	Salt	0.5
5	ADE mixture	0.5
	Total	*100*
B	**Nutritional values** [1]	**Amount**
1	Crude Protein (%)	11.75
2	ME (Kcal/kg)	2,600.00

[1] = Estimated according to ingredients and nutritional values in Monograph of National Institute of Animal Science (2001) [27].

Brahman genetic proportion on AFS, WFS, AFC, and DO. All statistical analyses were performed using SAS 9.4. Pairwise comparisons of least squares means was applied as the posthoc test with p-value < 0.05 considered as statistically significant.

Model 1

$$Y_{ijklm} = \mu + P_i + BG_j + (P*BG)_{ij} + YB_k + F_l(P_i) + e_{ijklm} \quad (1)$$

Where:

Y_{ijklm} is the observation (AFS, WFS or AFC) of the m^{th} heifer, kept in the l^{th} farm, born in the k^{th} year, belonging to the j^{th} breed group, kept in the i^{th} province,

μ is the overall mean

P_i is the fixed effects of the i^{th} province (i = 2: Dak-Lak and Gia-Lai provinces)

BG_j is the fixed effects of the j^{th} breed group (j = 10: LYC, RSC, and various crossbred groups; with j = 5 when analyzed based on breed groups according to different proportions of Brahman breed: group 0%, 25%, 50%, 75% and 87.5% of Brahman genetics).

$(P*BG)_{ij}$ is the interaction between provinces and breed groups

YB_k is the fixed effects of the k^{th} birth year of heifer (k=10: 2010 and before, 2011, …, 2019)

F_l is the effect of the l^{th} farm

$F_l(Pi)$ is the random effect of the l^{th} farm in the i^{th} province, assuming that $N(0, \sigma^2_F)$

e_{ijklm} is the random residual errors, assuming that $N(0, \sigma^2_e)$

Model 2.

$$Y_{ijklmno} = \mu + P_i + BG_j + (P*BG)_{ij} + Par_k + S_l + YC_m + F_n(P_i) + e_{ijklmno} \quad (2)$$

Where:

Y_{ijklmo} is the Days Open of the o^{th} cow, reared on the n^{th} farm, calved in the m^{th} year, in the l^{th} calving season, at the k^{th} parity, belonging to the j^{th} breed group, kept in the i^{th} province,

μ is the overall mean.

P_i is the fixed effects of the i^{th} province (i = 2: Dak-Lak and Gia-Lai provinces)

BG_j is the fixed effects of the j^{th} breed group (j = 10: LYC, RSC, various crossbred groups; with j = 5 when analyzed based on female groups according to various proportions of Brahman genetics: group 0%, 25%, 50%, 75% and 87.5% of Brahman genetics).

$(P*BG)_{ij}$ is the interaction between Provinces and breed groups

Par_k is the fixed effect of the k^{th} parity (K=6: Parity 1, 2, …, 6 and more).

S_l is the fixed effect of the l^{th} calving season (l=2: Summer-Autumn from April to September; Winter-Spring is left calving months)

YB_m is the fixed effects of the m^{th} birth year of heifer (m=10: 2010 and before, 2011, …, 2019)
$F_n(Pi)$ is the random effect of the n^{th} farm nested in the i^{th} province, assuming that N(0, σ^2_F)
e_{ijklm} is the random residual errors, assuming that N(0, σ^2_e)

Model 3.

$$Y = a + bx + e \quad (3)$$

Where:

Y is the dependent variable (AFS, WFS, AFC, and DO of various breed groups), a is a constant (basic performance), b is the slope (increment), x is an independent variable (proportion of Brahman genetics in breed groups from two dam groups: LYC and RSC and crossbred groups according to the different proportions of Brahman genetics)

e is the random residual errors assuming that N(0,σ^2_e).

3. Results

3.1 Performance of different breed groups from LYC and RSC

Age at first service (AFS): The results in Table 3 showed that AFS of LYC was significantly lower (P<0.05) than that of RSC (517.22±17.14 days versus 569.76±16.55 days). Among crossbreds, the higher the proportion of Brahman genetics is upgraded, the older the AFS would be. The 87.5% Brahman-LYC crosses were older by 152 days compared to LYC females, while 87.5% Brahman-RSC crosses were older by 92 days compared to RSC females. Within LYC dam resources, AFS of LYC (0% Brahman) was (517.22±17.14 days) significantly different from 25BrLYC (572.90±21.99 days) (P<0.05). AFS of 25BrLYC crosses significantly differed from 50BrLYC, 75BrLYC and 875BrLYC crosses (P<0.05). But differences among 50BrLYC, 75BrLYC and 875BrLYC crosses were not statistically significant (P>0.05). For RSC dam resources, AFS of RSC (0% Brahman) was (569.76±16.55 days) significantly different from 25BrRSC (627.00±31.95 days) (P<0.05). But AFS of crosses from 25% Brahman to 87.5% Brahman were not significantly different (P>0.05). When considering two crossbred groups with equal proportion of Brahman genetics, the breed group based on RSC dams usually had AFS lower than the group based on LYC dams, except for dam groups with 25% of Brahman genetics. However, the differences among them were not statistically significant (P>0.05).

