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ON THE COVER

The monomer form of the knob portion of the EDSV fiber protein is composed mostly of β-strands, which allow the fiber protein to self-assemble into a Trimer in its native state. This molecular form was modeled using the I-TASSER server(see page 41).

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Ecological studies and histopathological alterations caused by *Argulus japonicus* among goldfish in Mosul, Iraq

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ABSTRACT

Argulus japonicus (fish lice) is one of the the is one of the most economically significant *ectoparasites* infesting cultured and wild fish, predisposing hosts to secondary infections. This study aimed to investigated the ecological, epidemiological, and histopathological alterations caused by *A. japonicus* infestation in goldfish (*Carassius auratus*) in Mosul, Iraq. A total of 320 goldfish were obtained from fish local markets and carefully examined for infestation. Parasites were collected manually from the gills, fins, operculum, and skin using fine forceps, identified morphologically with standard entomological keys, and examined histologically using routine pathological procedures. Of the goldfish examined (320), 197 (61.56%; 95% CI: 56.13–66.72) were infested, yielding a total of 2,509 parasites. Infestation level ranged from 1 to 25, with a mean intensity of 12.73 and abundance values of 7.84. Histopathological examination revealed multiple lesions in the skin and gills of infested goldfish, including goblet cell hyperplasia, dermal edema, necrosis, hemorrhage, and gill lamellae destruction.

Keywords

Carassius auratus; Fish lice; Epidemiology; Pathological alterations

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Abbreviations

H&E: Hematoxylin and Eosin
CI: Confidence Interval
χ^2: Chi-Square value

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Introduction

Parasitic infection and infestation are recognized as one of the most significant challenges in aquaculture [1], often resulting in reduced fish growth, impaired vitality, and even death [2]. Among aquatic parasites, ectoparasites are of particular concern for aquatic animals raised in both aquaria and ponds. The major groups of ectoparasites include crustaceans, monogenes, and protozoans [3]. Of these *Argulus* species, a crustacean is a major ectoparasite with great economic importance in aquaculture [4].

Argulus species belong to the family Argulidae, a group of branchiuran parasites that infest and cause disease in fish and are commonly known as fish lice [5]. More than 100 species have been described, including *A. coregoni, A. foliaceus, A. inducus, A. japonicus,* and *A. siamenses* [6-8]. These parasites are known to infest a wide range of fish and amphibians, with particular affinity for goldfish, koi, centrarchids (sunfishes), cyprinids (minnows and carps), and salmonids (trout and salmon) [2-4].

Among them, *A. japonicus* causes the greatest economic impact among all ectoparasites that infest cultured and wild fishes. Infestation not only causes direct tissue damage but also causes susceptibility of its host to other infections such as bacterial (e.g., *Aeromonas* spp, *Pseudomonas* spp), parasitic (e.g., *Costia* spp), viral (e.g., *Rhabdovirus carpio*), and fungal (e.g., *Saprolegnia* spp) infections. Furthermore, *A. japonicus* can also play the role of being the intermediate host for numerous species of roundworms (nematodes) in fish populations [8, 9]. The economic losses resulting from *A. japonicus* infestation in fish are not only incurred due to the fish mortality but also from the costs of treatment and reduced growth rate during and after the outbreak of the disease, which affect the growth of fish farming worldwide [7, 9].

Ornamental fish such as goldfish (*Carassius auratus*) are highly valued for home decoration and entertainment due to their diversity and picturesque colors. The global ornamental fish industry has expanded rapidly, creating employment opportunities for many people all over the world [5, 6].

Given the importance of goldfish in the ornamental trade and the significant economic and pathological burden of *A. japonicus*, the present study aimed to investigate the ecological, epidemiological, and histopathological alterations caused by *A. japonicus* (fish louse) infestation in goldfish (*C. auratus*) in Mosul, Iraq.

Result

Out of the 320 goldfish examined, 197 (61.56%; 95% CI = 56.13–66.72) were infested with *A. japonicus*. A total of 2,509 *A. japonicus* were collected from the 197 infested goldfish. The infestation intensity ranged from 1 to 25, with a mean intensity of 12.73 and abundance values of 7.84 (Table 1). A statistically significant difference in infestation level of *A. japonicus* among goldfish was observed (χ^2 =34.88; p-value < 0.01). The distribution of *A. japonicus* prevalence and intensity among goldfish followed a normal distribution curve. The highest infestation rate was recorded in the 5 to <10 parasite category, with 59 goldfish (18.44%; 95% CI = 14.57–23.05). The lowest was in the < 5 parasite category, with 14 goldfish (4.38%; 95% CI = 2.62–7.21). The number of goldfish infested with *A. japonicus* in the other infestation level categories, ranged from 31 fish with 20 to 25 *A. japonicus* (9.69%; 95% CI = 6.91–13.42) to 48 fish with 10 to < 15 *A. japonicus* (15.00%; 95% CI = 11.50–19.33) (Table 2).

Table 1.
Ecological parameters of *A. japonicus* infestation among goldfish (*C. auratus*) in Mosul City, Iraq

Ecological indices	Occurrence
Number of fish examined	320
Number of fish infested	197
Total number of *A. japonicus* collected	2,509
Range of infestation	1-25
Prevalence (%)	61.56
Mean intensity	12.73
Abundance	7.84

Histopathological examination of goldfish skin infested with *A. japonicus* revealed severe lesions, including goblet cell hyperplasia, scale pocket forma-

Table 2.
Level of *A. japonicus* infestation among goldfish (*C. auratus*) in Mosul City, Iraq

Level of infestation	Number of fish	Prevalence (%)	95 % CI	χ2	p-value
<5	14	4.38	2.62 – 7.21		
5 to <10	59	18.44	14.57 – 23.05		
10 to <15	48	15.00	11.50 – 19.33	34.88 ¥	<0.01
15 to <20	45	14.06	10.68 – 18.30		
20 to 25	31	9.69	6.91 – 13.42		

tion and dermal edema, Zenker's necrosis of the muscular layer, myositis, hemorrhage, and actinic arrows of scales embedded within the dermis (Fig. 1).

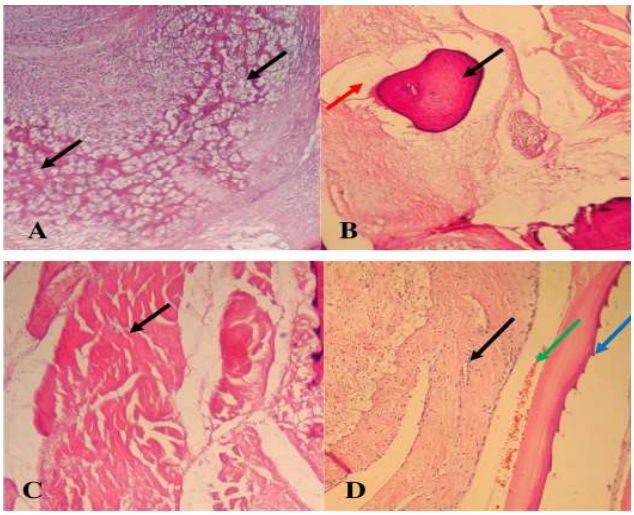

Figure 1.
Histopathological examination of goldfish (*C. auratus*) skin infested with *A. japonicus*; 1a, showing hyperplasia of goblet cells (black arrows). 1b, showing scale (black arrow) and edema (red arrow). 1c, showing Zenker necrosis of the muscular layer (black arrow). 1d, shows myositis (black arrow), hemorrhage (green arrow), and actinic arrows of scales embedded in the dermis (blue arrow) H&E X40.

The histopathological lesion observed in the gills was hemorrhage and necrosis of the gill filaments (Fig. 2).

Figure 2.
Histopathological examination of goldfish (*C. auratus*) gills infested with *A. japonicus*, showing hemorrhage (black arrow) and necrosis of gill filaments, H&E X40.

Discussion

In this study, we aimed at investigating the ecological, epidemiological, and histopathological alterations caused by *A. japonicus* infestation in goldfish in Mosul, Iraq. Prevalence is a valuable and informative measure for lice epidemiology and population dynamics in fish [10]. In the present study, *A. japonicus* infestation was recorded in 61.56% of examined, a prevalence higher than 35.20%, 51.18%, and 57.00% reported among goldfish in China [9], India [6], and Indonesia [11], respectively. *Argulus japonicus* has been documented to infest a wide range of fishes, with varying prevalences of 100.00% in pike-perch (Sander lucioperca) [12], 80.00% in common carp (Cyprinus carpio) [13], and 65.00% in Koi (Cyprinus rubrofuscus) [11]. In addition, prevalences of 42.12% in mandarin fish (Siniperca chuatsi), 36.07% in black carp (Mylopharyngodon piceus), 35.12% in silver carp (Hypophthalmichthys molitrix), 33.83% in rainbow trout (Oncorhynchus mykiss), 31.60% in perch (Perca fluviatilis), 20.00% in brown trout (Salmo trutta) [9], and 9.50% on Cirrhinus mrigala [4] have also been reported.

The high mean intensity (12.73) and abundance (7.84) observed in this study. Aalberg et al. [12] and Kismiyati et al. [11] also documented a high mean intensity and abundance in Indonesia and Slovakia, respectively. Such elevated mean intensity and abundance of *A. japonicus* may be attributed to the change in the natural abiotic and biotic factors in the aquatic system as recorded in cultured fishes, which strongly influence the outbreak of ectoparasites (including lice) in fishes [14].

The high prevalence and heavy infestation rate of *A. japonicus* in this study support its classification as a generalist parasite, as postulated by many authors who reported a high prevalence and heavy infestation rate of the fish louse [9].

Histopathological analysis revealed severe alterations in the musculoskeletal system of infested goldfish, which are characterized by goblet cell hyperplasia, scale pocket formation and dermal edema, Zenker's necrosis of the muscular fibers, myositis, hemorrhage, and actinic arrows of scales embedded within the dermis. Histological findings such as infiltration of inflammatory cells and Zenker necrosis of the myofibril with hemorrhage and edema were reported in the musculoskeletal system of goldfish (*C. auratus*) infested

with *A. japonicus* [15], also Ahamad et al. [16] and Al-Darwesh et al. [15] reported similar histological findings in Labeo rohita and Cirrhinus mrigala fishes, respectively.

Infestation of also led to the loss of the anatomical structure of goldfish. The histopathological finding revealed a total destruction of the secondary gill lamellae with disintegration of the gill filament, which was shown by the intensity of the louse (*A. japonicus*) infestation. Long-standing infestation with a large number of *A. japonicus* may negatively affect the structure of the gill in goldfish. The rakers of the gill were found blocked with too many debris particles, which could have resulted in the complete loss of the secondary gill lamellae. These findings are similar to the reports of Mamun et al. [17]. The pathological effects caused by argulosis are associated with the constant irritative behavior of infected fish, erratic swimming patterns and feeding disruption, caused by lice attachment [18].

The pre-oral stylet and modified mouthparts penetration of the host's skin, muscles, and gills causing localized hemorrhage and erythema, and numerous numbers of *Argulus* species feeding close to each other may cause localized swelling and edema. Feeding activity and constant piercing of lice with their stylet (the mouthpart adopted for sucking blood), introduces cytotoxic enzymes leading to inflammatory lesions that are characterized by hemorrhages, increased mucous secretion, and necrosis of the affected area [19, 20, 22]. The gill damage causes osmoregulation disturbances and reduces the ability of the fish to sustain normal oxygen uptake, which may lead to hypoxia and even the death of the infested fish [21].

In conclusion, our findings demonstrate that *A. japonicus* is highly prevalent in goldfish (*C. auratus*) populations in Mosul, Iraq, with heavy infestation intensities. The parasite induces profound histological lesions in both the skin and gills of the infested goldfish, underscoring its significant impact on host health and its potential to cause mortality in cultured fish.

Materials and Methods

Study area

This study was conducted in Mosul, Iraq, a city located on the west bank of the Tigris River, directly opposite the ancient Assyrian city of Nineveh on the east bank [23]. Mosul lies at 43.09 E and 36.19 N, with an elevation of 230 meters above sea level, and it covers a total land area of 70 sq mi (180 km2). The city has a climate that is characterized by cold winters with occasional snowfall and dry, hot summers [24].

Goldfish collection

A total of 320 goldfish were obtained from local fish markets and fish stores in Mosul. The fish were kept alive in water vats and transported to the laboratory of the Pathology and Poultry Diseases Department, College of Veterinary Medicine, University of Mosul, Mosul, Iraq, for parasitological, pathological and histopathological examinations.

Collection and identification of Argulus japonicus

Argulus japonicus was collected manually from the gills, fins, operculum, and skin of each goldfish using fine forceps following a thorough and careful external examination. *Argulus japonicus* from each goldfish was collected and preserved separately into sample bottles containing 70% ethanol for morphological identification, their count and intensity of their infestation also been record. Identification was performed under a stereomicroscope, using entomological keys described by Bykhovskaya-Pavlovskaya et al. [25] and Rushton-Mellor [26].

Histopathological analysis

Few of the goldfish infested with *A. japonicus* were anesthetized following the method described by AL-Taee et al. [27]. Tissue samples, including muscle, skin, and gill were excised and fixed in neutral buffered formalin (10%) for a at least 48 hours. Fixed tissues were trimmed into small parts of about 2 to 3 mm sections, dehydrated through ascending grades of ethanol (70%, 80%, 90%, and 100%; 15 minutes each), cleared in absolute xylene (100%) and embedding in paraffin wax. Sections of 5 µm were prepared and stained with H&E according to the procedure of Akanbi et al. [28]. Slides were examined under a compound light microscope at X40 magnification.

Statistical evaluation

The prevalence, abundance, and mean intensity of *A. japonicus* infestation in goldfish were calculated following the ecological indices (in terms of comparative parameters) for parasitology described by Margolis et al. [29] and Bush et al. [30].

$$\text{Prevalence (\%)} = \frac{\text{Number of infected fish} \times 100}{\text{Total number of fish examined}}$$

$$\text{Mean Intensity} = \frac{\text{Number of collected parasites}}{\text{Number of infected fish}}$$

Authors' Contributions

S.K.A., D.A.A., and N.S.A. conceived and planned the experiments. S.K.A. and N.S.A. carried out the experiments. S.K.A., D.A.A., and N.S.A. contributed to sample preparation. S.K.A., D.A.A., N.S.A., SDO, and FIG contributed to the interpretation of the results.

SDO took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Competing Interests

The authors hereby state that there are no conflicts of interest among them concerning the funding of this research and the publication of this manuscript.

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Growth indices, non-specific immune parameters, skin mucus protein profile and resistance to *Saprolegnia parasitica* in rainbow trout fed *Mentha longifolia*

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ABSTRACT

The development and implementation of nutritionally balanced diets is regarded as essential biological factors in improving growth and maintaining the health of aquaculture species. This study evaluated the effects of *Mentha longifolia* hydroalcoholic extract on growth performance, Hemato-immunological responses and skin mucus protein in rainbow trout (*Oncorhynchus mykiss*). A total of 360 fish (10.17 ± 0.18 g) were acclimated to the experimental environment and randomly distributed across 12 tanks (four treatment in triplicate; 30 fish per tank). Experimental diets were prepared by incorporating *M. longifolia* hydroalcoholic extract at 0 (control), 1, 2, and 3 g/kg into a basal diet and fed to the fish for a 60 day, after which they were challenged with *Saprolegnia parasitica*. Fish fed *M. longifolia* diets exhibited significantly higher specific growth rate and weight gain, along with reduced feed conversion ratio compared with the control. Red and white blood cells counts, serum respiratory burst activity, albumin, and total protein levels were also significantly higher in extract fed groups. Following challenge with *S. parasitica*, survival rate was markedly higher in treated fish compared to control. Overall, dietary supplementation with 1 g/kg *M. longifolia* hydroalcoholic extract is recommended to stimulate immune response, enhance growth performance, and improve resistance of rainbow trout to *S. parasitica*.

Keywords

Mentha longifolia; rainbow trout; Growth; Immunity; Blood; Oomycete; Oncorhynchus mykiss

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Abbreviations

M. longifolia: Mentha longifolia
S. parasitica: Saprolegnia parasitica
O. mykiss: Oncorhynchus mykiss
FCR: Feed conversion rate
WG: weight gain
SGR: specific growth rate
WBC: White Blood Cell
RBC: Red Blood Cell

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Introduction

Intensive Rainbow trout aquaculture is often associated with many stresses that predispose the fish to a variety of infectious diseases. Among the most important pathogens in freshwater systems are oomycetes, which can be found in various freshwater ecosystems and can pose risks to both farmed and wild fish populations [1]. Among them, *Saprolegnia parasitica* is a significant oomycete species that affects salmonids and other fish, leading to a disease known as saprolegniasis, which is considered one of the most crucial oomycete-related diseases in aquaculture. [1].

Chemical disinfectants are commonly used to control saprolegniasis. However, concerns regarding the negative impact of pesticides and harmful chemical compounds have encouraged researchers to seek for safer, natural and eco-friendly alternatives [2]. Plants have always been considered as a valuable source of biologically active compounds, with potential immunostimulatory properties [3,4]. Extensive research has highlighted the ability of medicinal plants to improve immunity and growth in aquatic animals [5,6]. Because immunostimulants primarily enhance the non-specific immune system, they are often considered more effective in aquatic animals than in warm-blooded animals [7,8].

Numerous studies have attentive on the use of medicinal plants to enhance fish growth, immunity, and resistance to pathogens [5,9]. For instance, Mehrabi et al. [10] reported that aloe vera (*Aloe brabadensis*) stimulated the immune system and improved resistance to *S. parasitica* in rainbow trout. Feeding *Mentha longifolia* hydroalcoholic extract to rainbow trout increased resistance to Yersinia ruckeri infection [11]. Similarly, Mehrabi et al. [12] reported that nettle (Urtica dioica) powder was shown to stimulate the immune system and increased survival in rainbow trout challenged with the *S. parasitica.*

Mentha longifolia, commonly known as horsemint, is one of the most important species in the Lamiacea family. The fresh buds and leaves possess antifungal, antimicrobial, anti-inflammatory, and antioxidant properties. These activities are attributed to its diverse biochemical compounds, including cinnamic acid, aglycone, acetylated flavonoids, glycoside steroids, carvone, menthol, piperitone oxide, limonene, 1.8-quinol, polgun, beta-caryophyllene, and trans-piperitol epoxide. [13,14].

As in other vertebrates, the immune system in fish includes innate and acquired immune system, with innate responses serving as the first line of defence against pathogens [15]. Mucus, a critical innate immune barrier, prevents pathogen binding through continuous secretion and excretion of dead tissues and stimuli [16,17].

To date, no study has investigated the effect of horsemint (*M. longifolia*) hydroalcoholic extract on rainbow trout resistance to saprolegniasis. Given *Mentha longifolia* contains compounds with antibacterial and antifungal properties, which help prevent microbial and fungal infections in fish [18]. Therefore, the present study was conducted to investigate, for the first time, the effects of *M. longifolia* extract on growth performance, immune responses and resistance of rainbow trout against *S. parasitica.*

Result

Growth parameters

The effects of feeding diets containing *M. longifolia* extract on the growth performance of rainbow trout after 60 days are presented in Table 1. The final mean weight increased significantly in all groups fed with *M. longifolia* ($p < 0.05$). The highest weight gain was observed in fish fed the diet containing 1 g/kg of *M. longifolia*. Similarly, this treatment also gained the highest SGR ($p < 0.05$). Overall, all extract fed groups showed significantly improved SGR compared to the control ($p < 0.05$). FCR decreased significantly in all groups receiving *M. longifolia* extract ($p < 0.05$), with the lowest FCR observed in the 1 g/kg treatment group ($p < 0.05$). Survival remained 100% across all treatments and the control after 60 days.

Blood parameters

Table 2 shows the hematological responses of trout after feeding *M. longifolia* extract for 60 days. RBC and WBC counts, hemoglobin concentrations, and hematocrit percentages all increased significantly in extract fed groups compared to the control ($p < 0.05$). Feeding with the minimum effective dose of the extract (1 g/kg) resulted in significant improvements

Table 1.
The growth factors (mean ± SD) of rainbow trout after feeding with M. longifolia for 60 days

groups	Initial weight (g)	Final weight (g)	Weight gain (g)	SGR (%)	FCR (%)	Survival rate (%)
0g/kg	10.25 ± 0.20^{a}	27.74 ± 1.68^{a}	17.49 ± 0.76^{a}	1.65 ± 0.11^{a}	1.49 ± 0.08^{c}	100a
1g/kg	10.20 ± 0.10^{a}	44.03 ± 2.16^{c}	33.83 ± 1.08^{c}	2.43 ± 0.19^{c}	0.89 ± 0.06^{a}	100a
2 g/kg	10.08 ± 0.23^{a}	38.77 ± 2.08^{b}	28.69 ± 1.02^{b}	2.24 ± 0.14^{b}	1.18 ± 0.07^{b}	100a
3 g/kg	10.16 ± 0.25^{a}	40.34 ± 1.98^{b}	30.18 ± 0.98^{b}	2.29 ± 0.10^{b}	1.21 ± 0.05^{b}	100a

In each column, values with different superscript letters are significantly different (P < 0.05).