Weight at first service (WFS): The results in Table 3 indicated that the body weight at first services of LYC was of 185.72±7.31 kg/heifer, significantly lower (P<0.05) than that of RSC (219.35±7.75 kg/heifer). Similarly, in crossbreds, a higher proportion of Brahman genetics corresponded to a heavier WFS. The 87.5% Brahman-LYC crosses were 158.17 kg heavier than LYC females, while the 87.5% Brahman-RSC crosses were 113.57 kg heavier than RSC females. For crosses based on the same LYC dam resources, the differences in WFS among them were statistically significant (P<0.05). Similarly, for crosses based on the same RSC dam resources, the differences in WFS were also statistically significant (P < 0.05); except between the RSC (0% Brahman) - 25 Brahman-RSC (25BrRSC) group with the 50% Brahman-RSC (50BrRSC) and the 75% Brahman-RSC (75BrRSC)-87.5%Brahman-RSC (875BrRSC) group. When comparing groups with the same Brahman genetic proportion, WFSs of breed groups based on LYC dams were lower than those based on RSC dams at the proportions of 25% and 50% Brahman genetics. However, at the proportions of 75% and 87.5% Brahman genetics, their WFSs were nearly equivalent, although WFSs based on LYC were a little bit higher than RSC. However, among them the differences were also not statistically significant (P>0.05). More specifically, in the crossbred group of 25% of Brahman genetics, the WFSs of crossbred groups based on LYC dams (216.62±8.65 kg/heifer), were lower than those of crossbreds based on RSC dams (229.85±14.03 kg/heifer). This difference was not significant (P>0.05).

Age at first calving (AFC): As shown in Table 3, AFC in LYC was averaged as 795.95±17.10 days, which was significantly lower (P<0.05) than that of RSC (849.01±16.61 days). Similar to the traits mentioned above, a higher proportion of Brahman genetics in crossbreds resulted in a longer AFC. Moreover, the 87.5% Brahman-LYC crosses were longer by 157.43 days to LYC females, while the 87.5% Brahman-RSC crosses were longer by 94.24 days to RSC females. Besides, the AFC of

Table 3. Performance of reproductive females by different breed groups.

Breed Groups	AFS		WFS		AFC		DO	
	n	LSM±SE	n	LSM±SE	n	LSM±SE	n	LSM±SE
LYC	153	517.22±17.14^{a}	128	185.72±7.31^{a}	156	795.95±17.10^{a}	307	83.09±4.07^{a}
RSC	105	569.76±16.55^{b}	81	219.35±7.75^{b}	105	849.01±16.61^{b}	333	86.59±3.27^{a}
25BrLYC	48	572.90±21.99^{bc}	44	216.62±8.65^{b}	48	852.26±22.12^{bc}	37	87.58±6.97^{a}
25BrRSC	36	627.00±31.95^{cd}	32	229.85±14.03^{bc}	36	905.95±32.06^{c}	27	95.64±8.51^{ac}
50BrLYC	46	650.47±18.50^{d}	44	256.53±6.82^{c}	42	930.93±19.01^{d}	75	116.07±5.75^{b}
50BrRSC	102	630.45±16.96^{cd}	101	280.61±6.66^{d}	102	909.75±17.03^{cd}	224	111.26±3.86^{bc}
75BrLYC	28	663.18±25.13^{d}	28	320.94±9.05^{e}	28	945.69±25.26^{d}	28	154.41±8.28^{d}
75BrRSC	68	643.70±25.93^{cd}	61	319.11±9.52^{ef}	68	916.74±25.99^{cd}	102	147.57±6.29^{d}
875BrLYC	22	669.38±26.88^{d}	22	343.89±9.76^{f}	22	953.38±27.01^{d}	20	144.10±9.40^{d}
875BrRSC	25	662.03±30.04^{d}	25	332.92±11.00^{ef}	25	943.25±30.11^{d}	25	159.62±9.25^{d}

Notes: AFS: Age at first service (days); WFS: Weight at first service (kg); AFC: Age at first calving (days); DO: Days open (days). In the same column, the LSM values with the same superscript are not significantly different (P>0.05).