Table 2.
The hematological factors (mean ± SD) of rainbow trout after feeding with *M. longifolia* for 60 days (pre-challenge) and 15 days after challenging with *S. parasitica* (post-challenge) are presented below

Factors	Time							
	Pre- challenge				Post- challenge			
	0.0%	0.1%	0.2%	0.3%	0.0%	0.1%	0.2%	0.3%
WBC ($\times 10^3$ µL)	15.62 ± 0.88^a	19.89 ± 0.34^b	20.06 ± 0.52^b	20.23 ± 0.31^b	19.28 ± 0.46^a	20.39 ± 0.61^b	21.12 ± 0.44^c	21.38 ± 0.63^c
RBC ($\times 10^6$ µL)	0.98 ± 0.19^a	1.47 ± 0.65^b	1.50 ± 0.26^b	1.53 ± 0.15^b	0.68 ± 0.49^a	1.28 ± 0.12^b	1.30 ± 0.17^b	1.31 ± 0.14^b
Hemoglobin (g dL^{-1})	9.13 ± 0.76^a	11.98 ± 0.32^b	12.21 ± 0.62^b	12.25 ± 0.49^b	6.41 ± 0.42^a	10.93 ± 0.59^b	11.10 ± 0.78^b	11.14 ± 0.61^b
Hematocrit (%)	28.20 ± 1.38^a	35.93 ± 1.00^b	36.19 ± 0.84^b	36.64 ± 1.10^b	28.90 ± 1.77^a	32.71 ± 1.41^b	33.13 ± 1.38^b	33.62 ± 1.65^b
MCV (fl)	287.75 ± 8.23^b	244.42 ± 7.51^a	241.26 ± 10.45^a	239.47 ± 8.62^a	425.00 ± 8.94^b	255.54 ± 8.24^a	254.84 ± 8.72^a	256.64 ± 9.81^a
MCH (pg)	93.16 ± 1.16^b	81.49 ± 2.24^a	81.4 ± 1.54^a	80.06 ± 1.356^a	94.26 ± 2.19^b	85.39 ± 2.28 ^{a}	85.38 ± 1.10^a	85.03 ± 2.45^a
MCHC (g dL^{-1})	32.37 ± 2. 42^a	33.34 ± 1. 37^a	33.73 ± 1. 80^a	33.43 ± 2. 48^a	22.17 ± 1. 56^a	33.41 ± 1.23^b	33.50 ± 1.12^b	33.13 ± 1.86^b
Lymphocyte (%)	73.22 ± 2. 28^a	81.63 ± 3.4^b	81.88 ± 2.94^b	82.10 ± 2.766^b	61.59 ± 2. 32^a	70.36 ± 1.14^b	75.91 ± 1.28^c	76.58 ± 1.49^c
Neutrophil (%)	22.10 ± 0.82^b	13.25 ± 0.17^a	11.18 ± 0.41^a	11.01 ± 0. 74^a	28.66 ± 0. 49c	23.32 ± 0. 35^b	17.21 ± 0. 21^a	16.73 ± 0. 40^a
Monocyte (%)	4.68 ± 0.75^a	5.12 ± 0. 33^a	6.94 ± 0.7^{9b}	6.89 ± 0. 65^b	9.75 ± 0. 28^b	6.32 ± 0.15^a	6.88 ± 0.36^a	6.69 ± 0.21^a

Red blood cells (RBC), white blood cells (WBC), Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). In each column, values with different superscript letters are significantly different ($P < 0.05$).

compared to the control. However, no significant difference were detected among the different treatments in RBC and WBC counts, hematocrit, and hemoglobin ($p < 0.05$). Monocyte and lymphocyte counts were significantly higher in the groups fed with 0.2% and 0.3% extract fed groups ($p < 0.05$), while neutrophil counts decreased significantly in all extract fed groups ($p < 0.05$). Additionally, MCH and MCV also decreased significantly in the extract fed groups ($p < 0.05$). After challenging with *S. parasitica*, blood parameters significantly increased in the extract fed groups compared to the control ($p < 0.05$). WBC counts increased with higher doses of *M. longifolia* extract, reaching their peak in the 2 g/kg and 3 g/kg groups. These values differed significantly from both the control and the 1 g/kg treatment. ($p < 0.05$). However, no significant differences were observed among the extract fed treatments regarding RBC counts, hemoglobin concentrations, and hematocrit percentages ($p > 0.05$). Furthermore, lymphocyte counts, MCHC, monocyte and neutrophil counts, as well as MCH and MCV, all showed significant increases in extract fed fish compared to the control ($p < 0.05$).

Serum biochemical indices

Serum biochemical parameters after 60 days of feeding with different doses of *M. longifolia* extract and post challenge with *S. parasitica*, are summarized in Table 3. Both albumin and total protein levels increased significantly in all extract fed treatments compared to the control ($p < 0.05$). The highest values were observed in the 1 g/kg treatment, which different significantly from the other groups ($p < 0.05$).

Immune response of fish

As shown in Table 4, neutrophil respiratory burst, lysozyme activity, and ACH50 of levels were significantly increased in extract fed groups compared to the control after 60 days ($p < 0.05$). However, no significant differences were detected between the extract fed treatments in lysozyme activity and ACH50 ($p < 0.05$). The highest neutrophil respiratory burst occurred in the 1 g/kg treatment, which differed significantly from the other groups ($p < 0.05$). Feeding fish with the minimum effective dose (1 g/kg) resulted in significant rises of all immune parameters compared to the control. Following the *S. parasitica* challenge, immune factors significantly increased in all extract fed groups, at all three levels, compared to the control ($p < 0.05$). The highest neutrophil respiratory burst was observed in the 2 g/kg treatment, which differed significantly from the other groups ($p < 0.05$).

Table 3.
The growth factors (mean ± SD) of rainbow trout after feeding with *M. longifolia* for 60 days

	Groups	Total protein (g dL-1)	Albumin (g dL-1)
Pre-challenge	0 g/kg	3.49 ± 0.12[a]	1.30 ± 0.10[a]
	1 g/kg	4.18 ± 0.25[c]	2.60 ± 0.35[c]
	2 g/kg	3.81 ± 0.19[b]	2. 20 ± 0.29[b]
	3 g/kg	3.80 ± 0.20[b]	2.10 ± 0.55[b]
Post-challenge	0 g/kg	3.10 ± 0.33[a]	1.00 ± 0.31[a]
	1 g/kg	3.80 ± 0.26[c]	2.35 ± 0.26[c]
	2 g/kg	3.44 ± 0.41[b]	1.93 ± 0.39[b]
	3 g/kg	3.41 ± 0.21[b]	1.88 ± 0.49[b]

In each column, values with different superscript letters are significantly different ($P < 0.05$).

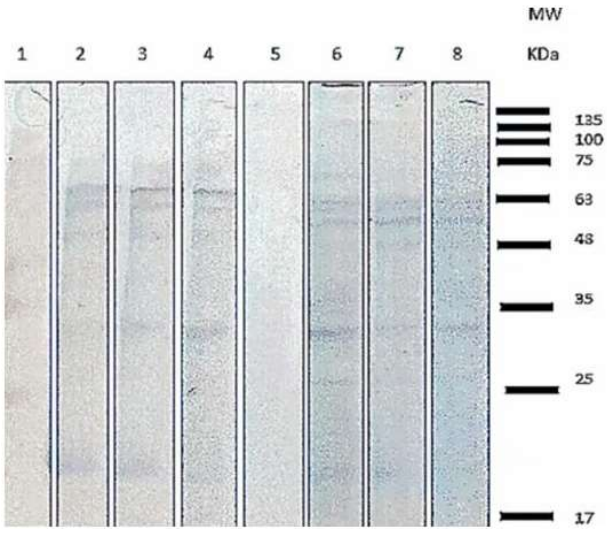

Figure1.
Mucus protein pattern of rainbow trout after feeding with *M. longifolia* for 60 days (pre-challenge) and 15 days after challenging with S. parasitica (post-challenge) (End of 60 days: 1 = Control, 2 = 0.1%, 3 = 0.2%, 4 = 0.3% and 15 days post-challenge: 5 = Control, 6 = 0.1%, 7 = 0.2%, 8 = 0.3%)

Mucus protein pattern

Figure 1 shows the protein profile of skin mucus in rainbow trout fed *M. longifolia* extract for 60 days, both before and 15 days after exposure to *S. parasitica.* The band density exhibited significant variations among the protein profiles of the different groups, with the identified proteins bands ranged from 17 to 75 kDa. The quantity and mass of bands within the 17-75 kDa molecular weight range were found to be greater in the groups receiving the extract compared to the control group. After the fungal challenge, the count and density of protein bands remained stable in extract fed groups, while the control group showed significant reduction. Survival results following the challenge are presented in Figure 2. After 15 days of exposure to *S. parasitica*, the fish that received *M. longifolia* extract exhibited significantly higher survival rates compared to the control ($p < 0.05$). However, no significant differences were detected among the groups fed with *M. longifolia* after the challenge with *S. parasitica* ($p < 0.05$).

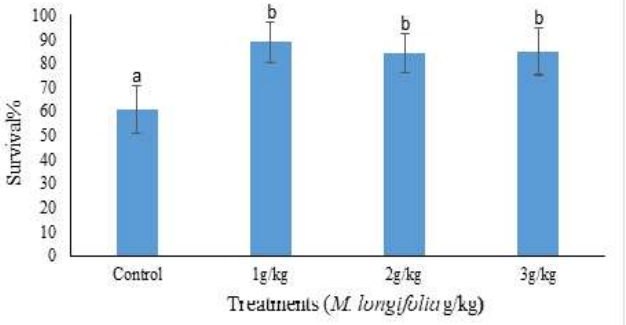

Figure 2.
Survival rate of rainbow trout fed with different levels of *M. longifolia* after 15 days of challenging with *S. parasitica*. The values with different superscript letters are significantly different ($p < 0.05$).

Table 4.
The immune factors (mean ± SD) of rainbow trout after feeding with *M. longifolia* for 60 days (pre-challenge) and 15 days after challenging with *S. parasitica* (post-challenge)

Time	Groups	Respiratory burst (OD at 540 nm)	Lysozyme (U/ml)	ACH50 (U/ml)
Pre-challenge	0 g/kg	0.493 ± 0.06[a]	429.21 ± 0.29[a]	38.09 ± 0.16[a]
	1 g/kg	0.551 ± 0.09[c]	430.17 ± 0.29[b]	42.12 ± 0.69[b]
	2 g/kg	0.530 ± 0.03[b]	430.00 ± 0.32[b]	42.44 ± 1.18[b]
	3 g/kg	0.529 ± 0.04[b]	430.45 ± 0.26[b]	42.31 ± 1.14[b]
Post-challenge	0 g/kg	0.464 ± 0.05[a]	430.00 ± 21[a]	128 ± 14[a]
	1 g/kg	0.572 ± 0.01[b]	431.17 ± 47[b]	138 ± 12[b]
	2 g/kg	0.739 ± 0.07[d]	431.14 ± 35[b]	139 ± 17[b]
	3 g/kg	0.680 ± 0.04[c]	431.62 ± 39[b]	140 ± 19[b]

Alternative complement activity (ACH50). In each column, values with different superscript letters are significantly different ($p < 0.05$).

Discussion

The use of immune system stimulants in aquaculture has become increasingly important for improving and stimulating the activity of non-specific immune system in fish [19,20]. Among these, the use of plants to control pathogens by stimulating the immune system and antioxidant activity have attracted considerable attention [5,8,9].

In the present study, supplementation with the lowest test dose (1 g/kg) of *M. longifolia* extract significantly improved SGR, weight gain, and FCR, as well as with other treatments containing *M. longifolia*, compared to the control. Several studies have documented the effects of plant extracts on various growth parameters in fish. Feeding Caspian kutum (*Rutilus frisii kutum*) with 1 g/kg *M. longifolia* led to increased body weight compare to controls [21]. Raissy et al. [22] showed that a combination of *Mentha longifo*lia, *Thymus carmanicus*, and *Trachyspermum copticum* improved weight gain and FCR in rainbow trout for 45 days. Likewise, Ghanbary et al. [23] demonstrated that incorporating *Thymbra spicata* hydroalcoholic extract significantly increased the final weight in rainbow trout on 8-week period. While, Mehrabi et al. [3] administered 5, 10, and 15 g of nettle (Urtica dioica) powder per kilogram of diet to rainbow trout over an 8-week period and found that fish fed 5 g/kg of dietary nettle showed significant enhancements in SGR and final growth, and reduction in FCR.

Blood parameters in fish change due to physiological and various external factors, such as diet [24]. Blood tissue, determination of blood factors, hematological tests, and biochemical analysis of blood plasma can be suitable indicators for diagnosis, determination of health, and infectious diseases in fish [25]. In this study, M. longifolia extract significantly improved blood parameters such as RBC, WBC, hemoglobin, and hematocrit levels compared to the control. These improvements may be linked to the vitamin C content of *M. longifolia*, which can increase intestinal absorption of vitamins and minerals from consumption of this plant, and thereby lead to improvement of hematological parameters [26]. Comparable results have been reported with M. *longifolia* [11], common mallow (*Malvae sylvestris*) [27], and *Thymbra spicata* [23], all of which improved hematological parameters in trout. Moreover, *Aloe barbadensis* also showed positive effects as an immunostimulants and significantly increased RBC count and hemoglobin concentrations in experimental groups of rainbow trout compared to the control [10]. In another study, feeding rainbow trout with Urtica *dioica*, extract for 4 weeks led to significant elevations of WBC and RBC counts [12]. Since WBCs, along with biochemical factors such as lysozyme, serum proteins, and ACH50, are crucial components of the fish innate immune system, their modulation is particularly relevant under stressful conditions such as illness, dietary imbalances, high stocking density, and environmental stressors [28]. In general, the evaluation of total protein and albumin is considered as an important indicator in response to environmental stresses. Stress from oxidative compounds in the liver, the primary organ for producing albumin and globulins, reduces serum total protein due to liver damage [29]. In contrary, elevated serum protein levels reflect enhanced non-specific immune stimulation, and in fact enhanced defensive response [30]. Our findings indicated that feeding rainbow trout with varying levels of *M. longifolia* hydroalcoholic extract significantly increased albumin and total protein levels. This aligns with the findings of Mehrabi et al. [10], who reported significant increases in serum albumin and total protein in rainbow trout fed aloe vera extract. Comparable results have also been reported regarding enhanced serum biochemical factors in rainbow trout when fed extracts of sweet basil (*Ocimum basilicum*) and Sargassum (*Sargassum angustifolium*) hot water extract [8,31].

Increasing serum lysozyme activity is indicative of an improvement in fish immunity and will help the immune system to cope better with infectious and stressful factors [32]. The results showed a significant rise in lysozyme activity after 60 days of feeding with *M. longifolia* extract. Similarly, the use of dietary aloe vera (*Barbados aloe*) hydroalcoholic extract for 30 days [3], *Stachys lavandulifolia* and greek juniper (*Juniperus excelsa)* significantly increased lysozyme activity in rainbow trout [33,34]. Phagocytic cells are able to produce

superoxide anions during their respiratory burst activity, which kill toxic oxygen, killing bacteria [35]. In fish, the respiratory burst is associated with the release of cytokines and the activation of inflammatory reactions [36].

In the present study, *leukocyte* respiratory burst after 60 days of feeding, increased significantly in the extract fed treatments compared to the control. This is similar to increased leukocyte respiratory burst activity as a result of feeding rainbow trout with ginseng (*Ginseng panax*) compared with the control group [37]. Similarly, feeding sea bass (*Dicentrarchus labrax*) with extracts from okra (*Abelmoschus esculentus*) seeds, fruits, and leaves [38], as well as Nile tilapia (*Oreochromis niloticus*) with *Artemisia annua* alcohol extract [39], and rainbow trout with *Aloe barbadensis* extract [10], has been associated with elevated leukocyte respiratory burst activity. Increase in complement system activity was also observed in this study, complement proteins are important non-specific immune factors that play a significant role in the immune response of fish. Increased complement activity has frequently been reported following the use of non-specific immunostimulants [7]. In general, the ability of medicinal plants has been proven in the activation and stimulation of complement activity, and it can be claimed that the present results are consistent with those of other researchers. Naderi Farsani et al. [40] studied the nutritional effects of coriander (Coriandrum sativum) on the immune system of rainbow trout and found that fish fed with this plant at 2% for 8 weeks exhibited significantly increased complement activity compare to the control.

The fish's skin is covered by a constantly replaced mucus layer, which acts as the first barrier against pathogens. Fish mucus is a vital source of components involved in the non-specific immune system, including lectins, immunoglobulins, antibacterial lipids, lysozyme, various proteins, and complement proteins [16]. Therefore, changes in mucus protein levels are considered a suitable indicator for assessing the non-specific immune status in fish. In this study, extract-fed trout exhibited an increased number and density of skin mucus bands compared to the control, both before and after fungal challenge.

Similar outcomes were reported by Heydari et al. [11], they recently studied the impact of hydroalcoholic extract of *M. longifolia* on the protein patterns of skin mucus in rainbow trout. Based on their study, feeding with a diet containing 3% *M. longifolia* extract for 30 days increased concentration and number of skin mucus bands compare to the control. In farmed aquatic animals, most bacterial, viral, fungal, and parasitic pathogens are secondary pathogens that present with stressful conditions and impaired aquatic health and immunity of aquaculture species. Therefore, strengthening the immune system is of paramount importance in maintaining the health of aquaculture species and preventing the spread of diseases among the population of farmed fish, thereby preventing disease induced economic losses and mortalities. In our study, results of challenge test with *S. parasitica* confirmed the protective effects of dietary *M. longifolia*, as survival rates significantly increased in all extract fed treatments compared to controls, *M. longifolia* extract enhanced the immune system by increasing blood and serum immunity factors, and subsequently improved the fish's resistance to *S. parasitica*. Comparable improvements in disease resistance have been also reported. The use of *Aloe barbadensis* powder at 15 g/kg [10] and 0.5% *Urtica dioica* [12] of rainbow trout diet for 8 weeks increased fish survival in challenge with *S. parasitica*, in addition to improvements in hematological and serum biochemical parameters. The dietary hot-water extract of *S. angustifolium* at a concentration of 400 mg/kg for 56 days resulted in a cessation of rainbow trout losses, improved serum and blood parameters, and significantly increased fish survival against *Yersinia ruckeri* compared to the control [8].

Conclusions

Medicinal plants represent as a viable alternative to pharmaceutical compounds for improving health and promoting growth in aquaculture species. The present study demonstrates that dietary inclusion of *M. longifolia* hydroalcoholic extract into the diet of *O. mykiss*, positively influences growth, hematological parameters, serum biochemistry, immune response, and mucus protein

patterns. Different levels of dietary *M. longifolia* extract had obvious impacts during 60 days of feeding, and on immunity and enhanced resistance of this species in challenge with *S. parasitica*. From an economic perspective, utilizing minimal doses of medicinal plants lowers production costs, including those associated with plant procurement and management. As a result, the most favourable outcomes in weight gain (33.83 ± 1.08g), final weight (44.03 ± 2.16g), SGR (2.43 ± 0.19%), FCR (0.89±0.06%), total protein (4.18 ± 0.25 g dL-1) and albumin (2.60 ± 0.35 g dL-1) of fish after the challenge with *S. parasitica* were observed in the treatment group receiving 1 g/kg *M. longifolia* in the basal diet. This difference was statistically significant for most parameters compared to other experimental groups. Therefore, *M. longifolia* hydroalcoholic extract, in particular 1 g/kg of diet, as the lowest effective dose can be recommended as an oral supplement to stimulate the growth and immune system of *O. mykiss*.

Materials and Methods

Extraction Solvent Preparation

Fresh leaves of *M. longifolia* (2 kg) were collected from mountainous areas of Semnan city, Iran, and approved by botanists at the University of Agricultural Sciences and Natural Resources, Sari, Iran. The leaves were shade dried at room temperature (25 °C), ground into powder using a mill, and used for the hydroalcoholic extraction following the method described by Heydari et al. [11].

Diet preparation

In order to prepare diet, the hydroalcoholic extracts were dissolved and homogenized in 6 mL of ethanol according to required concentrations (1, 2, and 3 g/kg diet) and then sprayed onto a commercial rainbow trout diet (Bayza Company, Fars, Iran). A control diet was prepared by adding only 6 mL of the solvent without extract [11]. The experimental diets were then dried for 24 h, after which the oil was sprayed on the feed in all groups to cover and preserve the plant extract lastly, the diets were stored at 4 °C until use.

Experimental fish

All procedures were conducted in accordance with standard ethical guidelines, protocols, and were approved by Animal Ethics Committee of the Sari Agricultural Sciences and Natural Resources University (Approval No.1274320). In the present study, rainbow trout were obtained from a local farm in Sari, clinically screened for potential diseases and the absence of any sores. The fish were subsequently maintained in a fish culture facility at the university for a two-week period to acclimatize to their new environment. This experiment consisted of four dietary treatments: 1, 2, and 3 g/kg of *M. longifolia* hydroalcoholic extract, and a control, each with three replications (30 juvenile fish per replicate; average initial weight 10.17 ± 0.18 g; length 10.27 ± 0.63 cm). Fish were stocked into 12 tanks (250 L) and fed three times daily (8:00, 12:00, and 16:00) at 3% of body weight [10,4038] for 60 days. All tanks were oxygenated using a central aeration system, and water was supplied from a well. The water physicochemical parameters were monitored daily and maintained as follows: temperature 14.01 ± 0.78 °C, dissolved oxygen 6.32 ± 0.33 mg/L, ammonia 0.05 mg/L, hardness 600.52 ± 22.26 mg/L, salinity 0.61 ± 0.01 ppt, electrical conductivity (EC) 1202.72 ± 46.84 µS/cm, and pH 7.2 ± 0.22. To ensure water quality, 50% of the water was replaced daily.