25BrLYC was (852.26±22.12 days) significantly different from LYC (0% Brahman) and crossbred groups (50BrLYC, 75BrLYC and 875BrLYC). However, the differences in AFC among 50BrLYC, 75BrLYC, and 875BrLYC were not significant (P>0.05). On the other hand, AFC of 25BrRSC was (905.95±32.06 days) significantly different from RSC (849.01±16.61 days) and 875BrRSC (943.25±30.11 days) (P<0.05), but not significantly different from 50BrRSC (909.75±17.03 days) or 75BrRSC (916.74±25.99 days) (P>0.05). For crossbred groups with the same Brahman genetic proportion but different dam sources, crossbred groups based on LYC dams were usually higher than the groups based on RSC dams, with exception of 25% Brahman's genetic group. However, the differences between each corresponding RSC and LYC pair of a given Brahman introgressed genetic population were not statistically significant (P>0.05). Similarly, for AFC of crosses based on the same dam resources, the differences among them were also statistically significant (P<0.05). Thus, when Brahman genetic resources were upgraded into crossbreds resulted in prolonging AFC, this upgrading negatively affected reproductive performance in the beef cattle population of the Western Highland.

Days open: The results from this investigation revealed that RSC had a higher DO than LYC (86.59±3.27 days versus 83.09±4.07 days), but this difference was not statistically significant (P>0.05). For the breed groups born from LYC dams, there was a strange change. When upgrading 25% Brahman genetics into crossbreds, DO in the group of 25% Brahman genetics (87.58±6.97 days) was lower than that in LYC dam (95.64±8.51 days). However, this difference was not statistically significant (P>0.05). Whereas, in the crossbred group, when increase of Brahman genetic proportions, DO was risen from 87.58±6.97 days in the group of 25% Brahman genetics, amounted to 154.41±8.28 days in the group of 75% Brahman genetics. It then decreased to 144.10±9.40 days in the crossbred group of 87.5% Brahman genetics. Besides, LYC and 25BrLYC had no significant difference of DO (P>0.05), but DOs from both were significantly differed from other crosses (50BrLYC, 75BrLYC and 875BrLYC) (P<0.05). The DO of 50BrLYC (116.07±5.75 days) was significantly different from all other groups (LYC, 25BrLYC, 75BrLYC and 875BrLYC) (P<0.05). The DOs of 75BrLYC (154.41±8.28 days) and 875LYC (144.10±9.40 days) showed no significant difference (P>0.05). In the crossbred groups born from RSC dam group, DO was gradually increased from RSC to crossbred groups when they were upgraded with Brahman genetics. Specifically, RSC got DO of 86.59±3.27 days; when they were upgraded with 25% Brahman genetics, their DO was extended to 95.64±8.51 days. When they were further increased to 50% Brahman genetics, their DO was increased to 111.26±3.86 days and achieved the plateau of 159.62±9.25 days in the crossbred group of 87.5% Brahman genetics. On the other hand, DO of 25BrRSC (95.64±8.51

days) was not significantly different from RSC (86.59±3.27 days) and with 50BrRSC (111.26±3.86 days) (P>0.05), but it was different from 75BrRSC (147.57±6.29 days) and 875BrRSC (159.62±9.25 days) (P<0.05). Besides, the findings indicated that there is no statistical difference between 875BrLYC and 875BrRSC (P>0.05).

From this outcome, we found that they were significantly different (P<0.05). Thus, DO was unusually high in LYC dam groups, but it was gradually increasing in RSC dam group. These results also confirmed that when upgrading Brahman genetics into crossbreds, DO tended to extend and had disadvantageous influence on reproductive performance as well as lifetime production and their overall herds.

3.2 Performance of pooled dam groups by different Brahman's breed proportions

Age at first services: In the dam group without Brahman genetics, AFS averaged as 542.01±12.50 days. This trait was increased to 586.70±18.03 days in the group of 25% Brahman genetics, and continuously increased along with increase of Brahman genetics, amounted to 663.87±19.97 days in the group of 87.5% Brahman genetics. The differences among them showed statistically significant (P<0.05). Specifically, the AFS of the crossbred group of 25% Brahman was significantly different from the group of 0% Brahman. The AFS of these two groups were apparently different from the groups of 50%, 75% and 87.5% Brahman. The AFS of three crossbred groups of 50%, 75% and 87.5% Brahman were not significantly different. According to these findings, when upgrading Brahman genetics in LYC or RSC, the AFS was prolonged, and affected unfavorably the reproductive efficiency of female herds (Table 4).