Challenge with S. parasitica

A pure culture of *S. parasitica* (accession NO. KC992717) was obtained from Sari Agricultural Sciences and Natural Resources Universit)SANRU(and used to perform the challenge test. First, the oomycete was grown on Potato Dextrose Agar and then circular tablets (1 cm in diameter) were prepared from the fungus with the culture medium and placed in sterile tubes containing distilled water at 18-20 °C for one week to induce sporogenesis. Zoospores were harvested by centrifuged (3000 rpm for 10 minutes) to separate the zoospores and counted using a hemocytometer slide [41]. At the end of the experimental period, the fish were challenged with *S. parasitica*. To perform challenge test, 30 fish were selected per treatment (10 pieces per replication). Before the fish were exposed to the zoospores, they were first anesthetized. Then, to increase the permeability of the zoospores to the fish's skin, the scales in the tail area (3cm) were removed. Afterward, to induce stress, the fish were placed in a net and gently shaken in the water for one minute. Finally, excess mucus was washed off with water, and the fish were immersed in tanks containing dose of 3 × 105 zoospores/L for 8 hours to undergo the bath. [12]. Fungal infected fish were confirmed microscopically.

Measurements of growth parameters

To investigate the result of *M. longifolia* on the growth performance of rainbow trout, biometry was performed measuring fish length and weight were recorded using a digital caliper (± 1 mm) and scale (± 0.01 g). Growth performance indices were calculated as follows:

Weight gain (g) = Final weight (g) - initial weight (g)

Specific growth rate (SGR) = [ln (final weight) - ln (initial weight)/days] × 100

Feed conversion rate (FCR) = Feed intake (g) / Weight gain (g)

Survival rate = [Number of survived fish/initial number of fish] × 100

Haematological and immune factors

At the end of 60 days of feeding the fish with *M. longifolia* extract and 15 days after the S. parasitica challenge, blood samples were collected from the peduncles of 12 fish (4 per replicate) to evaluate blood indices. The fish were fasted for 24 hours prior to sampling. Blood was collected into heparinized tubes (500 U/ml) for hematological analysis [11]. Hematocrit was measured using the microhematocrit method [42] and hemoglobin concentration was determined by the cyanmethemoglobin method [43]. RBC and WBC counts were obtained using a hemocytometer, after dilution with Natt–Herrick solution [42]. Serum was separated by centrifugation from non heparinized samples and stored at −20 °C until analysis.

Lysozyme levels were measured turbidimetrically at 450 nm [44]. ACH50 was determined photometrically at 414 nm [45]. Neutrophil respiratory burst was quantified using the nitroblue tetrazolium (NBT) assay [46]. Total protein and albumin were

measured using commercial kits (Parsa Azmoon) and an Autolyser (bs120, Manda, China), respectively.

Collection of mucus

Skin mucus was collected following Subramanian et al. [16]. After 60 days of feeding and 15 days post challenge, 12 specimens were sampled from each group, anesthetized with triacaine methanesulfonate (MS222) [41] and placed in cold water to remove contaminants. Each fish was placed separately in plastic bags containing 10 mL of 50 mM sodium chloride (NaCl) for 2 minutes. The mucus was collected and stored at −80 °C until analysis [11].

Protein profile

Protein patterns were analyzed using SDS-PAGE technique [47]. Mucus samples were combined with buffer (4% SDS, 5 mM tris hydrochloric acid, 2% mercaptoethanol, 12% glycerol, and 5% bromophenol blue) in a 4:1 ratio, heated at 95 °C for 5 minutes, centrifuged (1000 rpm, 3 minutes) at room temperature, and the filtered supernatant was utilized for electrophoresis. Subsequently, 25 µL of each sample, along with a molecular weight marker, was loaded onto an 18% polyacrylamide gel equipped with a 5% stacking gel. Electrophoresis was run at 120 V until the bromophenol blue indicator migrated past the stacking gel, then continued at 200 V for 7 hours using 5X electrophoresis buffer. To visualize the protein bands, the gels were stained with 5% Coomassie Blue (250 mg) for 5 hours, and destained using Coomassie Blue solution in two steps over 2 hours.

Statistical analysis

Data were analyzed using SPSS statistical software version 23. Normality of the data was assessed with the Kolmogorov-Smirnov test. A one-way ANOVA was used to evaluate treatment effects, followed by Tukey's to compare means. Differences were considered significant at $p < 0.05$.

Authors' Contributions

Mohadeseh Heydari and Farid Firouzbakhsh conceived and planned the experiments. Mohadeseh Heydari carried out the experiments. Mohadeseh Heydari contributed to sample preparation. Farid Firouzbakhsh contributed to the interpretation of the results. Farid Firouzbakhsh took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Abbreviations-Cont'd

ACH: Alternative hemolytic complement activity
MCV: Mean Corpuscular Volume
MCH: Mean Corpuscular Hemoglobin
MCHC: Mean Corpuscular Hemoglobin Concentration

Competing Interests

The authors declare that there is no conflict of interest.

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Isolation Rate and Antimicrobial Profiles of *Salmonella* from Captive Wild Animals at University of Ilorin Zoological Garden, Kwara State, Nigeria

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ABSTRACT

Salmonellosis, a globally distributed zoonotic disease, has poorly understood epidemiology in captive wildlife, particularly in developing countries. This study aimed to determine the isolation rate and antimicrobial susceptibility profiles of Salmonella species from captive wildlife at the University of Ilorin Zoological Garden, Kwara State, Nigeria. A cross-sectional study was conducted using 191 faecal samples collected from different species of captive wild animals. Samples were processed using standard bacteriological procedures and antimicrobial sensitivity testing was conducted using the Kirby-Bauer disk method. Out of the 191 samples analyzed, 19 (10.0 %) were positive for Salmonella. The frequencies of isolation varied among different animal classes, with the avian species showing the highest rate (5.24 %). Differences in isolation rates within and between different animal classes were not statistically significant ($p > 0.05$). The isolates generally showed a low level of antimicrobial resistance except against ampicillin (94.7%) and erythromycin (89.5%). Statistically significant resistance was observed for erythromycin ($p = 0.042$) and tetracycline ($p = 0.035$) among all the isolates. Similarly, resistance to ceftazidime was prominent among the primate species sampled ($p = 0.002$). We identified nine distinct resistance profiles, with 15.8% of resistant isolates exhibiting multidrug-resistant (MDR) phenotypes. Notably, 94.7% of Salmonella isolates demonstrated a multi-antibiotic resistance (MAR) index ≥ 0.2. These findings confirm the presence of Salmonella in captive wildlife at this facility, indicating a potential public health risk. Despite generally low antimicrobial resistance levels, ongoing surveillance is crucial to identify infection sources and prevent environmental contamination by MDR zoonotic pathogens.

Keywords

Captive wildlife, Salmonella, MDR, Zoo, Ilorin, MAR Index

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Abbreviations

MAR: multi-antibiotic resistance
MDR: multidrug-resistance
ONPG: ortho-nitrophenyl galactosidase
CLSI: Clinical and Laboratory Standard Institute

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Introduction

Salmonella is one of the leading causes of foodborne illnesses globally. Although the consumption of contaminated food has been identified as the major transmission pathway, direct or indirect contact with infected wild fauna, whether in captivity or in the natural environment, also plays a significant role in the epidemiology of salmonellosis [1,2]. Wild animals are important reservoirs of zoonotic pathogens, particularly *Salmonella* [1]. *Salmonella* is one of the major zoonotic pathogens transmitted from wildlife to humans due to its ubiquitous nature and remarkable ability to thrive under aFdverse environmental conditions [3,4]. *Salmonella* serovars are present in several species of wild animals in both captive and natural environment. However, epidemiological studies have been limited due to difficult access to the wild population [5]. Many wild animals may harbor *Salmonella* asymptotically, and shed the organism intermittently through faeces, thereby contaminate the environment. The bacterium may persist in harsh environmental conditions until the environment becomes favorable for growth and transmission to new susceptible host [3]. Modern zoological gardens are designed to mimic the natural environment of wild animals and have wide spaces for them to roam freely and display normal behaviours. However, this close simulation increases the likelihood of interaction between animals and visitors. Zoo visitors often approach fences or barriers and may come into contact with animals' faeces, resulting in possible transmission of zoonotic pathogens, including *Salmonella* [3,5]. *Salmonella* can persist in the environment for long period; hence, they can equally be transmitted to zoo visitors via contact with faecal-contaminated surfaces or exhibits [1,6].

Outbreaks of human salmonellosis associated with contact of visitors/zoo workers with wild animals in captivity have been documented in industrialized countries including those in Europe, Canada and Asia [3]. However, due to inadequate or absence of regular surveillance in Africa, there is no comprehensive data on the role of wild animals in the transmission pathway of salmonellosis. Moreover, the emergence of multidrug-resistant (MDR) *Salmonella* serovars from wild animals in captivity including reptiles and wild birds are available [2]. Therefore, the aim of the study is to determine the isolation rate and antimicrobial susceptibility profiles of *Salmonella* species recovered from captive wildlife at the University of Ilorin Zoological Garden, Kwara State, Nigeria.

Result

Rate of Isolation of Salmonella from Captive wildlife in Ilorin, Kwara State

In this study, a total of 191 samples were collected from different species of captive wild animals in the University of Ilorin zoological garden. Out of these, 19 samples (10.0 %) tested presumptive positive for *Salmonella* species. *Salmonella* was isolated from all classes of captive wildlife examined, though at different frequencies. The highest frequency of isolation was recorded among avian species (5.24 %), with geese showing the highest frequency (1.57 %) within this class. However, the differences in isolation rates within and between these groups were not statistically significant ($p > 0.05$). Only 1 (0.52 %) isolate each was obtained from reptiles and rodents. All isolates displayed typical biochemical characteristics consistent with *Salmonella* species: they were all Gram-negative rods, fermented glucose but not lactose, and reduced nitrate to nitrite (Table 1).

Distribution of Resistance phenotypes among Salmonella isolates from Captive Wildlife in Ilorin

The *Salmonella* isolates demonstrated varying frequencies of resistance to the antibiotics tested. The frequencies of resistance to ampicillin and erythromycin were 94.7 % and 89.5 % respectively. A single isolate (5.3 %) displayed resistance to cefotaxime, tetracycline, and neomycin while pan-susceptibility was observed to gentamicin by the isolates. The resistance rates to erythromycin (p= 0.042) and tetracycline (p= 0.035) were statistically significance among all the isolates. Similarly, resistance to ceftazidime was notably higher among isolates obtained from primate species (p = 0.002). Nine different resistance profiles were identified with ampicillin-erythromycin (AMP-E) phenotypes being the most prevalent (47.4 %). Three (5.8 %) isolates displayed multidrug-resistant (MDR) phenotypes, while 18 (94.7 %) isolates exhibited multi-antibiotic resistance indices ≥ 0.2 (fig. 1).

Discussion

The present study documented the occurrence of *Salmonella* among captive wildlife at the University of Ilorin Zoological Garden, highlighting the potential role of these animals as reservoir of *Salmonella*. The isolation rate of 10.0% observed in this study was higher than rates reported in India (3.1 %) [12], Trinidad (7 %) [13], and Tasmania (4.9 %) [14], but slightly lower than the 13.0 % reported among captive reptiles in Croatia [1].

Table 1.
Biochemical characterization of Salmonella isolates from captive wildlife at University of Ilorin Zoological Garden

Sample Source/Test	Ungulates	Carnivores	Avian	Reptiles	Rodents	Primates	Total
Gram reactions	Gram - rods	Gram - rods	Gram - rods	Gram - rods	Gram - rods	Gram - rods	
Urease	-	-	-	-	-	-	
Citrate	+	+	+	+	-	+	
H2S	+	+	+	+	+	+	
Glucose	+	+	+	+	+	+	
Lactose	-	-	-	-	-	-	
Sucrose	-	-	-	-	-	-	
Mannitol	+	+	+	+	+	+	
MR	+	+	+	+	+	+	
VP	-	-	-	-	-	-	
Indole	-	-	-	-	-	-	
ONPG	-	-	-	-	-	-	
Nitrate reduction	+	+	+	+	+	+	
No of positive	2.0	2.0	10.0	1.0	1.0	3.0	19.0
Detection	0.02	0.02	0.1	0.01	0.01	0.03	0.19

+= Positive, -=Negative, H2S= Hydrogen sulfide, VP=Voges Proskauer, MR= Methyl red, ONPG= ortho-nitrophenyl galactosidase

These variations could be attributed to differences in climatic conditions, management practices or sample sizes, as larger sample sizes increase the likelihood of recovering more isolates [15]. Among the different animal classes studied, the isolation rate was highest in captive wild birds, and this finding agrees with the previous studies identifying captive wild birds as major reservoirs of *Salmonella* within wildlife populations [5,11]. A high *Salmonella* rate among captive birds may indicate significant environmental contamination, increasing the risk of transmission to susceptible animals sharing the same environment. This could explain why *Salmonella* was isolated from all animal classes in the zoo. Inter-species transmission might also occur due to the close proximity of enclosures. Furthermore, flies could also serve as mechanical vectors, transmitting *Salmonella* from feacal droppings of infected animals to susceptible hosts via the feeds or water sources. This observation aligns with earlier findings suggesting that *Salmonella* survives longer in flies and beetles than many other zoonotic pathogens [4]. The presence of *Salmonella* in captive wild animals is of major zoonotic importance, as it poses a risk of human salmonellosis through direct contact with infected animals or indirectly via contaminated environment [1,3–5]. Interestingly, the frequency of *Salmonella* among captive reptiles (5.2 %) in this study was lower compared to previous reports, where reptiles are often considered highly susceptible to salmonellosis [5]. Such discrepancies may result from differences in geographic location, season, or sample size [4,5]. Lukac et al. [1] reported no *Salmonella* in captive reptiles in Croatia, suggesting that reptiles shed *Salmonella* intermittently, therefore the isolation rate at different times will vary based on the shedding rate at the period. The presence of *Salmonella* among carnivores (10.5 %) may be linked to their diet, as feeding on raw meat has been identified as a potential source of *Slmonella* [4]. In the current study, the frequency of isolation of *Salmonella* from primates is consistent with the

Class of wildlife	Source	No of isolate	Antibiotics									Patterns (N, %)	MARI
			CTX	CAZ	TE	E	FOX	CIP	N	CRO	AMP		
Equine	Horse	2										AMP-E-FOX (1, 5.3)	0.3
	Mule											AMP-E (3, 15.8)	0.2
Reptiles	Tortoise	1											
Rodents	Crested porcupine	1											
Carnivores	Hyena	2										AMP-E-TE (1, 5.3)	0.3
	Hyena											AMP-E (9, 47.4)	0.2
Avian	Emu	10											
	Geese												
	Geese												
	Geese												
	Pigeon												
	Pigeon												
	Crown dica												
	Peafowl												
	Guinea fowl											AMP-CIP-CRO-E-FOX (1, 5.3)	0.5
	Eagle											AMP-CIP-E-FOX (1, 5.3)	0.4
Primates	Monkey	3										AMP-CAZ-CRO-CTX (1, 5.3)	0.4
	Monkey											AMP-CAZ (1, 5.3)	0.2
	Monkey											CAZ (1, 5.3)	0.1
Total (%)		**19**	**1 (5.3)**	**3 (15.8)**	**1 (5.3)**	**17 (89.5)**	**3 (15.8)**	**2 (10.5)**	**1 (5.3)**	**2 (10.5)**	**18 (94.7)**	**MDR= 3 (15.8 %)**	
***P* value**			**0.34**	***0.002**	**0.11**	***0.042**	**0.067**	**0.85**	**0.34**	**0.80**	***0.035**		

Figure 1.
AMR profiles of Salmonella from captive wildlife in Ilorin. Black = resistance; White = Susceptible; Gray = MDR
CTX: Cefotaxime, CAZ: Ceftazidime, TE: Tetracycline, E: Erythromycin, FOX: Cefoxitin, CIP: Ciprofloxacin, N: Neomycin, CRO: Ceftriaxone, AMP: Ampicillin. * $p < 0.05$.

report of Gopee et al. [12] which reported that while it was rare for free-living wild primates to be infected, infection frequently occurs after they are placed in captivity. All isolates in this study showed distinct biochemical characteristics of *Salmonella* to biochemical reagents, consistent with previous reports [6]. Although biochemical characterizations are not commonly used for routine detection of *Salmonella* in the developed world because of their time consumption and low sensitivity, they are still the common methods available for routine diagnosis in developing countries [6,16].

Overall, the isolates displayed low resistance frequencies to most antimicrobials tested, except ampicillin (97.4 %) and erythromycin (89.5 %). These findings support previous studies reporting high rates of antimicrobial susceptibility among *Salmonella* isolates from wildlife [2,5]. For example, Farias et al. [2] reported that all *Salmonella* isolates from captive and exotic animal species in Ohio, USA, were pan-susceptible to all antimicrobial tested. The high resistance to ampicillin and erythromycin observed in this study could be due to selective pressure because of over-reliance on these antimicrobials in veterinary and animal production in the study area [14]. Although most isolates in the current study exhibited low resistance rates to antimicrobials, higher proportion of resistant isolates showed multidrug-resistant (MDR) phenotypes. This could be due to selective pressure on the antimicrobials in the environment [14,17]. In addition, a high proportion of the isolates have multiple antimicrobial resistance index (MARI) greater than 0.2, indicating that probably most of the isolates originated from high-risk sources and environments where overuse and abuse of antibiotics are common [10].

In conclusion, this study indicates that captive wildlife at the University of Ilorin zoological garden harbor *Salmonella* species at a prevalence rate of 10.0 %, with avian species showing the highest frequency of isolation. The isolates show low antimicrobial resistance levels, though some showed MDR phenotypes. These findings underscore the importance of continuous surveillance for foodborne pathogens among captive wild fauna to prevent cross species and zoonotic transmission.

Materials and Methods

Ethical Consideration

The study was approved by the Faculty of Veterinary Medicine, University of Ilorin the Ethical Review Committee with code UREC/FVM/15/32TA002.

Study area

The study was conducted at the University of Ilorin Zoological Garden located in Ilorin, the capital city of Kwara State, Nigeria. The zoo was originally established as a biological garden on the University's mini-campus in 1975 and was upgraded to a zoological garden in 1985 to support the teaching and research needs of the Department of Biological Sciences. The zoo is located near the main gate of the University, approximately between Lat. 80° 17" N & Long. 40° 82" [7].

Sample Collection

Fecal samples, were collected from overnight droppings of captive wild animals. For each animal, a sterile swab was inserted into the center of the fecal mass within the pen of each animal, as directed by the zoo attendants who helped restrain the animal when necessary. One sample was collected from each animal, on three separate visits, resulting in a total of 191 samples collected from ungulates (n= 25, 13.1 %), carnivores (n=31, 16.2 %), Avian (n= 83, 69.7 %), reptiles (n= 25, 13.1 %), rodents (n= 6, 3.1 %), and primates (n= 21, 11.0 %) (Table 2). All samples were transported to the Veterinary Microbiology Laboratory, University of Ilorin, within one hour of collection under a cold chain. Sample processing was initiated within 24 hours of their collection.

Sample Processing

The sample in swab stick was inoculated into 10 ml of peptone water (Oxoid, Hampshire, UK) and incubated at 35 ± 2°C for 22 ± 2 hours for pre-enrichment. Subsequently, enrichment was performed in Selenite-F broth (Oxoid, Hampshire, UK), prepared according to the manufacturer's instructions, by adding 1 mL of pre-enriched culture to 9 mL of the enrichment broth (ratio of 1:9) and then the mixture was incubated at 35 ± 2°C for 20 ± 2 hours [8]. After enrichment, samples were then selectively plated onto xylose lysine deoxycholate (XLD) agar (Oxoid, Hampshire, UK) and incubated at 35 ± 2°C for 20 ± 2 hours. Discrete pink colonies with black centers, suggestive of *Salmonella*, were sub-cultured on blood agar (Oxoid Ltd, Hampshire, UK) plates for purification and incubated at 35 ± 2°C for 20 ± 2 hours. The isolates were subjected to biochemical tests including IMVC tests (indole, methyl red, Voges Proskauer and citrate tests), urease test, triple sugar iron test and motility test. Presumptive *Salmonella* isolates were stored in Mueller Hinton broth containing 20 % glycerol at -20°C for further analysis.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of the presumptive *Salmonella* isolates was performed using the Kirby-Bauer agar diffusion method as previously described [14]. The panel of antibiotics discs (Oxoid, Hampshire, UK) used in the assay contained following antibiotics and concentration: ceftriaxone (30 µg), erythromycin (10 µg), cefotaxime (30 µg), gentamicin (10 µg), neomycin (30 µg), ceftazidime (30 µg), tetracycline (30 µg), ampicillin (10 µg), cefoxitin (30 µg), and ciprofloxacin (5 µg).