Weight at first services: In the group of no Brahman genetics, their WFS was of 204.89±5.93 kg/heifer, and was enhanced in the group of 25% Brahman genetics (229.52±7.46 kg/heifer), continued grading-up in the group of 50% Brahman genetics achieved 267.37±4.99 kg, increased continuously in the group of 75% Brahman genetics and acquired 317.98±6.67 kg/heifer, and in the group of 87.5% Brahman genetics with 335.56±7.63 kg/heifer. These differences were statistically significant (P<0.05). Thus, when LYS or RSC were upgraded using Brahman genetic resources, reproductive female herds tended to improve their body weights and statures, thereby being more effective as base females for crossbreeding with exotic high-yielding breeds (Table 4).

Age at first calving: Derived from these results, for the breed groups of LYC or RSC (the groups of zeroed Brahman genetics), the average AFC was 819.96±12.54 days. Crossbreeding and introducing 25% Brahman genetics increased the AFC to 864.43±18.18 days, while upgrading to 50% Brahman genetics further increased it to 912.78±13.03 days. With 75% Brahman genetics, the AFC rose to 929.01±17.69 days, and at 87.5% Brahman genetics, it reached 945.82±20.08 days.

Specifically, AFC of the crossbred group of 25% Brahman genetics was significantly different from the 0% Brahman group, and the AFCs from both groups were significantly different compared to all other groups (P<0.05). AFCs of the 50%, 75% and 87.5% Brahman crossbred groups were not significantly different. Thereby, upgraded crossbred females using Brahman genetic

Table 4. Performance of breed groups by various Brahmans' breed proportions on pooled dam group.

Percentage of Brahman	AFS		WFS		AFC		DO	
	n	LSM±SE	n	LSM±SE	n	LSM±SE	n	LSM±SE
0	258	542.01±12.50^{a}	209	204.89±5.92^{a}	261	819.96±12.54^{a}	640	86.15±3.05^{a}
25	84	586.70±18.03^{b}	76	229.52±7.46^{b}	84	864.43±18.18^{b}	64	88.93±5.59^{a}
50	148	634.35±12.74^{c}	145	267.37±4.99^{c}	144	912.78±13.03^{c}	299	110.74±3.39^{b}
75	96	652.04±17.55^{c}	89	317.98±6.67^{d}	96	929.01±17.69^{c}	130	146.72±5.14^{c}
87.5	47	663.87±19.97^{c}	47	335.56±7.63^{e}	47	945.82±20.08^{c}	45	148.80±6.92^{c}

In the same column, LSM values with different letters are significantly different (P<0.05).

resources increased their AFC and affected reproductive performance (Table 4).

Days open: Similarly, gradual upgrading of LYC or RSC with Brahman genetic resources increased their DO with significant difference observed from 50 – 87.5%. Specifically, the DO of 0%, 25%, 50%, 75%, and 87.5% Brahman are as follows 86.15±3.05 days, 88.93±5.59 days, 110.74±3.39 days. 146.72±5.14 days, and 148.80±6.92 days. Thus, 50 – 87.5 % upgraded LYC or RSC with longer DO lowers their reproductive performance (Table 4).

3.3 Increments in body weight and reproductive performance

The results in Table 5 indicated that all the estimated parameters were statistically significant (P<0.05).

improved but affected reproductive performance negatively.

Specifically, in the groups of LYC dams, the average of AFS in the population was 529.54±17.43 days. When crossbreeding with Brahman genetics, for every increase of 1% Brahman genetics, their AFS was prolonged to 1.79±0.30 days. For WFS, the overall mean was 176.44±8.52 kg, and when they were crossbred with Brahman genetics, for every 1% increase of Brahman genetics, WFS was enhanced by 1.86±0.15 kg. Regarding AFC, the population mean of the group with 0% Brahman genetics was 807.73±16.96 days, but every 1% increase in Brahman genetics added 1.85±0.30 days to AFC. Furthermore, female herds with 0% Brahman genetics, the DO was 76.43±9.26 days, but for every 1% increase in Brahman genetics, DO was prolonged by 0.86±0.16 day. These results showed that there was an increase in body weight but

Table 5. Base values and increment of performance trait of reproductive female crossbred by various Brahman genetic proportions in two dam's groups.