Briefly; the presumptive isolates from stock were cultured on freshly prepared nutrient agar (Oxoid, Hampshire, UK) and incubated overnight at 35 ± 2 °C. Discrete colonies from the nutrient agar were inoculated into 10 ml of sterile normal saline in test

Table 2.
The rate of Isolation of *Salmonella* from captive wildlife at University of Ilorin Zoological Garden

Class of animal	Species of animal	No of sample (%)	Rate of isolation (%)
Ungulates	Horse	6 (3.1)	1 (0.52)
	Mule	2 (1.0)	1 (0.52)
	Donkey	10 (5.2)	0 (0.0)
	Camel	7 (3.7)	0 (0.0)
	Subtotal	25 (13.1)	2 (1.05)
Carnivores	Warthog	4 (2.1)	0 (0.0)
	Lion	4 (2.1)	0 (0.0)
	Hyena	10 (5.2)	2 (1.05)
	Leopard	5 (2.6)	0 (0.0)
	African civet cat	8 (4.2)	0 (0.0)
	Subtotal	31 (16.2)	2 (1.05)
Avian	Emu	4 (2.1)	1 (0.52)
	Geese	13 (6.8)	3 (1.57)
	Pigeon	11 (5.8)	2 (1.05)
	Crown dica	2 (1.0)	1 (0.52)
	Peafowl	7 (3.7)	1 (0.52)
	Guinea fowl	5 (2.6)	1 (0.52)
	Eagle	8 (4.2)	1 (0.52)
	Marabou stork	6 (3.1)	0 (0.0)
	Ostrich	6 (3.1)	0 (0.0)
	Duck	7 (3.7)	0 (0.0)
	Vulture	6 (3.1)	0 (0.0)
	White Indian fowl	4 (3.4)	0 (0.0)
	Black-crowned crane	4 (3.4)	0 (0.0)
	Subtotal	83 (69.7)	10 (5.24)
Reptiles	Tortoise	6 (3.1)	1 (0.52)
	Crocodile	9 (4.7)	0 (0.0)
	Puff adder	4 (3.4)	0 (0.0)
	Royal python	6 (3.1)	0 (0.0)
	Subtotal	25 (13.1)	1 (0.52)
Rodents	Crested porcupine	6 (3.1)	1 (0.52)
	Subtotal	6 (3.1)	1 (0.52)
Primates	Monkey	16 (8.4)	3 (1.57)
	Baboon	5 (2.6)	0 (0.0)
	Subtotal	21 (11.0)	3 (1.57)
	Total	**191 (100.0)**	**19 (10.0)**

tubes using a sterile wire loop. The turbidity of the inoculum was adjusted to 0.5 McFarland standards using a Nephelometer (Oxoid, Hampshire, UK). The inoculum was poured on the Mueller Hinton agar plate (Oxoid, Hampshire, UK) and it was uniformly spread until the surface of the agar plate was covered by the inoculum. Excess inoculum was discarded after 30 seconds. Plates were partly left open for 3-5 minutes on the sterilised working bench until they dried. Antibiotic sensitivity discs were dispensed on each plate using a disc dispenser (Oxoid, Hampshire, UK). Plates were then incubated at 35 ± 2°C for 18 ± 2 hours. The inhibition zones of each antimicrobial were measured using a vernier calliper (Hi-Media, Mumbai, India) and recorded according to CLSI standards [9,10]. *E. coli* ATCC 25922 was used as a control strain. The multiple antimicrobial resistance index (MARI) was determined according to standard methods as previously described [11].

Statistical Analysis

The data were computed in a Microsoft Excel 2019 database. The overall isolation rate and the frequency of *Salmonella* from each wild animal sampled was determined. Statistical estimates were made using Graphpad Prism statistical package, San Diego, Califonia, U.S.A (www.Graphpad.Com) at confidence interval of 95 %. Probability values less than 0.05 ($p < 0.05$) were considered significant. Chi-square was used to determine the level of significance in the rates of isolation between and within different classes of wild animals under the study.

Authors' Contributions

Conceptualization and Study design: AOA and RAI. Material preparation, data collection, and analysis: KAA, SAA, ALF, SBA AA, and AMI. Manuscript draft, editing, and reviewing: AOA, RAI, KAA, SAA, ALF, SBA, AA, and AMI. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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The effect of Vitamin D Supplementation on its serum level and energy metabolic profile in Grey Shirazi ewes

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ABSTRACT

The present study aimed to evaluate the effects of vitamin D supplementation on serum vitamin D levels and the metabolic profile of Grey Shirazi ewes, during pregnancy. A total of sixty healthy Grey Shirazi ewes were divided into three groups: Group 1 (control); Group 2, which received a single intramuscular dose of 10,000 IU vitamin D at the time of insemination; and Group 3, which received a single dose of 10,000 IU vitamin D at mid-pregnancy. Blood samples were collected at four stages: insemination, mid-pregnancy, late pregnancy, and post-lambing. Vitamin D levels in the control group decreased significantly during pregnancy. In contrast, no such decrease occurred in the two supplement groups, so that the highest vitamin D concentrations were observed in late pregnancy and postpartum in group 3. In terms of energy balance, serum β-hydroxybutyrate (BHBA) and non-esterified fatty acid (NEFA) levels were lowest in the mid-pregnancy supplemented group. Additionally, in group 3, insulin levels increased during the final stages of pregnancy, while serum calcium and phosphorus concentrations were higher in this group compared to the other groups, reaching their maximum levels after delivery. All groups increased triglycerides and cholesterol, but Group 3 had the highest triglycerides postpartum. Additionally, serum protein and albumin levels were significantly higher in Group 3 during the postpartum period, reflecting improved nutritional status and protein synthesis. The results of this study suggest that vitamin D supplementation during mid-pregnancy has the potential to improve metabolic health in Grey Shirazi ewes, having significant implications for both maternal and neonatal health outcomes.

Keywords

Vitamin D, Energy balance, metabolic biomarkers, Pregnancy, Grey Shirazi sheep

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Abbreviations

BHBA: β-hydroxybutyrate
NEFA: non-esterified fatty acid
25(OH)D: 25-hydroxyvitamin D
1,25(OH)2D: 1,25-dihydroxyvitamin D
VDR: vitamin D receptor
NEB: negative energy balance

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Introduction

Vitamin D is an essential nutrient for regulating calcium levels and maintaining skeletal health in ruminants [1]. However, there is increasing evidence that vitamin D plays additional roles, including effects on metabolism and overall health [2]. The body obtains the vitamin D it needs through oral intake of cholecalciferol or through its production in the skin through exposure to ultraviolet radiation. After production or consumption, vitamin D is hydroxylated in the liver to 25-hydroxycholecalciferol (calcidiol), and then converted in the kidneys to the active form 1,25-dihydroxycholecalciferol (calcitriol). The active form functions as a hormone, binding to the vitamin D receptor (VDR) to control calcium and phosphorus metabolism, which is necessary for bone formation and maintenance. The primary functions of vitamin D and its effects on calcium homeostasis are achieved by increasing calcium absorption from the intestines, regulating calcium release from bones by acting on osteoblasts and osteoclasts, and promoting calcium reabsorption from the kidneys [3].

Vitamin D deficiency has been reported in sheep. It can occur due to inadequate sunlight exposure, ineffective shearing, or insufficient vitamin intake, which can have significant implications for calcium absorption and balance, as well as overall health [4]. Published studies show that cholecalciferol supplementation in sheep can effectively increase serum vitamin D levels and is therefore effective in preventing or treating vitamin D deficiency and its subsequent complications, including calcium balance disorders, and is also associated with positive effects on the metabolic profile of these animals [5, 6]. Other evidence that can be considered for the more widespread effects of vitamin D is the presence of receptors for this vitamin in numerous tissues, such as the endocrine pancreas, liver, muscle, and adipose tissue, indicating its possible role in regulating energy metabolism [7]. Supplementation of vitamin D can result in an improvement in insulin sensitivity and glucose tolerance in ruminants, highlighting its potential role in energy metabolism [6].

The timing of vitamin D supplementation, whether it occurs during mating or throughout the gestation phase, makes a great difference in its effects [8]. Cholecalciferol supplementation should be considered during two critical stages of sheep reproduction. Administration of this vitamin at the time of mating can be effective in improving ovulation and increasing reproductive efficiency [9, 10]. In the later stages of pregnancy, due to fetal growth, multiple births, and preparation for lactation, as well as the overall increase in metabolic needs of the ewe, the use of this supplement is justified. Supplementation during this period can improve serum vitamin D concentrations, support optimal energy metabolism, reduce the likelihood of metabolic disorders in pregnant ewes, and aid fetal development [11].

Pregnancy toxemia is a common metabolic disease in ewes, which occurs especially in high-producing animals and ewes with multiple births in late pregnancy and coincides with the rapid fetal growth phase due to a mismatch between energy intake and expenditure, negative energy balance (NEB). This condition, which is associated with multiple metabolic disorders, including increased fatty acid levels and insulin resistance, can lead to significant economic losses in the sheep husbandry. Therefore, finding effective strategies that promote energy balance and metabolic health of ewes seems essential to reduce these risks [12].

Gray Shirazi breed is a native Iranian sheep breed originating from the Shiraz region of Fars Province in southern Iran. The breed is known for its high quality wool and adaptability to the arid and semi arid environments of the region, displaying strong resilience to harsh weather conditions and low quality forage. Grey Shirazi sheep are medium sized animals with a typical bluish-gray coat, hence the name. In addition to their contributions to wool production, Grey Shirazi sheep are also raised for their meat, thereby supporting the livelihoods of local farmers. Their resilient characteristics, coupled with moderate reproductive rates, position them as a vital genetic resource for promoting sustainable farming practices in the region. Even though the importance of this breed cannot be underestimated, there is a decline in their population, raising concerns over the conservation of this unique breed [13].

Regardless of known metabolic benefits, the effect of vitamin D supplementation on pregnancy-induced metabolic changes in ewes has not been adequately demonstrated. Therefore, this study was conducted to evaluate the effects of vitamin D supplementation on serum concentrations of 25-hydroxyvitamin D and energy-related metabolic biomarkers in pregnant Grey Shirazi ewes.

Result

Vitamin D

Table 1 presents changes serum vitamin D concentrations as determined within groups across time

Abbreviations-Cont'd

FGF-23: fibroblast growth factor-23
HMGCR: hydroxymethylglutaryl-CoA reductase

Table 1.
Mean ± SE of serum Vitamin D (ng/ml) in various groups at different sampling times.

	mating	mid-pregnancy	Late pregnancy	Post-partu-rition
Group 1	53.16 ± 14.07	30.36 ± 13.69	39.71 ± 10.18	34.31 ± 13.12
	a	b	b	b
		A	A	A
Group 2	54.58 ± 10.27	73.24±14.52	53.25±9.31	44.09 ± 14.29
	a	b	a	a
		B	B	A
Group 3	47.13 ± 12.01	52.20 ± 13.39	67.14±13.99	50.81 ± 16.98
	a	a	b	a
		C	C	B

*Group 1: control, Group 2: vit D supplementation at insemination, Group 3: vit D supplementation at mid-pregnancy.
*Distinct capital letters within each column indicate a statistically significant difference between the groups ($p < 0.05$).
* Distinct lowercase letters within each row indicate a statistically significant difference between the sampling times ($p < 0.05$).

points. In Group 1 (control), a significant reduction in serum vitamin D levels was observed throughout pregnancy and postpartum compared with pre-pregnancy period (e.g., mating: 53.16 ± 14.07 ng/mL vs. mid-pregnancy: 30.36 ± 13.69 ng/mL; $p < 0.05$). In Group 2 (supplementation at insemination), a significant increase was detected at mid-pregnancy (73.24 ± 14.52 ng/mL) compared to mating (54.58 ± 10.27 ng/mL; $p < 0.05$), with levels remaining stable thereafter. In Group 3 (supplementation at mid-pregnancy), a significant rise in vitamin D was observed from mid-pregnancy (52.20 ± 13.39 ng/mL) to late pregnancy (67.14 ± 13.99 ng/mL; $p < 0.05$).

Between-group comparisons at each sampling point showed that Groups 2 and 3 had significantly higher serum vitamin D levels than Group 1 during the middle of pregnancy (Group 2: 73.24 ± 14.52 ng/mL; Group 3: 52.20 ± 13.39 ng/mL; Group 1: 30.36 ± 13.69 ng/mL; $p < 0.05$) and during late pregnancy (Group 3: 67.14 ± 13.99 ng/mL; Group 2: 53.25 ± 9.31 ng/mL; Group 1: 39.71 ± 10.18 ng/mL; $p < 0.05$). The highest postpartum level of serum vitamin D was recorded in Group 3 (50.81 ± 16.98 ng/mL), which was significantly higher than both Group 2 (44.09 ± 14.29 ng/mL; $p < 0.05$) and Group 1 (34.31 ± 13.12 ng/mL; $p < 0.05$).

BHBA and NEFA

Within-group comparisons of serum BHBA levels over time are presented in Table 2. In all three

Table 2.
Mean ± SE of serum Vitamin D (ng/ml) in various groups at different sampling times.

			mid-pregnancy	Late pregnancy	Post-parturition
BHBA (mmol/l)	Group 1	0.21 ± 0.02	0.45 ± 0.06	0.79 ± 0.29	0.44 ± 0.18
		a	b	c	b
	Group 2	0.18 ± 0.02	0.40 ± 0.03	0.91 ± 0.11	0.38 ± 0.05
		a	b	c	b
	Group 3	0.20 ± 0.02	0.38 ± 0.02	0.63 ± 0.12	0.42 ± 0.08
		a	b	b	b
NEFA (mmol/l)	Group 1	1.18 ± 0.49	0.85 ± 0.16	1.78 ± 0.09	2.22 ± 0.62
			A	AB	A
	Group 2	1.44 ± 0.17	1.61± 0.07	2.41 ± 0.30	1.73 ± 0.22
			B	A	B
	Group 3	1.31 ± 0.28	0.77 ± 0.06	1.09 ± 0.18	1.15 ± 0.29
			A	B	C

*Group 1: control, Group 2: vit D supplementation at insemination, Group 3: vit D supplementation at mid-pregnancy.
* Distinct capital letters within each column indicate a statistically significant difference between the groups ($p < 0.05$).
* Distinct lowercase letters within each row indicate a statistically significant difference between the sampling times ($p<0.05$).
* Distinct lowercase letters within each row indicate a statistically significant difference between the sampling times ($p < 0.05$).

groups, BHBA concentrations increased significantly as pregnancy progressed. In Group 1, BHBA levels rose from 0.21 ± 0.02 mmol/L at the time of mating to 0.79 ± 0.29 mmol/L at late pregnancy ($p < 0.05$). Similarly, Group 2 exhibited a significant increase from 0.18 ± 0.02 mmol/L at the time of mating to 0.91 ± 0.11 mmol/L at late pregnancy ($p < 0.05$). In Group 3, the increase was more moderate, with levels rising from 0.20 ± 0.02 mmol/L to 0.63 ± 0.12 mmol/L by late pregnancy ($p < 0.05$).

Between-group comparisons across each time point (Table 2) revealed no significant differences in BHBA levels ($p > 0.05$).

Table 2 shows that within-group comparisons of NEFA levels did not present any significant differences ($p > 0.05$).

Between-group comparisons exhibited significant differences in NEFA levels at several time points. During mid-pregnancy, Group 2 (1.61 ± 0.07 mmol/L) had significantly higher NEFA concentrations than Groups 1 (0.85 ± 0.16 mmol/L) and 3 (0.77 ± 0.06 mmol/L; $p < 0.05$). At late pregnancy, Group 3 had significantly lower NEFA levels (1.09 ± 0.18 mmol/L) than Group 2 (2.41 ± 0.30 mmol/L; $p < 0.05$). In the postpartum period, NEFA was also lowest in Group 3 (1.15 ± 0.29 mmol/L), significantly differing from Group 1 (2.22 ± 0.62 mmol/L; $p < 0.05$). Insulin and glucose

Table 3 presents the within-group changes of serum insulin levels throughout the experiment. In the control group (Group 1), insulin levels increased significantly with the progression of pregnancy and postpartum, rising from 1.39 ± 0.14 ng/mL at the time of mating to 3.36 ± 1.33 ng/mL postpartum ($p < 0.05$). A similar increase was observed in the supplemented groups, with Group 2 from 1.44 ± 0.64 ng/mL at the time of mating to 1.99 ± 1.09 ng/mL postpartum, and Group 3 from 1.34 ± 0.16 ng/mL at the time of mating to 6.06 ± 1.06 ng/mL postpartum ($p < 0.05$ for Group 3).

Between group comparisons at each time point showed significant differences in insulin levels during late pregnancy and postpartum. At late pregnancy, insulin levels in Group 3 (4.00 ± 1.82 ng/mL) were significantly higher than in Group 1 (2.39 ± 1.44 ng/mL) and Group 2 (2.96 ± 1.42 ng/mL; $p < 0.05$). This difference became more pronounced postpartum, with Group 3 reaching the highest insulin level (6.06 ± 1.06

Table 3.
Mean ± SE of serum insulin and glucose in various groups at different sampling times.

		mating	mid-pregnancy	Late pregnancy	Post-parturition
Insulin (ng/ml)	Group 1	1.39 ± 0.14	1.86 ± 0.82	2.39 ± 1.44	3.36 ± 1.33
		a	a	a	b
	Group 2	1.44 ± 0.64	1.64 ± 0.51	A	A
				2.96 ± 1.42	1.99 ± 1.09
	Group 3	1.34 ± 0.16	1.08 ± 0.79	A	A
		a	a	4.00±1.82	6.06±1.06
Glucose (mg/dl)	Group 1	111.90 ± 7.65	82.71 ± 5.42	b	b
		a	ab	B	B
	Group 2	99.14 ± 5.97	85.00 ± 7.89	71.42 ± 4.40	47.00 ± 6.96
		a	ab	b	c
	Group 3	109.00 ± 6.58	97.61 ± 5.14	75.85 ± 4.56	43.53 ± 2.95
		a	ab	b	c

*Group 1: control, Group 2: vit D supplementation at insemination, Group 3: vit D supplementation at mid-pregnancy.
*Distinct capital letters within each column indicate a statistically significant difference between the groups ($p < 0.05$).
* Distinct lowercase letters within each row indicate a statistically significant difference between the sampling times ($p < 0.05$).
* Distinct capital letters within each column indicate a statistically significant difference between the groups ($p < 0.05$).
* Distinct lowercase letters within each row indicate a statistically significant difference between the sampling times ($p < 0.05$).
* Distinct lowercase letters within each row indicate a statistically significant difference between the sampling times ($p < 0.05$).

ng/mL), significantly exceeding both Group 1 (3.36 ± 1.33 ng/mL) and Group 2 (1.99 ± 1.09 ng/mL; $p < 0.05$).

Within-group comparisons of serum glucose levels over time showed a significant downward trend during pregnancy and postpartum (Table 3). In all groups, glucose concentrations decreased significantly from mating to postpartum. For example, in Group 1, glucose declined from 111.90 ± 7.65 mg/dL at mating to 47.00 ± 6.96 mg/dL postpartum ($p < 0.05$). Similar reductions were observed in Group 2 (99.14 ± 5.97 mg/dL to 43.53 ± 2.95 mg/dL) and Group 3 (109.00 ± 6.58 mg/dL to 45.71 ± 4.50 mg/dL; $p < 0.05$ in both).

Between-group comparisons did not show significant differences in glucose levels at any stage of sampling ($p > 0.05$).

Calcium and Phosphorous

Within-group comparisons over time showed stage-specific elevations in calcium levels (Table 4). In Group 2, the highest calcium concentration was recorded during mid-pregnancy showing a value of 10.41 ± 0.09 mg/dL, which was significantly higher than values at other stages of this group ($p < 0.05$). In Group 3, the maximum level of calcium was observed in the postpartum stage (10.55 ± 0.09 mg/dL), which was also significantly higher than the previous stages in this group ($p < 0.05$). In contrast, calcium levels in Group 1 showed no significant variations throughout the sampling period, and remained relatively stable ($p > 0.05$).

Between-group comparisons of serum calcium levels at each time point are presented in Table 4. At the postpartum stage, Group 3 (vitamin D supplementation at mid-pregnancy) showed a significantly higher calcium concentration (10.55 ± 0.09 mg/dL) compared to Group 1 (10.07 ± 0.12 mg/dL) and Group 2 (9.86 ± 0.10 mg/dL; $p < 0.05$). No significant intergroup differences were observed during the earlier stages of sampling (mating, mid-pregnancy, or late pregnancy; $p > 0.05$).

Table 4 presents between-group comparisons of serum phosphorus levels over time revealed a significant decline during late pregnancy and postpartum in all groups. For example, in Group 1, phosphorus decreased from 6.04 ± 0.20 mg/dL at mating to 5.03 ± 0.38 mg/dL postpartum ($p < 0.05$). Similar trends were observed in Group 2 (6.68 ± 0.28 mg/dL to 4.60 ± 0.26 mg/dL) and Group 3 (6.12 ± 0.16 mg/dL to 4.81 ± 0.33 mg/dL), with statistically significant reductions in each case ($p < 0.05$).

Between-group comparisons of phosphorus concentrations at each stage revealed no statistically significant differences among the three groups ($p > 0.05$).

Lipids and proteins

Table 5 shows the comparisons of serum triglyceride concentrations among study periods within each group. In all groups, triglyceride levels increased significantly during pregnancy. For instance, Group 1

Table 4.
Mean ± SE of serum Calcium and Phosphorous in various groups at different sampling times.

		mating	mid-pregnancy	Late pregnancy	Post-parturition
Calcium (mg/dl)	Group 1	10.13 ± 0.06	10.28 ± 0.25	9.82 ± 0.19	10.07 ± 0.12
					A
	Group 2	9.90 ± 0.13	10.41 ± 0.09	10.08 ± 0.12	9.86 ± 0.10
		a	b	a	A
					a
	Group 3	10.11 ± 0.09	10.13 ± 0.12	10.09 ± 0.13	10.55 ± 0.09
		a	a	a	B
					b
Phosphorous (mg/dl)	Group 1	6.04 ± 0.20	6.48 ± 0.57	5.32 ± 0.80	5.03 ± 0.38
		a	a	b	b
	Group 2	6.68 ± 0.28	5.97 ± 0.35	5.54 ± 0.43	4.60 ± 0.26
		a	a	b	b
	Group 3	6.12 ± 0.16	6.67 ± 0.31	5.29 ± 0.26	4.81 ± 0.33
		a	a	ab	b

*Group 1: control, Group 2: vit D supplementation at insemination, Group 3: vit D supplementation at mid-pregnancy.
*Distinct capital letters within each column indicate a statistically significant difference between the groups ($p < 0.05$).
* Distinct lowercase letters within each row indicate a statistically significant difference between the sampling times ($p < 0.05$).

Table 5.
Mean ± SE of serum triglycerides (mg/dl) in various groups at different sampling times.

		mating	mid-pregnancy	Late pregnancy	Post-partu-rition
Tri-glycerides (mg/dl)	Group 1	36.45 ± 4.00	41.85 ± 2.35	58.42 ± 7.39	46.57 ± 2.56
		a	ab	b	AB
					ab
	Group 2	28.21 ± 3.80	48.00 ± 2.67	61.28 ± 9.70	40.60 ± 2.58
		a	b	b	A
					b
	Group 3	30.75 ± 4.73	38.46 ± 2.65	55.70 ± 5.24	55.71 ± 2.62
		a	a	b	B
					b
Cholesterol (mg/dl)	Group 1	72.18 ± 2.76	64.00 ± 1.78	61.57 ± 4.39	64.42 ± 5.42
		a	b	A	b
			70.85 ± 2.35	b	
	Group 2	69.14 ± 3.29	70.85 ± 2.35	76.85 ± 3.43	70.46 ± 3.20
		a	a	B	a
				b	
	Group 3	65.62 ± 3.45	65.00 ± 2.36	74.30 ± 4.05	69.28 ± 3.23
				B	
Protein (g/dl)	Group 1	7.52 ± 0.14	6.88 ± 0.20	6.15 ± 0.42	6.98 ± 0.29
		a	ab	b	ab
	Group 2	7.22 ± 0.15	6.81 ± 0.17	6.21 ± 0.18	6.69 ± 0.12
		a	ab	b	b
	Group 3	7.55 ± 0.18	6.80 ± 0.20	6.40 ± 0.24	6.85 ± 0.22
		a	b	b	ab
Albumin (g/dl)	Group 1	3.50 ± 0.05	3.18 ± 0.06	2.71 ± 0.14	3.31 ± 0.10
		a	b	A	a
				b	
	Group 2	3.41 ± 0.06	3.15 ± 0.07	3.04 ± 0.09	3.41 ± 0.03
		a	ab	AB	a
				ab	
	Group 3	3.53 ± 0.07	3.30 ± 0.08	3.18 ± 0.05	3.42 ± 0.13
		a	b	B	ab
				b	

*Group 1: control, Group 2: vit D supplementation at insemination, Group 3: vit D supplementation at mid-pregnancy.
*Distinct capital letters within each column indicate a statistically significant difference between the groups ($p < 0.05$).
* Distinct lowercase letters within each row indicate a statistically significant difference between the sampling times ($p < 0.05$).

increased from 36.45 ± 4.00 mg/dL at the time of mating to 58.42 ± 7.39 mg/dL at late pregnancy ($p < 0.05$), Group 2 from 28.21 ± 3.80 mg/dL to 61.28 ± 9.70 mg/dL ($p < 0.05$), and Group 3 from 30.75 ± 4.73 mg/dL to 55.70 ± 5.24 mg/dL ($p < 0.05$). Postpartum, triglyceride levels declined in Groups 1 and 2 but remained elevated in Group 3.

Between-group comparisons revealed a significant difference only at the postpartum stage. At this time, Group 3 exhibited a significantly higher triglyceride concentration (55.71 ± 2.62 mg/dL) compared to Group 1 (46.57 ± 2.56 mg/dL) and Group 2

(40.60 ± 2.58 mg/dL; $p < 0.05$).

Within-group comparisons over time showed different patterns in cholesterol concentrations across groups. In Group 1 (control), cholesterol levels declined significantly from mating (72.18 ± 2.76 mg/dL) to mid-pregnancy (64.00 ± 1.78 mg/dL) and remained low through late pregnancy (61.57 ± 4.39 mg/dL; $p < 0.05$). In contrast, Group 2 showed a significant increase in cholesterol concentrations from mating (69.14 ± 3.29 mg/dL) to late pregnancy (76.85 ± 3.43 mg/dL; $p < 0.05$), followed by a slight decrease postpartum.

Between-group comparisons of serum cholesterol concentrations at late pregnancy are also shown in Table 5. At this stage, both Group 2 (76.85 ± 3.43 mg/dL) and Group 3 (74.30 ± 4.05 mg/dL) had significantly higher cholesterol levels compared to the control Group 1 (61.57 ± 4.39 mg/dL; $p < 0.05$). No significant differences were observed between groups at other sampling points ($p > 0.05$).

Comparisons of serum total protein concentrations over time resulted a significant decrease in all groups during pregnancy. For example, in Group 1, protein levels decreased from 7.52 ± 0.14 g/dL at the time of mating to 6.15 ± 0.42 g/dL at late pregnancy ($p < 0.05$). Similar trends were observed in Group 2 (7.22 ± 0.15 to 6.21 ± 0.18 g/dL) and Group 3 (7.55 ± 0.18 to 6.40 ± 0.24 g/dL).

Between-group comparisons at each time point did not show significant differences in serum protein concentrations ($p > 0.05$).

Serum albumin concentrations followed a consistent within-group trend across all groups. A significant reduction in albumin was observed during pregnancy in all groups. For instance, in Group 1, albumin declined from 3.50 ± 0.05 g/dL at mating to 2.71 ± 0.14 g/dL at late pregnancy ($p < 0.05$). This pattern was also noted in Group 2 (3.41 ± 0.06 to 3.04 ± 0.09 g/dL) and Group 3 (3.53 ± 0.07 to 3.18 ± 0.05 g/dL). Postpartum, albumin levels increased in all groups, returning to near-baseline levels ($p < 0.05$).

Between-group comparisons during late pregnancy indicated significantly higher serum albumin levels in Group 3 (3.18 ± 0.05 g/dL) compared to Group 1 (2.71 ± 0.14 g/dL; $p < 0.05$).

Discussion

This study investigated the effect of vitamin D supplementation at the insemination stage compared to mid-gestation on metabolic indices of pregnant Grey Shirazi ewes. Given the extensive physiological roles of vitamin D, especially in regulating metabolism, this study investigated the effect of supplementation on serum levels of this vitamin and metabolic changes associated with pregnancy and postpartum.

According to the results, in the group that did not receive vitamin D, serum levels of this vitamin decreased significantly during pregnancy and postpartum, such that in the control group it decreased from 53.16 ± 14.07 ng/ml at the time of mating to 30.36 ± 13.69 ng/ml at mid-pregnancy and 34.31 ± 13.12 ng/ml postpartum. In contrast, in the groups receiving supplementation, vitamin D levels remained relatively stable. Ewes supplemented at insemination (Group 2) showed a peak of 73.24 ± 14.52 ng/mL at mid-pregnancy ($p < 0.05$ vs. control), while those supplemented at mid-pregnancy (Group 3) reached 67.14 ± 13.99 ng/mL at late pregnancy and maintained the highest postpartum levels (50.81 ± 16.98 ng/mL, $p < 0.05$ vs. control).

Group 3 showed higher vitamin D levels than group 2 in late pregnancy and postpartum, however the difference between the two groups was not significant ($p > 0.05$). Therefore, although supplementation in mid-pregnancy was associated with more pronounced effects, this effect should be interpreted cautiously as a clear benefit.

The decline in serum vitamin D of the control group ewes is in agreement with previous studies, demonstrating that pregnancy decreases circulating 25-hydroxyvitamin D due to increased utilization by the mother and fetus [4,14]. Comparable patterns have been reported in both humans and livestock, where maternal vitamin D status decreases as gestation progresses, particularly in the absence of supplementation [15,16].

The rise in vitamin D concentration following supplementation in mid-pregnancy may reflect increased metabolic demands of pregnancy, particularly for regulating calcium and phosphorus levels through improved intestinal absorption and renal reabsorption, which are essential for fetal skeletal formation [17]. Due to the increased maternal calcium requirement during pregnancy, upregulation of vitamin D processing occurs during this period [18]. Therefore, the possible reason why supplementation in mid-pregnancy is more effective in maintaining serum vitamin D

levels is the increased physiological requirement for this nutrient during this period of fetal development.

The observed difference in response observed between the timing of supplementation (mid-pregnancy versus insemination) may also be related to the varying expression of vitamin D receptors (VDR) and the activity of enzymes responsible for vitamin D metabolism in maternal tissues and the placenta as pregnancy advances. Studies in humans and other animals have shown that placental expression of VDR and 1α-hydroxylase, in charge of converting 25-hydroxyvitamin D to its active form, calcitriol, increases during pregnancy [19]. This may help explain why vitamin D supplementation in mid-pregnancy had a more pronounced effect, as the physiological machinery needed to convert and utilize vitamin D is more active at this stage.

Comparing these results with other studies on livestock, the impact of vitamin D supplementation on serum concentrations and metabolic indices has been well-documented in various species. According to the results of a study conducted by Rodney et al. (2018), vitamin D supplementation to pregnant dairy cows increases serum levels of this vitamin and improves calcium metabolism, which in turn can prevent many important postpartum complications such as hypocalcemia [20]. It has been shown that providing of vitamin D to sheep improves the balance of calcium and phosphorus, thereby supporting better fetal bone development and maternal health [21].

Furthermore, the timing of vitamin D supplementation appears to play a significant role in maximizing its advantages. Poindexter et al. (2023) showed that administering vitamin D supplementation in late pregnancy in goats is much more effective in increasing maternal serum levels of this vitamin than when administered at earlier stages of pregnancy, which is consistent with the results of the present study [22].

In this study, the rise in serum β-hydroxybutyrate (BHBA) and non-esterified fatty acids (NEFA) noted in the control group ewes suggests a negative energy balance (NEB), which is frequently seen during late pregnancy due to increased energy requirements and limited nutrient consumption. During negative energy balance, due to reduced energy availability, increased fat catabolism occurs, and the rise in BHBA and NEFA is a marker of this condition [23]. Previous studies have also reported a similar trend in late pregnancy, indicating that the energy requirements for fetal growth are higher than the maternal dietary intake [24].

Vitamin D supplementation had a significant influence on these metabolic parameters, with BHBA and NEFA levels being lower in ewes that received vitamin D in comparison to the control group. Ewes supplemented with vitamin D during mid-pregnancy exhibited the lowest BHBA levels, although the differences among groups were not significant. On the contrary, changes in NEFA levels were more noticeable, with the mid-pregnancy supplementation group displaying significantly reduced NEFA levels both in late pregnancy and after delivery. These results indicate that vitamin D supplementation, especially when given during mid-pregnancy, may help alleviate NEB and decrease fat mobilization during late gestation.

The mechanisms underlying this effect may be associated with the regulatory role of vitamin D in energy metabolism. Vitamin D has been shown to enhance insulin sensitivity and glucose utilization, potentially leading to better energy partitioning and reducing reliance on fat stores during periods of high energy demand [25]. The reduction in NEFA levels in the supplemented groups is also likely due to improved insulin function and reduced NEFA release from adipose tissue. Decreases in NEFA and BHBA and improved energy balance during the transition period have been previously reported in dairy cows receiving vitamin D supplementation [25].

In the present study, the selected dose of 10,000 IU vitamin D administered at mid-pregnancy (Group 3) was sufficient to maintain stable serum 25(OH) D concentrations and showed a tendency to improve certain metabolic parameters. BHBA levels in late pregnancy were lower in group 3 (0.63 ± 0.12 mmol/L) than in group 1 (0.79 ± 0.29 mmol/L) and group 2 (0.91 ± 0.11 mmol/L), but

these differences were not significant ($p > 0.05$). This suggests that the administered dose may have had a partial effect, and it is plausible that a higher dose or repeated administration could exert a stronger and more consistent influence on ketone body regulation and energy balance in late gestation.

In this study, a significant rise in serum insulin concentrations was found in ewes supplemented with vitamin D during mid-pregnancy, while supplementation at insemination did not have an effect. Previous studies have shown that during metabolic stress, such as pregnancy, vitamin D stimulates insulin secretion by improving pancreatic beta cell function [25]. Increased insulin levels, in turn, can prevent excessive fat mobilization and the development of NEB by increasing glucose uptake by maternal and developing fetal tissues. This finding reinforces the concept that vitamin D prevents NEB in pregnant ewes by a mechanism involving improvement in glucose metabolism and insulin activity.

On the other hand, serum glucose levels significantly declined as pregnancy advanced in all ewes, which aligns with the heightened use of glucose for fetal growth. Although vitamin D supplementation during mid-pregnancy was associated with an increase in serum glucose levels, this difference did not reach statistical significance. Although vitamin D supplementation is associated with increased insulin sensitivity, the lack of correspondence between changes in serum glucose and insulin suggests that it cannot significantly alter glucose regulation in the late stages of pregnancy, when significant glucose is consumed for fetal growth [6].

In total, the observed changes in insulin, glucose, and NEFA levels suggest a potential improvement in insulin sensitivity in Group 3. This group had the highest postpartum insulin concentrations (6.06 ± 1.06 ng/mL), while having relatively higher glucose levels and lower NEFA concentrations during late pregnancy and postpartum, which may indicate reduced insulin resistance. These findings align with previous studies reporting that vitamin D enhances insulin receptor expression and modulates energy metabolism in ruminants and other mammals. Although direct measures of insulin resistance were not directly measured in this study, the metabolic profile observed in Group 3 supports the hypothesis that vitamin D supplementation during mid-pregnancy may positively influence insulin action.

In this study, the highest serum calcium level, especially in postpartum sampling, were in group 3, receiving vitamin D supplementation in mid-pregnancy. Given the role of vitamin D in regulating serum calcium levels through increasing intestinal absorption and renal reabsorption and calcium transport from bone, such an effect is expected, especially during times of increased demand, including late pregnancy and lactation [17, 26].

The absence of a significant difference in serum calcium levels between the control and the group supplemented at the time of insemination may be explained by the timing of vitamin D administration. Considering the lower calcium requirements in early pregnancy compared to late pregnancy and lactation [27], vitamin D supplementation in mid-pregnancy may have been more effective in meeting the higher calcium requirements in later pregnancy and postpartum.

Conversely, the lack of significant differences between groups in serum phosphorus concentrations at any of the sampling times is likely due to the tight regulation of phosphorus levels in ruminants to support metabolic processes such as energy production and bone mineralization [28]. On the other hand, since phosphorus levels are regulated primarily by fibroblast growth factor-23 and parathyroid hormone, the concentration of this mineral is less affected by vitamin D than calcium [29]. In addition, phosphorus absorption is usually efficient in ruminants, and dietary intake may have been sufficient to maintain serum levels, regardless of vitamin D administration [28].

In this study, the administration of vitamin D to ewes was associated with a significant increase in serum cholesterol levels at the end of pregnancy and increased triglyceride levels observed after delivery. These findings propose that vitamin D may play a part in lipid metabolism, especially during periods of heightened metabolic demand,

such as late pregnancy and lactation.

Vitamin D influences cholesterol homeostasis by controlling the expression of essential enzymes involved in cholesterol production and absorption, such as hydroxymethylglutaryl-CoA reductase (HMGCR) [30]. Cholesterol is an important part of cell membranes and is required for synthesizing steroid hormones, which are particularly important during pregnancy for supporting fetal development and preparing for lactation [31,32]. The rise in cholesterol levels observed at the conclusion of pregnancy in ewes that were given vitamin D supplements may suggest improved availability of cholesterol for these essential physiological functions.

In the same vein, the rise in serum triglyceride levels after lambing corresponds with earlier research conducted on other livestock species. Since milk is rich in fat, triglycerides serve as the main source of energy for both mother and infant during lactation [33]. The increase in triglycerides could be related to the mobilization of maternal fat stores to meet the energy needs of lactation and may also reflect increased insulin-stimulated lipogenesis. The elevation in triglyceride levels in ewes receiving vitamin D after delivery suggests that vitamin D supplementation may support energy metabolism throughout the shift from pregnancy to lactation.

In this study, total protein and serum albumin levels in all ewes decreased as pregnancy progressed. Previous studies have also shown that pregnancy can lead to changes in plasma protein concentrations due to increased fluid retention and altered protein metabolism [34]. During late pregnancy, as the demand for nutrients by the developing fetus increases, maternal protein reserves may be mobilized to support fetal growth, causing a reduction in serum protein levels [35].

Vitamin D supplementation did not result in significant alterations in total serum protein. However, it is noteworthy that the ewes receiving vitamin D injections during mid-pregnancy had higher serum albumin levels by the end of pregnancy. Albumin is a major plasma protein, which has a fundamental role in preserving oncotic pressure and facilitating the transport of different substances, such as hormones, fatty acids, and drugs [36]. The significant increase in serum albumin concentrations in the supplemented group may reflect the function of vitamin D in enhancing liver performance and protein synthesis. Vitamin D has been shown to influence the synthesis of several proteins, including albumin, and may improve the general nutritional status of the animal [37,38].

Additionally, improved serum albumin levels are often associated with better health outcomes and less incidence of metabolic disorders. This can be especially advantageous for pregnant ewes during the crucial transition phase around parturition [39].

Although vitamin D administration increased albumin levels, total protein levels did not undergo significant changes following vitamin D administration, probably due to the effect of several factors such as dietary protein intake and physiological status of the animal in ewes during late pregnancy on this analyte [40].

In conclusion, this study demonstrated that supplementing with vitamin D, especially in mid-pregnancy, considerably boosts serum vitamin D levels and favorably affects several metabolic indices in pregnant ewes of the Grey Shirazi breed. Ewes receiving vitamin D supplementation had lower serum BHBA and NEFA levels, indicating better energy balance, along with increased serum cholesterol and triglyceride levels, supporting lactation requirements. In addition, higher serum albumin levels in these ewes indicated improved protein synthesis and overall nutritional status. These findings highlight the potential of vitamin D administration to improve outcomes for both the ewe and lamb during pregnancy and lactation.

Materials and Methods

Ethical Considerations

All animal procedures were conducted in accordance with institutional guidelines and were approved by the Animal Ethics Committee of Shahid Chamran University of Ahvaz.

Animals

The research was conducted at the Fars Agricultural and Natural Resources Research Center, where 60 healthy ewes of the

Grey Shirazi breed were randomly selected. All ewes received a diet consisted of a total mixed ration containing conserved forage (corn silage and legume haylage) and commercial concentrates formulated to meet maintenance and gestational nutritional requirements. On a dry matter basis, the diet contained approximately 12.5% crude protein, 2.4 Mcal/kg metabolizable energy, 32% neutral detergent fiber (NDF), and 4.2% crude fat. Mineral and vitamin premixes were included to provide balanced micronutrient intake. Clean drinking water was available ad libitum throughout the study period.

All animal procedures were conducted in accordance with institutional guidelines and were approved by the Animal Ethics Committee of Shahid Chamran University of Ahvaz.

Estrus Synchronization

All ewes were synchronized by inserting a 40 mg progesterone-containing sponge (Esponjavet, Hipra, Spain) into the vaginal vault for 12 days. Following the removal of the sponges on the twelfth day, 400 units of PMSG (Gonaser, Hipra, Spain) were administered. The ewes were laparoscopy inseminated about 46 hours after PMSG injection to ensure control and uniformity in the reproductive process of the study.

Experimental Design

The ewes were randomly allocated into three experimental groups (n = 20 per group), ensuring balance across groups in terms of age, body weight, and parity to minimize confounding effects.

Group 1 (Control): Ewes in this group were not given any vitamin D supplement.

Group 2: Ewes in this group received a 10,000 IU vitamin D injection during the insemination period [41].

Group 3: Ewes in this group received a 10,000 IU vitamin D injection at 2.5 to 3 months of pregnancy.

The ewes which did not lamb during the trial period or suffered from any disorder or disease were excluded from the study.

Blood Sampling

Blood sampling was conducted at four stages: at the time of insemination, at 2.5 to 3 months of pregnancy, and two weeks before and after lambing. Approximately 10 ml of blood was collected through the jugular vein of each ewe at each time point using disposable syringes and subsequently transferred into plain gel tubes for analysis. Following centrifugation at 4000 rpm for 10 minutes, the serum was collected and subsequently stored at -20°C for further analysis.

Laboratory assessments

Laboratory assessments were performed to determine serum levels of various metabolites and hormones.

- Vitamin D3 and insulin concentrations were measured using ELISA kits (MonoKit, Iran).

- Nonesterified fatty acids (NEFA) and β-hydroxybutyric acid (BHBA) were quantified using commercially available enzymatic colorimetric assay kits (Randox Laboratories Ltd., Ardmore, UK). For the NEFA assay, the detection limit was 0.06 mmol/L, with an intra-assay CV of <5% and an inter-assay CV of <7%. The BHBA assay had a detection limit of 0.07 mmol/L, with intra- and inter-assay CVs of <5% and <6%, respectively. All measurements were performed in duplicate following the manufacturer's instructions.

- Glucose, Triglycerides, Cholesterol, Total Protein, Albumin, Calcium, and Phosphorus were measured using colorimetric methods with commercial kits from Biorex Fars (Iran) and a biochemical autoanalyzer (Biotecnica BT-1500, Italy).