Traits	Parameters[1]	LYC dams		RSC dams		Pooled dams	
		DF	E±SE$_E$	DF	E±SE$_E$	DF	E±SE$_E$
AFS	a	1	529.54±17.43	1	584.07±12.10	1	549.75±8.68
	b	1	1.79±0.30	1	0.90±0.21	1	1.39±0.15
WFS	a	1	176.44±8.52	1	209.61±8.44	1	197.48±6.23
	b	1	1.86±0.15	1	1.41±0.15	1	1.55±0.11
AFC	a	1	807.73±16.96	1	863.21±13.09	1	827.07±8.28
	b	1	1.85±0.30	1	0.88±0.23	1	1.42±0.14
DO	a	1	76.43±9.26	1	78.83±7.17	1	77.61±7.69
	b	1	0.86±0.16	1	0.87±0.13	1	0.81±0.13

Notes: [1]: All the estimated parameters are statistically significant.
a is intercept coefficient – basic performance, b is slope – increment of performance when increase of 1% Brahman genetics into crossbreds. E: Estimates of parameters; SE$_E$: Standard errors of Estimates. DF: Degree of freedom.-smaller font

In the pooled dam group, the results also showed that AFS, WFS, AFC, and DO in the herds without Brahman genetics were averaged as 549.75±8.68 days, 197.48±6.23 kg, 827.07±8.28 days and 77.61±7.69 days, respectively. Nevertheless, for every 1% of Brahman genetics was increased into crossbred groups, these traits were increased by 1.39±0.15 days, 1.55±0.11 kg, 1.42±0.14 days and 0.81±0.13 days, respectively. These results further supplemented that in crossbreeding and upgrading LYC or RSC with Brahman cattle, their body weight was favorably lowered reproductive traits in LYC dams with increasing Brahman genetics resources.

Similarly, in the breed groups of RSC dams, females with 0% Brahman genetics had an average AFS of 584.07±12.10 days in the base population. For every 1% Brahman genetic upgrade, AFS was increased by 0.90±0.21 day. Moreover, the WFS for RSC dams with 0% Brahman genetics was 209.61±8.44 kg, but for every 1% Brahman genetic upgrade, their WFS was enhanced by 1.41±0.15 kg. Similarly, with AFC in basic female herds, AFC averaged 863.21±13.09 days, and for every

1% Brahman genetic upgrade, AFC was prolonged by 0.88±0.23 days. For DO, RSC females averaged 78.83±7.17 days, but in crossbreeding, for every 1% Brahman genetic upgrade, DO was prolonged by 0.87±0.13 days. These results also additionally confirmed that when Brahman genetic resources were increased in RSC dams, there was also an increase in body weight but their reproductive performance tended to lessen in relation to increasing input to produce a calf.

4. Discussion

Brahman genetics positively affected growth performance of their crossbreds from birth to 12 months old [28]. This concurs with our current findings where WFS increases for every increase of 1% in Brahman genetics. WFS was enhanced from 1.41±0.15 kg to 1.86±0.15 kg, depending on the base dam groups. Recently, in order to produce commercially high-yielding crossbreds, cattlemen in the Western Highlands and adjacent regions usually use the basic reproductive female herds as LYC, RSC or 50% Brahman genetic crossbreds. These females are artificially inseminated with frozen semen of exotic breeds as Brahman, Red Angus, Charolais, and Limousine cattle [29,]. Body weight and carcass performance of the crossbreds of 50% Brahman genetics were enhanced by 20% to 50% as compared to existent beef cattle herds, thus, economic efficiency was clearly improved [22,24,26]. However, small-sized basic reproductive females would be difficult to apply for mating or AI with large-sized high-yielding beef bulls, due to declining reproductive performance such as increasing difficulty in calving. But when large-sized cows were introduced, they would encountered difficulties resulting from limited proper knowledge and economic potential capacity for nourishment.