Statistical Analysis

The statistical evaluation was performed using version 22 of the SPSS software (SPSS Inc., Chicago, IL, USA). The findings were expressed as means ± standard error (SE) across various groups. Data distribution was assessed for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test. The data underwent statistical assessment utilizing Repeated Measures Analysis of Variance (ANOVA), One-way ANOVA, and Tukey's post hoc tests. The level of significance was taken at $p < 0.05$ in all analyses.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors declare that they have used AI-based writing assistance tools available in Grammarly (Grammar Check, Clarity, and Writing Enhancement features) to improve the readability, and language of the manuscript.

Authors' Contributions

S.M.J., M.M., and M.R.H. conceived and planned the experiments. A.B. and M.M. carried out the experiments. S.M.J. and Z.D. carried out the laboratory analysis. S.M.J. and M.M. contributed to the interpretation of the results. S.M.J. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript..

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Evaluation of Egg Drop Syndrome Virus Fiber Protein as a Vaccine Candidate: In Silico Analysis, Expression, Purification and Its Stability

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ABSTRACT

The Egg Drop Syndrome Virus (EDSV), an avian adenovirus, triggers a sharp decline in both egg production and quality in infected chickens, leading to substantial economic losses in poultry industry. Previous studies have suggested that the EDSV fiber protein may serve as a candidate for subunit vaccine. The present research focused on the expression, purification, and thermal stability evaluation of recombinant fiber protein as a vaccine against EDSV. Using an in *silico* approach, we investigated the fiber protein structure and its expression in *Escherichia coli* (*E. coli*). We also evaluated the thermal stability of the expressed protein. The protein was expressed predominantly as soluble trimeric proteins in *E. coli* and purified using nickel-affinity purification method, yielding approximately 15 mg/L of purified protein. Structural analysis using immunological and bioinformatics tools Confirmed retention of the native trimeric conformation in the recombinant protein. Based on the thermal stability evaluation on this recombinant protein, the protein showed good thermal stability, highlighting its potential as a subunit vaccine candidate for a vaccine against EDSV.

Keywords

Egg drops syndrome virus, Fiber protein, In silico, Purification, Vaccines

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Number of Pages: 12

Abbreviations

BSA: Bovine serum albumin
CAI: Codon adaptation index
CE: Conformational epitopes
EDSV: Egg drop syndrome virus
GRAVY: Grand average of hydropathicity
MFE: Minimum free energy
PBS: Phosphate-buffered saline
TMB: Tetramethylbenzidine

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Introduction

The Egg drop syndrome virus (EDSV), a member of the Atadenovirus genus [1], poses a significant threat to poultry health worldwide. First identified in the Netherlands in 1976 [2], this non-enveloped, double-stranded DNA virus causes substantial economic losses through its characteristic clinical manifestation: the production of thin-shelled, soft, or shell-less eggs in laying hens. The virus demonstrates broad host specificity, infecting not only chickens but also turkeys, geese, and quails, making it a particularly challenging pathogen to control [3]. The viral capsid of EDSV is comprises of 11 structural proteins, among which the fiber protein plays a pivotal role in viral-host interaction and cell entry [4]. This trimeric protein consists of three distinct domains: tail, shaft, and knob, with the knob domain being particularly crucial for initial host cell binding [5]. Due to its immunodominant nature and its essential function in viral pathogenesis, the fiber protein has emerged as an ideal candidate for recombinant vaccine development [6]. Current vaccination strategies rely on inactivated vaccines produced using duck eggs, these vaccines presents several limitations. Dependence on non-specific pathogen-free duck eggs carries inherent risks of pathogen contamination and viral spread, given the high susceptibility of ducks to various avian pathogens [7, 8]. These challenges highlight the urgent need for alternative vaccine production methods that can ensure both safety and efficacy. Subunit vaccines comprising viral surface protein components can circumvent these limitations [9, 10]. Advances in recombinant DNA technology have greatly expanded the potential for vaccine development, as evidenced by the successful commercialization of numerous recombinant viral vector vaccines against major avian diseases. As reported in a review by Hein et al, more than 15 commercially available recombinant viral vector vaccines have already been developed against key avian diseases, including Newcastle disease, infectious laryngotracheitis, infectious bursal disease, avian influenza, and Mycoplasma gallisepticum [11]. The potential of subunit vaccines, particularly those targeting key viral surface proteins like the fiber protein, offers a promising solution to the limitations of traditional vaccine production methods [12]. This study addresses current limitations in EDSV vaccine development through three primary objectives: 1) Comprehensive in silico analysis of the fiber protein structure to identify key immunogenic elements, 2) Development of an optimized expression and purification system for the recombinant fiber protein, and 3) Evaluation of the thermal stability properties of the purified protein. By combining computational biology with experimental validation, this research aims to establish a foundation for developing a safer, more effective alternative to current EDSV vaccines. The broader significance of this work extends beyond EDSV control, as the methodologies developed may be applicable to other avian adenoviruses. Furthermore, adopting recombinant production methods can significantly improve vaccine safety profiles while reduce production costs and complexity. Overall, this study aligns with global shift in modern in vaccinology toward rational, structure-based vaccine design

Result

Physicochemical parameter evaluation

The molecular weight, the number of amino acids, and numbers of positively and negatively charged residues of the fiber protein were 28659.33 Da, 268, 15 and 16, respectively. The extinction coefficient of was 34630 M−1 cm−1 at 280 nm measured in water. The aliphatic index and Grand Average of Hydropathicity (GRAVY) values of the chimeric protein were 77.16 and 0.007, respectively. The bio computed half-life was 5.5 hours in mammalian reticulocytes (*in vitro*), 3 minutes in yeast (*in vivo*), and 2 minutes in *E. coli* (in vivo). Moreover, the instability index, and isoelectric point (pI) were 23.39, and 6.14, respectively (Table 1). The protein solubility, predicted using SOLpro was estimated to be 0.840895.

Secondary structures Evaluation

The secondary structural composition of the fiber protein, analysis using the GOR IV server, revealed 7.46% α-helix, 35.07% β-sheet, and 57.46% coiled-coil. According to GOR IV results, it can be seen that random coils are dominant in the fusion protein sequence, followed by extended strands and alpha helices (Fig. 1). Also, using the PSIPRED server, were shown the secondary structure and graphical representation of our protein.

3D structures prediction of the fiber protein and validation

Three modeling servers, Phyre2, I-TASSER and Galaxy, were used for 3D structure modeling. The evaluation of the results of different servers showed that the I-TASSER server is suitable for modeling the 3D structure of our protein. The scoring system of I-TASSER server is based on C-score, and the C-score range is usually between -5 and 2. Models with higher C-score (-1.23) were selected for further evaluations. (Fig. 2). Fiber protein refinement was done using GalaxyRefine server to optimize quality. Regarding the role of trimer structure in antigenicity and binding to host cell, the trimer structure of the mentioned construct was modeled using the Galaxy Web server. Ramachandran diagram showed that, 81.2 of residues

Table 1.
Parameters analyzed by the Expasy ProtParam server

Sequence length	Mw*	T pI	-R	+R	EC	II	AI	GRAVY
268	28659.33	6.14	16	15	34630	23.39	77.16	0.007

*Mw, Molecular weight; T pI, Theoretical Isoelectric point; -R, Number of negative charged residues; +R, Number of positive charged residues; EC, Extinction coefficient at 280 nm; II, Instability index; AI, Aliphatic index; GRAVY, Grand Average Hydropathy

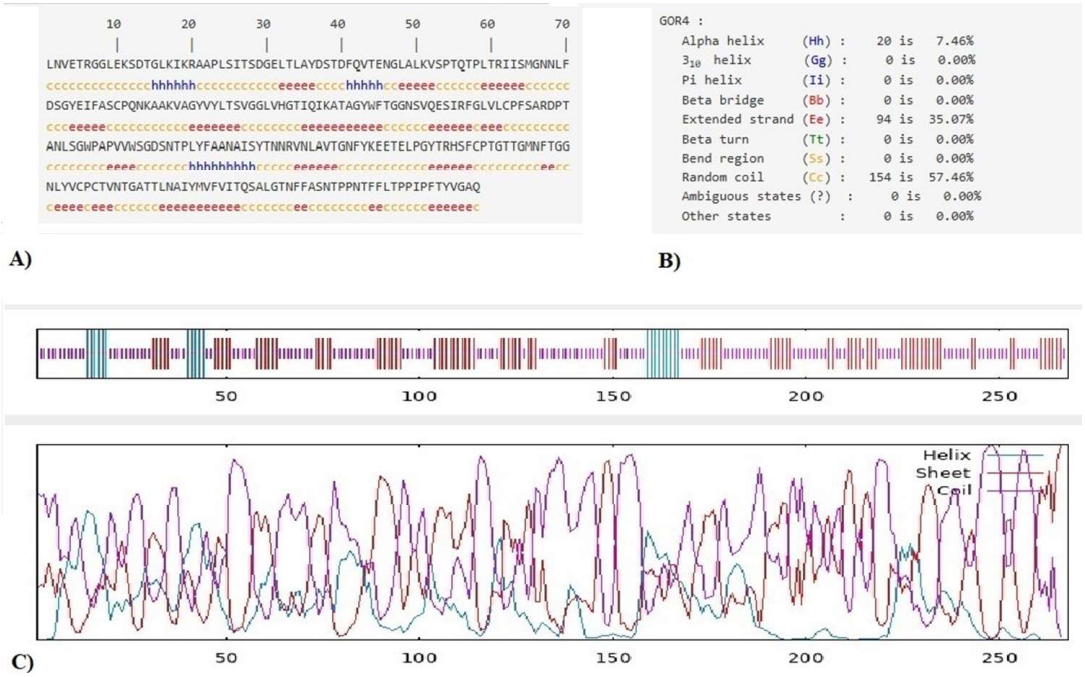

Figure 1.
Fiber protein secondary structure predicted by GOR4 server. (A) The composition of the secondary structure in percent (B) fiber protein secondary structure map (h: alpha-helix; e: extended strand; c: coil). (C) diagram of fiber protein secondary structure (the blue color represents the alpha-helices; the red color represents beta strands, and the purple color was the coils)

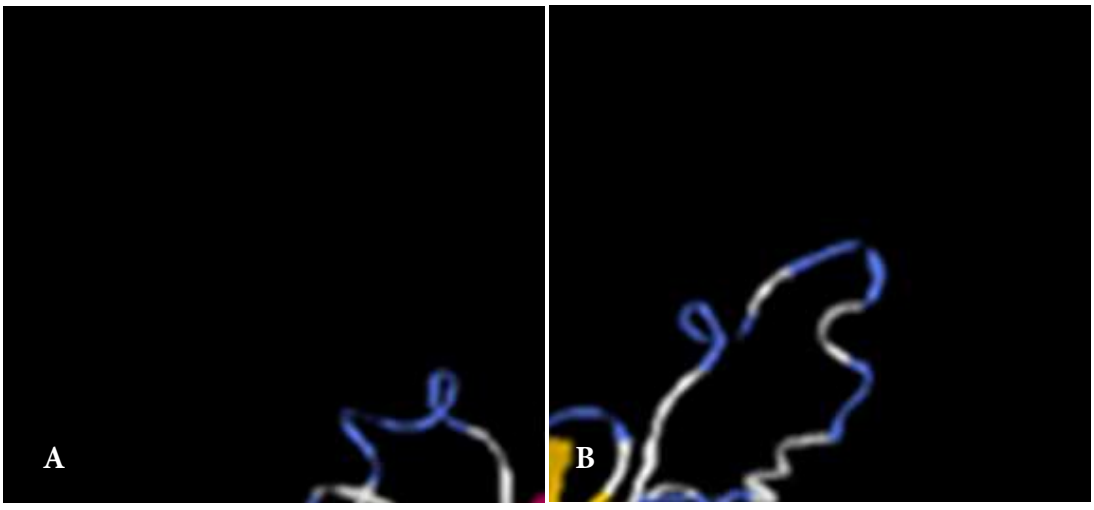

Figure 2.
Tertiary structure prediction. A probabilistic structural model for fiber protein by online servers: A) Monomer prediction by I-TASSER server; B); prediction of trimer structure by Galaxyhomomer

were in favored regions, 15.7 in allowed regions, 1.3 in generously allowed regions and 1.8% in disallowed regions. In order to determine the potential errors in the 3D structures, the analysis of the models has been done by ProSA-webserver (Wiederstein & Sippl, 2007). ProSA z-score of input model was -4.38 (Fig. 3).

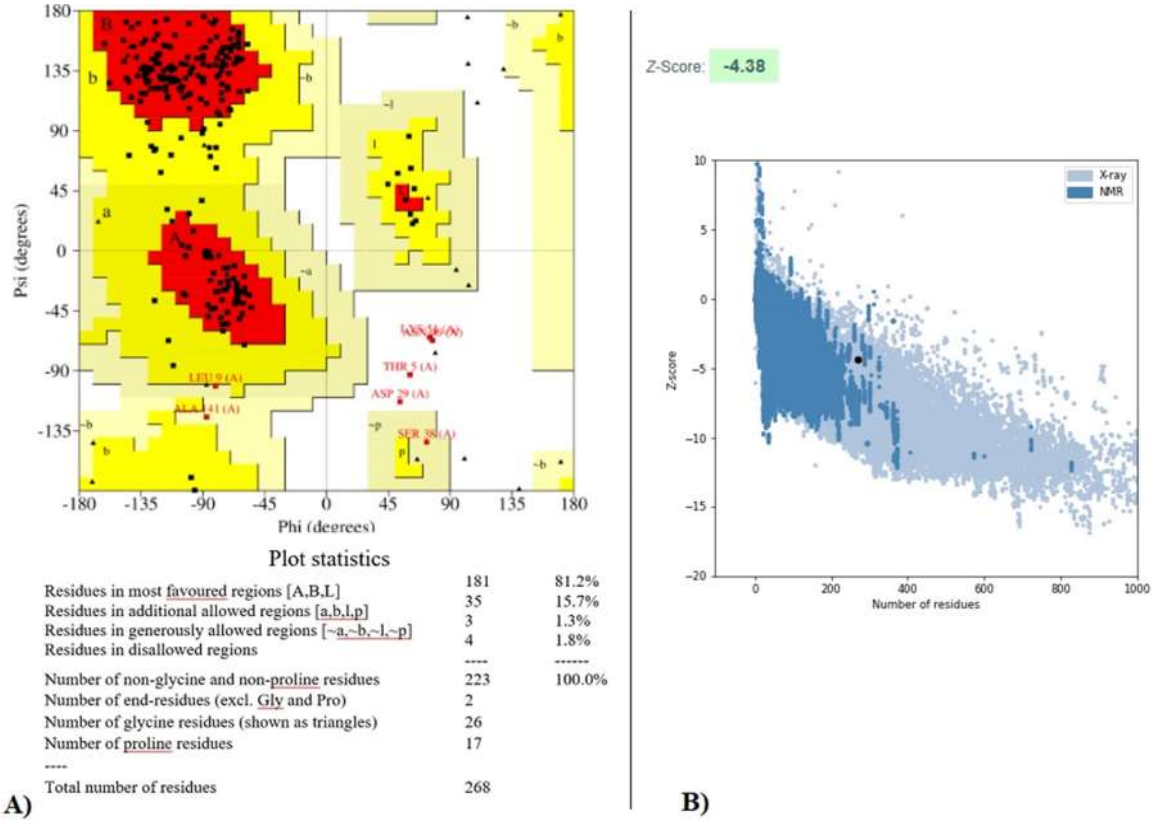

Figure 3.
Model stability was evaluated based on Ramachandran plot for fiber protein: A) Ramachandran plots for trimer structure fiber protein by PROCHEC; B) Fiber protein structure analysis by ProSA- web server

Antigenicity evaluation

Antigenicity prediction using the ANTIGENpro and Vaxijen servers predicted high antigenicity probabilities of 93% and 100%, respectively. These results suggest that, engineered fiber protein is highly antigenic.

Table 2.
Linear B-cell epitope of fiber protein predicted using the SVMTriP server

Rank	Location	Epitope	Score
1	91 – 110	YVYLTSVGGLVHGTIQIKAT	1
2	173 – 192	RVNLAVTGNFYKEETELPGY	0.807
3	131 – 150	LCPFSARDPTANLSGWPAPV	0.605

Prediction of B-cell epitopes

Linear epitope prediction results of the target protein were predicted using the Ellipro and SVMTriP servers, and are shown in the Tables 2 and 3. B-cell epitopes were computationally ranked by prediction scores obtained from ABCpred's neural network model (Table 4). Prediction scores directly correlate with epitope probability (threshold= 0.5), with all shown

Table 3.
Linear B-cell epitope of fiber protein predicted using the ElliPro server

No.	Start	End	Peptide	Residues No.	Score
1	35	59	AYDSTDFQVTENGLALKVSPTQTPL	25	0.79
2	236	254	TQSALGTNFFASNTPPNTF	19	0.79
3	218	226	TVNTGATTL	9	0.784
4	200	208	TGTTGMNFT	9	0.737
5	135	141	SARDPTA	7	0.663
6	110	124	TAGYWFTGGNSVQES	15	0.661
7	165	192	NAISYTNNRVNLAVTGNFYKEETELP-GY	28	0.646
8	68	75	NLFDSGYE	8	0.597
9	9	24	LEKSDTGLKIKRAAPL	16	0.591
10	153	158	SGDSNT	6	0.567
11	26	29	ITSD	4	0.548

Table 4.
Predicted Epitope by ABCpred Prediction Server

Rank	Sequence	Start position	Score
1	GTTGMNFTGGNLYVCP	201	0.93
2	GNSVQESIRFGLVLCP	118	0.92
3	SGDSNTPLYFAANAIS	153	0.9
3	LSGWPAPVVWSGDSNT	143	0.9
4	ANAISYTNNRVNLAVT	164	0.89
5	GNLYVCPCTVNTGATT	210	0.88
5	KATAGYWFTGGNSVQE	108	0.88
6	GELTLAYDSTDFQVTE	30	0.87
7	VSPTQTPLTRIISMGN	52	0.86
7	NFFASNTPPNTFFLTP	243	0.86
7	TQSALGTNFFASNTPP	236	0.86
8	SGYEIFASCPQNKAAK	72	0.85
8	VETRGGLEKSDTGLKI	3	0.85
8	HGTIQIKATAGYWFTG	102	0.85
9	RIISMGNNLFDSGYEI	61	0.83
10	PFSARDPTANLSGWPA	133	0.8

peptides exceeding this cutoff value. Discontinuous epitope were also identified using the Ellipro server and are shown in Table 5. Predicted conformational epitopes including: conformational epitopes 1 (CE1) and conformational epitopes 2 (CE2), conformational epitopes 3 (CE3), conformational epitopes 4 (CE4), which having score greater than 0.5. Three discontinuous epitopes were identified with substantial surface accessibility: CE1 (68 residues, 68.5% exposed), CE2 (35 residues, 65.9% exposed), and CE3 (55 residues, 63.7% exposed). The significant solvent accessibility of these regions (scores > 0.63) supports their inclusion in vaccine design.

MHC-I binding prediction analysis

The MHC-I processing tool identified 259 potential nonamer peptides from the fiber protein that may bind to MHC-I and trigger immune responses. Among these, three peptide fiber protein sequence, i.e., QESIRFGLV and GELTLAYDS and TENGLALKV, were determined, which are likely to be to bind with MHC-I alleles, with percentage rank ≤ 2 (Table 6).

MHC-II binding prediction analysis

For MHC class II binding prediction, the fiber protein sequence was segmented into 254 overlapping 15-mer peptides using NetMHCIIpan 4.0 server. The peptide LTRIISMGNNLFDSG was identified as a strong MHC-II binder, with an optimal peptide binding core of IISMGNNLF (Table 7).

Prediction of mRNA structure

The predicted mRNA secondary structure, analyzed using the RNAfold server, exhibited a minimum free energy of -255.60 kcal/mol, indicating that the gene structural sequence can form a stable RNA sec-

Table 5.
Conformational B-cell epitope of fiber protein vaccine predicted using the ElliPro server

No.	Peptide regions and residues number	Residues No.	Score
1	A:N67, A:N68, A:L69,A:F70, A:D71, A:S72, A:G73, A:Y74, A:E75, A:Q82, A:N83, A:K84, A:T110, A:A111, A:G112, A:Y113, A:W114, A:F115, A:T116, A:G117, A:G118, A:N119, A:S120, A:V121, A:Q122, A:E123, A:S124, A:I125, A:A177, A:V178, A:T179, A:G180, A:N181, A:F182, A:Y183, A:K184, A:E185, A:T187, A:E188, A:P199, A:T200, A:G201, A:T202, A:T203, A:G204, A:M205, A:N206, A:F207, A:T208, A:T236, A:Q237, A:S238, A:A239, A:L240, A:G241, A:T242, A:N243, A:F244, A:F245, A:A246, A:S247, A:N248, A:T249, A:P250, A:P251, A:N252, A:T253, A:F254	68	0.685
2	A:F134, A:S135, A:A136, A:R137, A:D138, A:P139, A:T140, A:A141, A:N165, A:A166, A:I167, A:S168, A:Y169, A:T170, A:N171, A:N172, A:R173, A:N175, A:L189, A:P190, A:G191, A:Y192, A:T218, A:V219, A:N220, A:T221, A:G222, A:A223, A:T224, A:T225, A:L226, A:N227, A:G266, A:A267, A:Q268	35	0.659
3	A:N2, A:T5, A:R6, A:G7, A:G8, A:L9,A:E10, A:K11, A:S12, A:D13, A:T14, A:G15, A:L16, A:K17, A:I18, A:K19, A:R20, A:A21, A:A22, A:P23, A:L24, A:S25, A:I26, A:T27, A:S28, A:D29, A:G30, A:E31, A:L32, A:T33, A:A35, A:Y36, A:S38, A:T39, A:D40, A:F41, A:Q42, A:V43, A:T44, A:E45, A:N46, A:G47, A:L48, A:A49, A:L50, A:K51, A:V52, A:S53, A:P54, A:T55, A:Q56, A:T57, A:P58, A:L59, A:T60	55	0.637
4	A:V151, A:S153, A:G154, A:D155, A:S156, A:N157, A:T158	7	0.548

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Table 6.
MHC-I binding epitopes predicted by the NetMHCcons 1.1 server

Allele	Start	Length	Peptide	1-log50k (aff)	Affinity (nM)	% Rank
HLA-B40:06	29	9	GELTLAYDS	0.247	3441.22	2.00
HLA-B40:06	43	9	TENGLALKV	0.330	1407.46	1.50
HLA-B40:06	121	9	QESIRFGLV	0.287	2244.37	1.50

Table 7.
MHC-II binding epitopes predicted by the NetMHC 4.0 server.

Allele	Start	Peptide	Core Sequence	Score EL	PR*
DRB1_1454	13	DTGLKIKRAAPLSIT	LKIKRAAPL	0.367271	3.55
DRB1_1454	14	TGLKIKRAAPLSITS	IKRAAPLSI	0.340573	4.11
DRB1_1454	15	GLKIKRAAPLSITSD	IKRAAPLSI	0.419096	2.66
DRB1_1454	58	PLTRIISMGNNLFDS	IISMGNNLF	0.393941	3.04
DRB1_1454	59	LTRIISMGNNLFDSG	IISMGNNLF	0.464146	2.10
DRB1_1454	60	TRIISMGNNLFDSGY	IISMGNNLF	0.358547	3.73
DRB1_1454	101	VHGTIQIKATAGYWF	IQIKATAGY	0.336206	4.22
DRB1_1454	102	HGTIQIKATAGYWFT	IQIKATAGY	0.370812	3.48
DRB1_1454	116	TGGNSVQESIRFGLV	VQESIRFGL	0.370514	3.48
DRB1_1454	117	GGNSVQESIRFGLVL	VQESIRFGL	0.367819	3.54

ondary structure. (Fig. 4).

Gene expression and protein purification

Codon optimization of the fiber protein gene was performed using the Java codon adaptation tool (JCAT) for expression in a eukaryotic system. The optimized gene sequence consisted of 804 nucleotides, encoding a 268 amino acid protein. CAI value of 0.99, and CG-content of 53%, suggest the possibility of expression of protein in eukaryotic system (Fig. 5). The optimized construct was cloned into the pET and then transformed into *E. coli* SHuffle cells, a strain engineered for enhanced disulfide bond formation

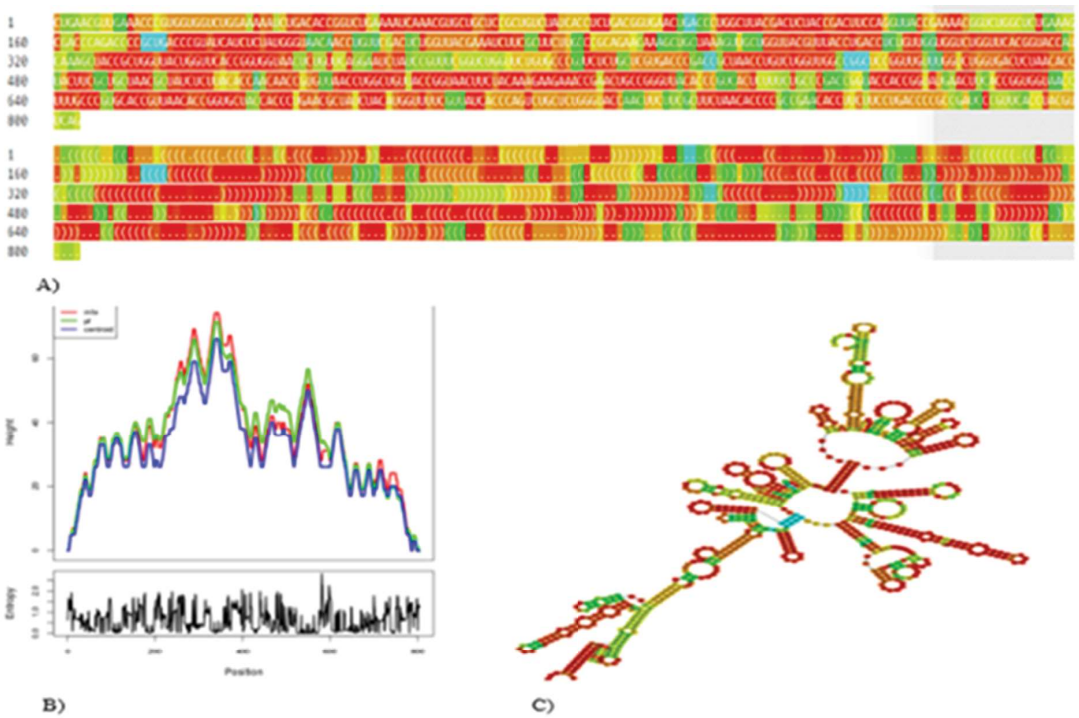
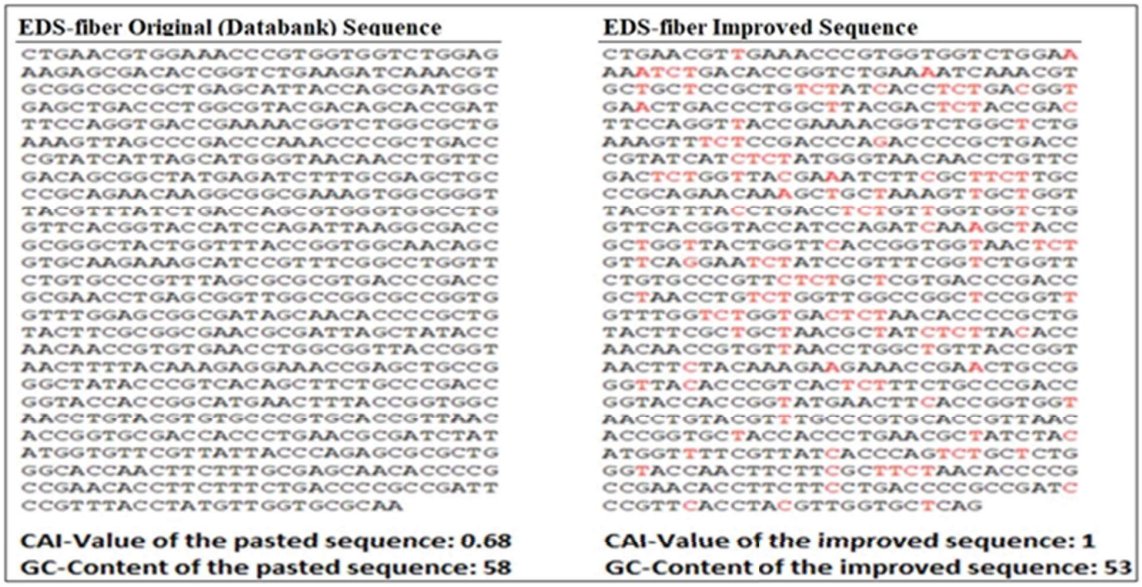


Figure 4.
Predicted mRNA secondary structure by RNA fold server: A) Evaluation of the minimum free energy. The optimal secondary structure with the minimum free energy is indicated in the dot-bracket notation, which is colored by the positional entropy. The shown interactive drawing represents the RNA structure with the minimum free energy, colored based on the probabilities of base pairing. [((())) stem; . loop; ,,,,,, internal loop]; B) The mountain plot of the MFE structure, RNA structures thermodynamic ensemble, and the centroid structure. Plateaus correspond to loops in the mountain plot (with hairpin loops representing peaks), while slopes represent helices. (The red, green and blue lines referred to the minimum free energy (MFE), the probability of the pair (PF) and, the centroid algorithm, respectively. Also, the positional entropy was indicated below; C) The mRNA secondary structure.


EDS-fiber Original (Databank) Sequence
CTGAACGTGGAAACCCGTGGTGGTCTGGAG
AAGAGCGACACCGGTCTGAAGATCAAACGT
GCGGCGCCGCTGAGCATTACCAGCGATGGC
GAGCTGACCCTGGCGTACGACAGCACCGAT
TTCCAGGTGACCGAAAACGGTCTGGCGCTG
AAAGTTAGCCCGACCCAAACCCCGCTGACC
CGTATCATTAGCATGGGTAACAACCTGTTC
GACAGCGGCTATGAGATCTTTGCGAGCTGC
CCGCAGAACAAGGCGGCGAAAGTGGCGGGT
TACGTTTATCTGACCAGCGTGGGTGGCCTG
GTTCACGGTACCATCCAGATTAAGGCGACC
GCGGGCTACTGGTTTACCGGTGGCAACAGC
GTGCAAGAAAGCATCCGTTTCGGCCTGGTT
CTGTGCCCGTTTAGCGCGCGTGACCCGACC
GCGAACCTGAGCGGTTGGCCGGCGCCGGTG
GTTTGGAGCGGCGATAGCAACACCCCGCTG
TACTTCGCGGCGAACGCGATTAGCTATACC
AACAACCGTGTGAACCTGGCGGTTACCGGT
AACTTTTACAAAGAGGAAACCGAGCTGCCG
GGCTATACCCGTCACAGCTTCTGCCCGACC
GGTACCACCGGCATGAACTTTACCGGTGGC
AACCTGTACGTGTGCCCGTGCACCGTTAAC
ACCGGTGCGACCACCCTGAACGCGATCTAT
ATGGTGTTCGTTATTACCCAGAGCGCGCTG
GGCACCAACTTCTTTGCGAGCAACACCCCG
CCGAACACCTTCTTTCTGACCCCGCCGATT
CCGTTTACCTATGTTGGTGCGCAA
CAI-Value of the pasted sequence: 0.68
GC-Content of the pasted sequence: 58
EDS-fiber Improved Sequence
CTGAACGTTGAAACCCGTGGTGGTCTGGAA
AAATCTGACACCGGTCTGAAAATCAAACGT
GCTGCTCCGCTGTCTATCACCTCTGACGGT
GAACTGACCCTGGCTTACGACTCTACCGAC
TTCCAGGTTACCGAAAACGGTCTGGCTCTG
AAAGTTTCTCCGACCCAGACCCCGCTGACC
CGTATCATCTCTATGGGTAACAACCTGTTC
GACTCTGGTTACGAAATCTTCGCTTCTTGC
CCGCAGAACAAAGCTGCTAAAGTTGCTGGT
TACGTTTACCTGACCTCTGTTGGTGGTCTG
GTTCACGGTACCATCCAGATCAAAGCTACC
GCTGGTTACTGGTTCACCGGTGGTAACTCT
GTTCAGGAATCTATCCGTTTCGGTCTGGTT
CTGTGCCCGTTCTCTGCTCGTGACCCGACC
GCTAACCTGTCTGGTTGGCCGGCTCCGGTT
GTTTGGTCTGGTGACTCTAACACCCCGCTG
TACTTCGCTGCTAACGCTATCTCTTACACC
AACAACCGTGTTAACCTGGCTGTTACCGGT
AACTTCTACAAAGAAGAAACCGAACTGCCG
GGTTACACCCGTCACTCTTTCTGCCCGACC
GGTACCACCGGTATGAACTTCACCGGTGGT
AACCTGTACGTTTGCCCGTGCACCGTTAAC
ACCGGTGCTACCACCCTGAACGCTATCTAC
ATGGTTTTCGTTATCACCCAGTCTGCTCTG
GGTACCAACTTCTTCGCTTCTAACACCCCG
CCGAACACCTTCTTCCTGACCCCGCCGATC
CCGTTCACCTACGTTGGTGCTCAG
CAI-Value of the improved sequence: 1
GC-Content of the improved sequence: 53
A)

Index	Description	Range	Value
GC3	GC Content at the Third Position of Synonymous Codons	0~1	0.76
GC	GC Content		0.59
GC1	GC Content at the First Position of Synonymous Codons		0.51
CAI	Codon Adaptation Index	0~1	0.91
CFD	Codon Frequency Distribution	0~1	0
FOP	Frequency of Optimal Codons	0~1	0.74
CBI	Codon Bias Index	-1~1	0.61

B)

Figure 5.
Optimization of the synthetic gene for expression of the recombinant protein: A) Optimization by Jcat; B) Analysis by GenRCA Rare Codon Analysis Tool.

in cytoplasmic proteins. Following induction, SDS-PAGE analysis revealing a prominent band at approximately 30 kDa, consistent with the predicted molecular weight of the monomeric fiber protein subunit (Fig. 6).

Thermal stability evaluation

SDS-PAGE analysis of the recombinant fiber protein under different treatment conditions revealed distinct oligomeric states (Figs. 6A, 7). Under native (non-boiled) conditions, the protein migrated as

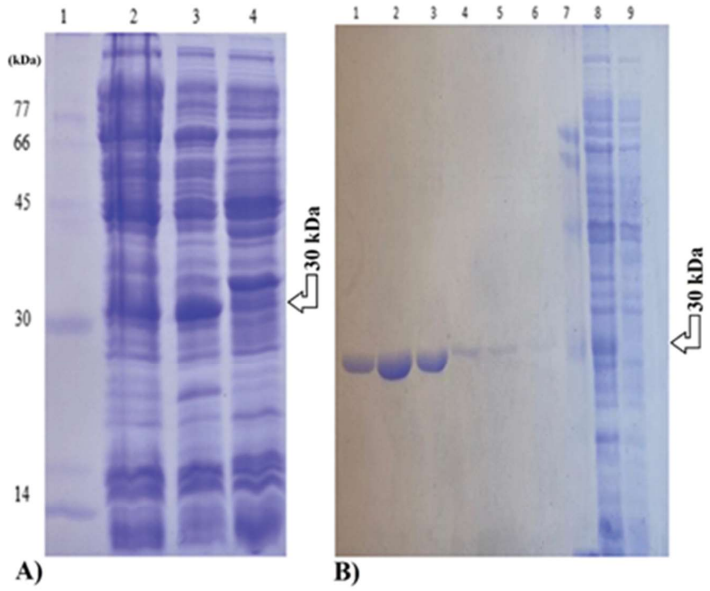


Figure 6.
Protein pattern of the LB crude extract and purified fiber protein in the SDS-PAGE: A) SDS-PAGE analysis of level of expression of fiber protein in LB broth, Lane1= Protein molecular weight marker (77-14 kDa), lane2= Soluble part of supernatant containing fiber protein, Lanes3 and 4= Pellet of E. coli shuffle including pET-fiber not and induced with IPTG respectively, grown in LB broth; B) Expression and purification of fiber protein (PET28-fiber) as a candidate vaccine in E. coli cells by SDS-PAGE, Lane 7= Protein molecular weight marker; lane 9= Non induced, lane8= Induced E. coli cells bearing fiber protein-PET28 plasmid, lane 1 to 6= Elution

two predominant bands corresponding to molecular weights of 30 kDa (monomeric form) and 65 kDa (trimeric form), with the trimeric form representing the primary conformation. This trimeric structure demonstrated remarkable stability, maintaining its oligomeric state even in the presence of SDS at room temperature. Upon thermal denaturation by boiling prior to electrophoresis resulted in complete dissociation of trimers, yielding exclusively monomeric 30 kDa band. Notably, pre-incubation at 4°C and 37°C, prior to analysis did not affect these migration patterns, suggesting temperature-independent stability of the protein's oligomeric states under non-denaturing conditions. Parallel ELISA results further confirmed that the purified recombinant fiber protein maintained strong antigenic properties after storage at both temperatures (4°C and 37°C), as shown in the Figure 7B.

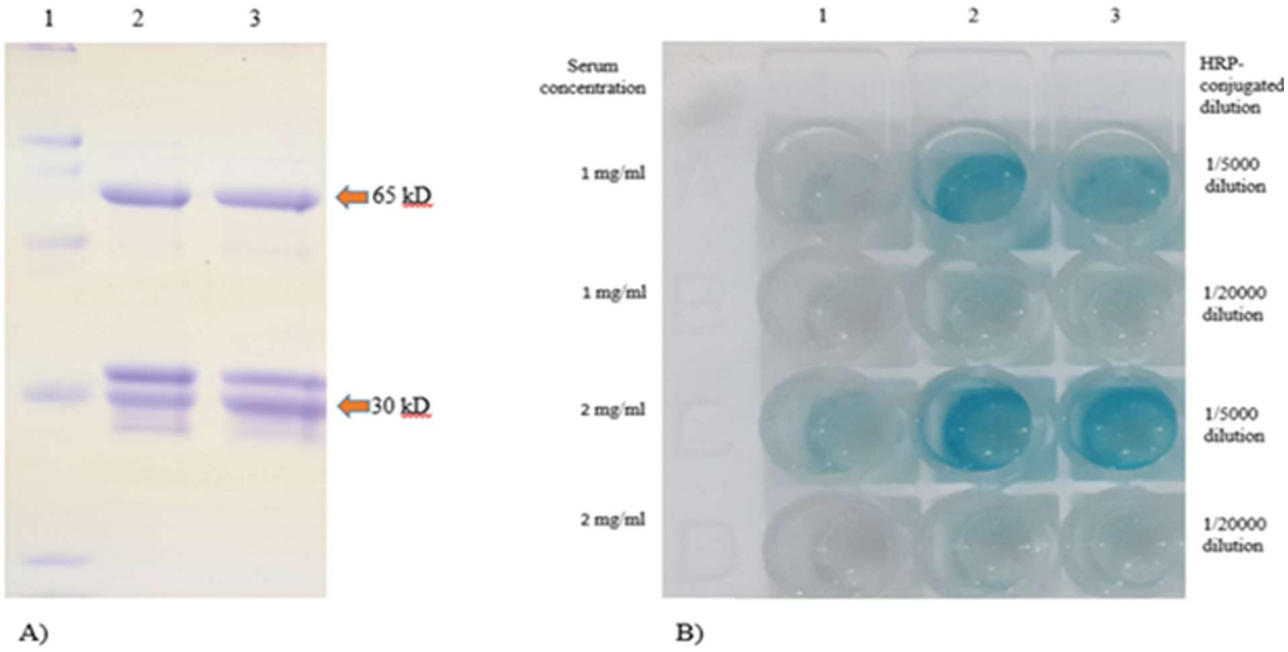


Figure 7.
Thermal stability evaluation by SDS-PAGE and ELISA. A) SDS-PAGE of purified fiber protein: lane 1, Protein molecular weight marker (77-14 kDa), lane2; Protein fiber stored in 4° C, lane3; Protein fiber stored in 37° C. B) ELISA assay in order to evaluate antigenicity. lane1; negative control, lane2; Protein fiber stored in 4°C that coated as antigen, lane3; Protein fiber stored in 37° C that coted as antigen.

Discussion

Developing effective vaccines against avian pathogens remains a major challenge due to the time-consuming and costly nature of traditional vaccine production methods [13]. Recent advances in computational immunology and structural biology have enabled more rational vaccine design approaches. In this study, we combined comprehensive bioinformatics analyses and experimental characterization of the EDSV fiber protein as a potential vaccine candidate. Our findings are consistent with the emerging trends in epitope-based vaccine design, building upon successful examples such as immunoinformatics approach for MARV and cross-protective vaccine for Mpox and EBOV. These studies collectively highlight the transformative potential of computational tools in identifying stable, immunogenic vaccine candidates while significantly reducing development timelines [14-16]. Our study provide crucial insights for vaccine development, one of the key outcomes of our work is the confirmation of trimeric stability through both computational modeling using the Galaxy server [17] and experimental validation via SDS-PAGE, with the protein maintaining structural integrity at physiological conditions (37°C for 14 days). This stability was further supported by favorable physicochemical parameters, including an aliphatic index of 77.16 and instability index of 23.39. The design of our construct, strategically incorporating both the knob domain and partial shaft region (268amino acid), was guided by previous studies demonstrating their critical role in maintaining proper trimer formation and antigenicity [7, 18].

Preservation of conformational epitopes (CE1-CE3), each exhibiting more than 63% surface exposure, underscores potential of this protein to elicit a strong and protective immune responses. From a production standpoint, the recombinant protein demonstrated several advantageous characteristics. The *E. coli* Shuffle [19] strain facilitated efficient expression with high yield, producing both monomeric (30 kDa) and trimeric (65 kDa) forms. The mRNA stability (MFE= -255.60 kcal/mol) and retained antigenicity post-temperature challenge (as validated by ELISA) further support the practical utility of this vaccine candidate. These results are particularly encouraging given the well-documented challenges of maintaining adenovirus fiber protein stability during production and storage. However, several important questions and limitations remain that should be addressed in future. The compatibility of this antigen with various adjuvants requires systematic evaluation, as adjuvant selection can significantly impact both immunogenicity and stability [20]. Additionally, comprehensive in vivo studies in chicken models will be essential to confirm the protective efficacy suggested by our in silico and in vitro results. Long-term storage stability and large-scale production optimization must also be thoroughly assessed to support commercial applications [10]. Structural refinement through techniques like cryo-EM could provide atomic-level insights into the trimer conformation and potential stabilization strategies [21]. This study makes significant contributions to multiple aspects of vaccine development. It demonstrates how integrating computational predictions with experimental approaches can streamline rational vaccine design, particularly for veterinary applications. By focusing on thermal stability, we address two critical limitation in vaccine distribution, especially relevant for resource-limited settings. Moreover, the design framework established here for EDSV could be readily adapted for other avian pathogens, potentially transforming poultry vaccine development pipelines [22]. Despite promising results, we acknowledge certain limitations. The absence of in vivo immunogenicity data remains a key gap. Likewise, the effects of adjuvants on both stability and immunogenicity remain to be characterized, and scale-up production parameters need optimization. Nevertheless, our multidisciplinary approach exemplifies how modern computational tools can accelerate and improve vaccine development while maintaining rigorous scientific standards [14]. By combining robust bioinformatics predictions with systematic experimental confirmation, this study establish a strong foundation for developing an effective EDSV vaccine while minimizing animal use in preliminary testing stages.