In recent years, many farmers have their female cattle artificially inseminated with frozen semen of Belgian Blue Breed (BBB), and thus, it is compulsory to improve both stature and frame of their reproductive females. The results from this research showed that LYC, although possessing a small size, expressed slightly better reproductive performance than RSC. Similarly, when upgrading to 25% Brahman genetics, their crossbreds based on LYC dam still maintained this advantage. However, when Brahman genetics exceeded 50%, the advantage for reproductive performance shifted and favored crossbreds based on the dam derived from RSC genetic resources. This phenomenon was actually observed in intensive farms in the Western Highlands, where ranchers often prefer crossbreds with high proportions of Brahman genetics derived from RSC as base females for commercial beef production [30]. The results from this research also indicated that increasing the proportion of Brahman genetics in female herds enhanced body weight but had a negative impact on reproductive performance. Farmers in the Western Highlands expect the beef cattle industry to acquire high economic efficiency, each cow should annually produce a calf and their calves need to adapt and grow up well within locally available conditions and based on crossbreeding from their own females. However, the large-framed reproductive females are not the choice of the majority of small-scaled beef cattle farmers in the Western Highlands because they would require much more inputs including feeds. In the Western Highlands, many farmers would like to keep small-sized females because these cows consume less feed but they prefer to have their cows inseminated with high-yielding beef breeds (BBB, Charolais). This is antagonistic with the actual practice because the large-sized reproductive females can be well utilized with exotic high-yielding beef breeds as their expectation. Meanwhile, the results from this research showed that although LYC and RSC possessed more outstanding reproductive performance than all crossbreds, their stature was smaller and the WFSs were quite low (176.44±8.52kg and 209.61±8.44 kg per female). The crossbreds with 25% Brahman genetics are also difficult to be mated or artificially inseminated with large-sized beef breeds due to smaller body weight. Crossbreds of 50% Brahman genetics relatively improved their body weights since their WFS were from 256.53±6.82 kg to 280.61±6.66 kg. In addition, their reproductive performance was likely better than that of the crossbred groups with higher Brahman genetic proportions. The crossbred group with 75% and 87.5% Brahman genetics have good enough (more than 300 kg) WFS thus, they were quite appropriate for crossbreeding with large-sized beef breeds such as Charolais or BBB, aligning with the current cattlemen's trend. However, their DOs were prolonged from 144.10±9.40 to 159.62±9.25 days, which reduced the lifetime productive efficiency of the females.

Several scientists stated that utilization of 50% Brahman genetic crossbred females for producing commercially high-yielding crossbreds would result in efficient growth performance and considerable economic efficiency [20,22,26]. On the other hand, utilization of crossbreeding would keep hybrid vigour in the herds. Applying breeding management strategies to maintain heterosis in a herd offers numerous economic benefits to the beef cattle efficiency [2].

From our findings, crossbred group of 50% Brahman genetics should be utilized as base reproductive females for smallhold farms, where they were lowly invested and applied technically simple knowledge. In order to be inseminated with large-frame beef breeds (BBB), the female must weigh at least 280 kg [31-33]. Therefore, crossbreds with the proportions of 75% or 87.5% Brahman genetics have larger body weight and would be effective for crossbreeding with exotic breeds, but their reproductive performance was worse, and thus, they need to be applied the appropriately updated technologies for feeding and management. They would be more appropriate to be applied in intensive farms, where they were well invested and with the advanced technologies. It is necessary to introduce moderate Brahman fractions, from 25% to 50% into female herds for small-scaled farms, and the fraction over 50% for intensive beef production systems in the Western Highlands of Vietnam.

From all mentioned above, females that were upgraded by Brahman genetics with moderate proportion, from 25 to 50% Brahman genetics, would be appropriate in small scaled farms. Those with over 50% of Brahman genetics seemed to be reasonable and appropriate for reproductive females in intensive beef farms because that body weight of reproductive females would not be so heavy and their reproductive performance would not be greatly affected.

5. Conclusions

It was concluded that incorporating Brahman genetics into reproductive female herds favorably improved body weight. However, traits such as age at first service, age at first calving, and days open were negatively affected. It is recommended to introduce moderate Brahman fractions (25% to 50%) for small-scale farms, while fractions exceeding 50% are better suited for intensive beef breeding herds in the Western Highlands of Vietnam.

Availability of Data and Materials

All data used in this research are available.

Author Contributions

Conceptualization, P.V.G., S.T.L., and N.V.T.; Methodology, P.V.G., N.T.D., N.V.T., and S.T.L.; Investigation, P.V.G., and N.V.T.; Writing – Original Draft, P.V.G., S.T.L., and N.T.D.; Writing – Review & Editing, P.V.G., and N.T.D.

Ethics Approval and Consent to Participate

The current research has been permitted from NIAS in the research project from Agricultural Ministry at the beginning. Finally, it was approved by the ethics committee of Vietnam Animal Welfare Association (Approval no. 2024-01/QĐ-VAWA).

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Conflict of Interest Statement

The authors declare no conflicts of interest. All of us have seen and agreed on the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not published in any other journal.

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