Conclusion

In this study, we investigated the EDSV fiber protein using a comprehensive in silico and experimental method. The result of structural and immunological analyses indicate that this recombinant protein possesses strong potential as a preventive vaccine candidate. Its demonstrated stability, antigenicity, and expression efficiency support its feasibility for further vaccine development. Although more validations are needed, to fully establish its protective efficacy, the EDSV fiber protein can be used as a vaccine candidate against EDSV.

Materials and Methods

Sequence selection and design of construct

The amino acid sequence of the partial fiber protein A0A482J640, comprising the knob domain and a segment of the shaft region, was obtained from the UniProt Knowledgebase (https://www.uniprot.org/) in FASTA format.

Prediction of physicochemical properties

Various physicochemical parameters of the designed fiber protein were predicted using the ProtParam tool. Parameters assessed included molecular weight, theoretical isoelectric point (pI), amino acid composition and count, atomic composition, chemical formula, extinction coefficients, estimated half-life, aliphatic index, instability index, and grand average of hydropathicity (GRAVY). Additionally, protein solubility was evaluated using the SOLpro (http://scratch.proteomics.ics.uci.edu/) and Protein-Sol (https://protein-sol.manchester.ac.uk/) servers.

Secondary structure prediction of fiber protein

Secondary structure elements of the fiber protein were performed using GOR IV and PSIPRED servers. Both algorithms calculated probabilities for α-helix, β-sheet, and random coil at each amino acid position. The conformation with the highest probability structure was chosen as the predicted conformation [23, 24].

3D structures prediction

Protein 3D structure prediction was performed using three computational servers, Phyre2, I-TASSER, and GalaxyWeb, [25-27]. Among these, I-TASSER server, which provides a confidence

score (C-score) for model evaluation, was chosen for final modeling based on comparative results. The Galaxy server generated the trimer structure. Model quality was verified through Ramachandran plot analysis and ProSA-web evaluation [28, 29].

Antigenicity prediction

Antigenicity Evaluation of the designed fiber protein was performed using VaxiJen version 3.0, (https://www.ddg-pharmfac.net/vaxijen3/home/) and ANTIGENpro servers. Both tools predict antigenicity based solely on Physicochemical properties of antigens [30].

B-cell epitopes prediction

Computational prediction of B-cell epitopes, both continuous (linear) and discontinuous (structural) was performed using multiple servers to ensure cross-validation. The EliPro server scoring was used to identify linear and structural epitopes based on a score, ranging from 0 to 1 and a cutoff of 0.5, where scores higher than 0.5 were considered antigenic [31]. Additional epitope prediction was conducted using SVMTriP [32] and ABCpred servers. . The ABCpred Prediction Server predict B cell epitopes in a protein sequence [33], using artificial neural network.

MHC class I and II binding prediction

The NetMHCcons 1.1 server was employed to predict MHC class I binding affinities. The threshold for strong binding peptides was determined as an IC50 value of less than 2 nM, and cutoff of 50 nM has been set for weak binders, that is, peptides with IC50< 2 nM were considered as strong binders and those with IC50 < 50 nM as weak binders [34]. Fiber protein sequences (shaft + knob) were submitted to the NetMHCcons 1.1 server. The NetMHCIIpan 4.0 algorithm was employed to identify potential MHC-II binding epitopes. The fiber protein was cleaved into peptides with a length of 15 amino acids in the server. Among these peptides, only those peptides that were strong binders (%Rank < 2) for MHC alleles were selected [35]. In this study, based on previous studies the allele (HLA*B 40:06) was used for MHC class I and the allele (DRB1:1482) was used for MHC class II [36]. Using MHCcluster software, Thomsen et al showed that human MHC-I/II alleles are the best substitute for its homologues in chicken [37].

mRNA structure prediction

To evaluate transcript stability, the RNAfold server was used to analyze the minimum free energy (MFE), which is a loop-based energy model presented by Zucker et al., for the secondary structures formed by RNA molecules [38].

Optimization of the synthetic gene

Codon optimization and reverse translation were conducted using the Java Codon Adaptation Tool (JCat) online software. Optimization parameters were set to maximize the codon adaptation index (CAI) for cloning and expression in *E. coli* K12. [39]. The NEB Cutter Server (New England Biolabs) was used to identify and confirm the presence of common restriction enzyme sites in the nucleotide sequence of the designed construct (http://www.labtools.us/nebcutter-v2-0/). Parameters such, codon adaptation index (CAI), and GC content were also analyzed and codon adaptation index (CAI), and GC content are also evaluated.

Protein expression and purification protocol

Following gene synthesis by ShineGene Co., the fiber protein gene was subcloned into the pET-28a vector (Novagen, 2023) to create recombinant pET-fiber plasmids. This constructs were subsequently transformed into *E. coli* SHuffle strain competent cells [19] using the heat shock method [40]. Transformed colonies were selected through overnight growth at 37°C in LB medium supplemented with 50 µg/mL kanamycin. For protein expression, starter cultures were diluted 1:100 into fresh LB-kanamycin medium and grown at 37°C with shaking until reaching mid-log phase (OD600= 0.4-0.6), typically requiring 3-5 hours. Protein expression was then induced by adding 0.5 mM, IPTG [41], followed by overnight incubation at 17°C with 150 rpm shaking to enhance proper protein folding. After induction, following centrifugation at 5,000 ×g for 10 min, cell pellets were collected and resuspended in lysis buffer for subsequent protein extraction. Initial expression analysis was performed by resolving whole cell lysates on 12.5%, SDS-PAGE gels. The recombinant protein was subsequently purified under native conditions using Ni-NTA Sepharose Fast Flow resin (ARG Biotech, Iran) to isolate the His-tagged fusion protein."

Thermal stability of the recombinant protein

Thermal stability analysis of the purified recombinant protein was assessed under two storage conditions: 1-incubation at 37°C for 14 days to simulate physiological temperature stress, and 2- refrigeration at 4°C as a stability control. Protein integrity was assessed through 12.5%, SDS-PAGE under both conditions. Antigenic properties were examined using a standardized ELISA protocol with the following steps: 96-well microplates were coated overnight at 4°C with 1 µg/mL of recombinant fiber protein in carbonate-bicarbonate coating buffer (100 mM $NaHCO_3$, pH 9.6). After four washes with PBS, plates were blocked for 1 hour with 1%, bovine serum albumin (BSA) in PBS-T (PBS containing 0.05%, Tween 20). Serial dilutions (1 mg/mL and 2 mg/mL) of chicken immune serum (containing EDSV-specific antibodies from inactivated vaccine immunization) were added and incubated for 1 hour at room temperature. Post-washing with PBS (3×5 min), plates were incubated with horseradish peroxidase-conjugated goat anti-chicken IgG secondary antibody for 60 minutes at 25°C with gentle shaking (Razi Biotech Co. Iran) at two working dilutions (1:5000 and 1:20000 in PBS-T). Tetramethylbenzidine (TMB) substrate (0.01% 3,3',5,5'-Tetramethylbenzidine, 99% Water, 0.1% Hydrogen peroxide, pH 3.3-4.0), which is a common substrate for horseradish peroxidase (HRP) and suitable for enzyme-linked immunosorbent assay (ELISA), was added for color development. Then, this reaction was stopped with 2M, H_2SO_4, and absorbance was measured at 450 nm using a microplate spectrophotometer.

Authors' Contributions

We confirmed that all authors were involved in writing this article. AHC as a supervisor of the Ph.D. candidate, in the conceptualization, and methodology; JN as a Ph.D. candidate, performed the experiment and data collection, analysis and interpretation of results, writing- original draft preparation; AM Managed the preparation of laboratory tools, experimental data collection and analysis.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript..

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Effect of Vasectomy on the Testosterone Levels and Testicular Structures in Bucks for Long Term

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ABSTRACT

This study was conducted on 12 adults local crossbreed Bucks to investigate the long-term effect of the vasectomy on Testosterone levels, sexual efficiency, and testicular structures. The bucks were divided into 4 groups according to the duration after the surgery. Following surgery, testosterone hormonal fluctuations were observed in association with stressed factors such as operation pains. Initially, surgical discomfort increased sexual arousal time during the first week post-operation. As healing progressed, arousal time gradually declined by the fourth week. However, ejaculate volume showed a continuous decline throughout the study. A similar pattern of decline, followed by an absolute lack, is noted in sperm motility in all the bucks by the end of the fourth week. Testosterone levels evaluated by enzyme-linked immunosorbent assay (ELISA), demonstrated fluctuations. Post operation testicular parenchyma sections during the first month indicated injury to seminiferous tubules due to increase in the count of Leydig cells. By the second month tubules had separated and shrunken, accompanied by a decline in Leydig cells. During the 3rd month, the seminiferous tubules disappeared, and the interstitial tissue extended with renewed proliferation of the Leydig cells. However, by the fourth month, the interstitial tissue suffered from mild shrinkage, and Leydig apoptosis were evident. In conclusion, vasectomized bucks demonstrated the suitability to use the males for teasers at the first period. But the libido declined during the second period due to the scrotal pains. Then these pains became chronic and less severe in the third month, which improved the sexual desire.

Keywords

Leydig cells; oxidative stress; Seminiferous tubules; Sertoli cells; Sexual desire; Spermatogonia; Vasectomised buck.

Number of Figures: 4
Number of Tables: 3
Number of References:: 27
Number of Pages: 7

Abbreviations

SPSS: Statistical Package for Social Sciences
M±SE: Standard Error.
ANOVA: Analysis of Variance.
LSD: Least significant difference.

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Introduction

Vasectomy is a common and reliable method of male contraception; however, the impact of the surgical intervention on sexual efficiency and libido disorders is unknown [1]. Testosterone is the hormone responsible for libido and overall sexual behaviour in males [2].

Testosterone hormone effects on the brain by neurohormonal pathway, modulating neurotransmitter activity and influencing several brain functions and behaviors. Moreover, androgen hormones modify different stages in neurogenesis of adult, by induced alterations at neurogenesis. as well as the steroids selectively improve the maintenance of recently generated nerve cells, although they have simple influence on cell proliferation [3].

Testosterone-secreting Leydig cells are derived from peritubular stem cells, which transform into spindle-shaped progenitor cells. Once they proliferate and differentiate into immature cells, their cytoplasm contains numerous droplets with a small amount of Testosterone. Immature Leydig cells eventually differentiate into adult cells, which contain the androgen hormones, mainly the testosterone hormone [4].

So far, the research that highlights the relationship between sexual behaviour and testicular structure in vasectomized bucks for a long term is severely lacking [5]. To narrow this gap, the current study was designed to examine the long-term association among the testosterone levels, sexual efficiency and testicular changes in vasectomized bucks.

Result

Sexual Assessment

Sexual behavior evaluation revealed that sexual arousal time increased during the first week post-operation in all bucks. This was followed by a gradual decline until the fourth week, when the values approached the level prior to operation, returning to nearly normal level. Conversely, ejaculate volume showed a decline until the last weeks of evaluation. In all the vasectomised males, sperm was absent in the ejaculate samples, as a result of sperms retention at epididymis. Microscopic examination showed a reduction in the percentage of sperm motility, eventually sperm motility had disappeared by the third and fourth periods in all bucks, as summarized in Table 1.

The different superscript letters (a, b, c, d) within columns show statistically significant differences ($p < 0.05$).

Testosterone Assessment

Testosterone levels appeared to be elevated during the first month post vasectomy, compared with the control and zero period (pre-vasectomy) values. However, in the second month testosterone levels significantly dropped ($p < 0.05$), in contrast to the control group. By the third month, levels elevate again, although the increase was not significant. In the fourth month, testosterone levels decreased once more, showing a significant difference ($p < 0.05$) compared with the zero period, as summarized in Table 2.

Table 1.
The sexual assessment appeared weeks prior and post vasectomy operations.

Week	Desire time / minute	Ejaculate volume/ ml	Individual motility %
Zero	2.25 ± 0.5^{a}	0.752 ± 0.1^{a}	5.00 ± 2.04^{a}
1st	9.24 ± 0.5^{c}	0.31 ± 0.09^{b}	36.25 ± 3.4^{b}
2nd	4.42 ± 0.4^{b}	0.25 ± 0.08^{b}	1.55 ± 1.2^{c}
3rd	3.35 ± 0.6^{a}	0.23 ± 0.07^{b}	0.00^{d}
4th	2.2 ± 0.2^{a}	0.21 ± 0.07^{b}	0.00^{d}

The different superscript letters (a, b, c, d) within columns show statistically significant differences ($p < 0.05$).

The different superscript letters (a, b) within the row show statistically significant differences ($p < 0.05$).

Histopathological Assessment

Histological evaluation of vasectomised tests at the first month post-operation revealed degradation of the seminiferous epithelium with apoptosis among spermatocytes, spermatids and Sertoli cells. A small portion of the spermatogonia of tubules were observed separating from the germinal layer and migrating toward the lumen. On the other hand, the interstitial tissue size increased, likely due to the proliferation of Leydig cells, at the expense of shrinking seminiferous tubules (Figure 1).

By the second month, all seminiferous tubules appeared shrunken and separated from the interstitial tissue toward the central lumen, with nearly all their cells undergoing apoptosis. The interstitial tissue size and Leydig cells population also decreased, resulting in prominent empty spaces between degenerated tubules (Figure 2).

In the third month, the pattern shifted again. Due to the proliferation of Leydig cells, the interstitial tissue completely disappeared from the seminiferous tubules, and the interstitial tissue formed distinct islands surrounded by the empty spaces (Figure 3).

During the fourth month, the histopathological features were similar to the third month, but with

Table 2.
. The effects of testosterone levels at prior and post vasectomy operations.

Group/Month	Zero	1st	2nd	3rd	4th	LSD
Testosterone level (pg/ml)	180.2 ± 20.13[a]	186.03 ± 19.28[a]	149.27 ± 15.5[b]	187.5 ± 23.6[a]	143.77 ± 13.77[b]	169.4 ± 8.63

The different superscript letters (a, b) within the row show statistically significant differences ($p < 0.05$).

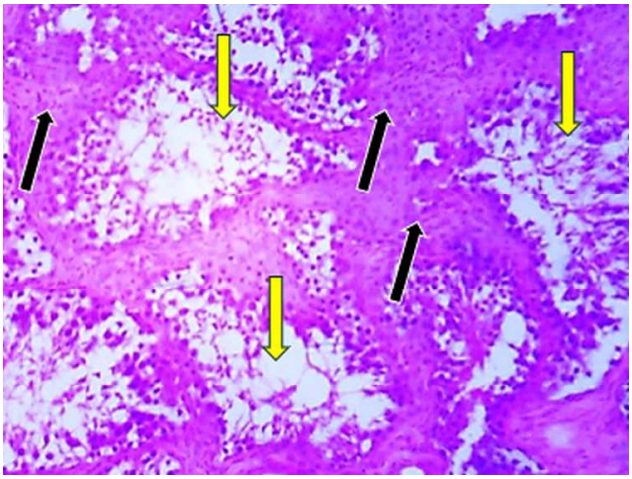

Figure 1.
Parenchymal tests at first month post vasectomy showed destruction of the seminiferous epithelium and their cells suffering from apoptosis, and tubules are initiated to separate from the germinal layer and pushed toward their lumen (yellow arrows). The interstitial tissue size increased, with proliferation of Leydig cells (black arrows).

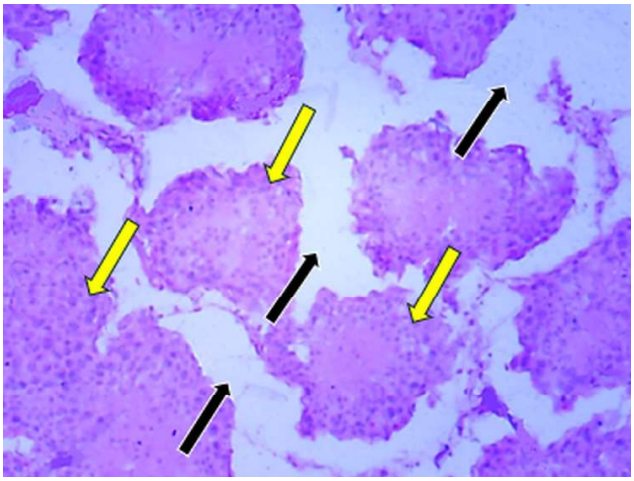

Figure 3.
Parenchymal tests at third month post vasectomy expanded the interstitial tissue with proliferation of Leydig cells (yellow arrows), also disappeared the seminiferous tubules features completely (black arrows).

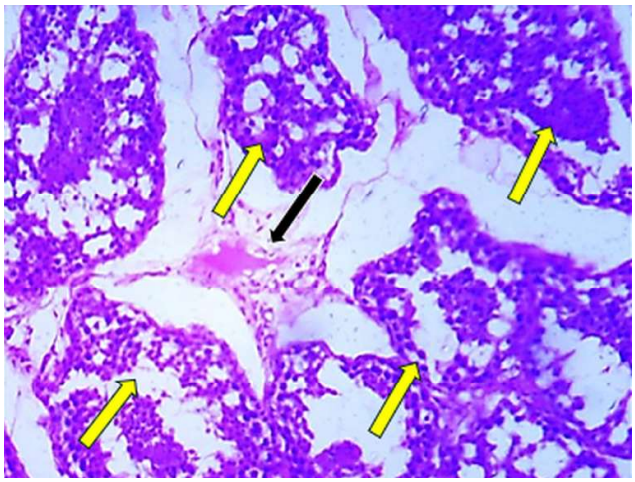

Figure 2.
. Parenchymal tests at second month post vasectomy appeared the seminiferous tubules were separated and shrank toward the central lumen, and all their cells suffering from apoptosis (yellow arrows), with decreased the interstitial tissue size and Leydig cells account (black arrow).

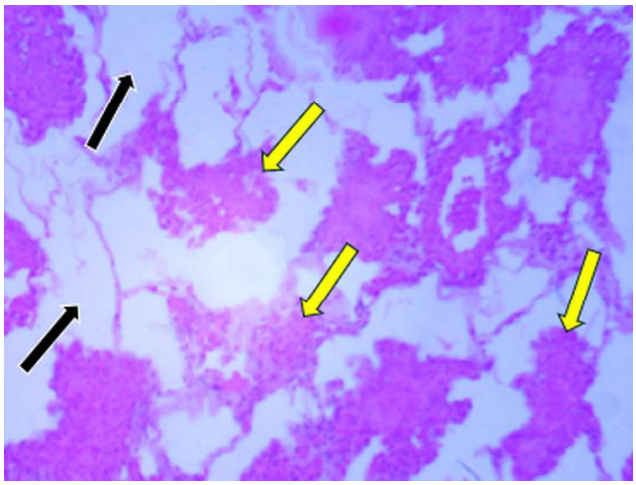

Figure 4.
Parenchymal tests at fourth month post vasectomy expanded mild shrinkage of the interstitial tissue and some of Leydig cells having undergone apoptosis (yellow arrows), with extended the empty spaces among the islands of interstitial tissues (black arrows).

minor differences. Slight shrinkage of the interstitial tissue and mild apoptosis among Leydig cells were observed, accompanied by more extensive empty spaces surrounding the islands of interstitial tissues (Figure 4).

Leydig cell counts was recorded. The average number of Leydig cells among three seminiferous tubules was 45.39 ± 1.4 in the first month. This number decreased to 36.25 ± 0.8 in the second month, then returned to 47.33 ± 1.3 in the third month. By the fourth month, Leydig cell numbers decreased significantly ($p < 0.05$) compared with the first month, as shown in Table 3.

The different superscript letters (a, b) within the row show statistically significant differences ($p < 0.05$).

Table 3.
The leydig cells count at post vasectomy operations

Group/Month	1st	2nd	3rd	4th	LSD
Leydig No.	45.39 ± 1.4[a]	36.25 ± 0.8[b]	47.33 ± 1.3[a]	33.03 ± 1.3[b]	40.5 ± 0.3

The different superscript letters (a, b) within the row show statistically significant differences (p <0.05).

Discussion

The results of this study demonstrate that, sexual arousal time in bucks decreases during the first week following vasectomy, most likely due to postoperative pain associated with the surgical procedure . The arousal time gradually decreased in the later weeks, until it returned to normal levels (similar to the pre-operation levels) by the fourth week. Similar observations were reported by several researchers, such as [9]. As a result [10, 11] and [12], who stated that pain may persist for up to one week post operation, before gradually decreases in later weeks. Therefore, the vasectomised bucks should be given resting period of 2-3 weeks before being used as teasers. This period allows complete recovery from stress and psychological factors associated with surgical operation such as anesthesia administration, incision, dissection of sensitive tissue and the pain that can continue for several days after the operation [13].

The ejaculate volume showed a significant ($p<0.05$) decline beginning in the first week post-vasectomy operation until the fourth week, in contrast to its value prior operation. This reduction can be attributed to the occlusion of the reproductive tract, which prevents the testicular secretions, although a small amount of ejaculate remained likely originated from the accessory sex glands outside the testicles. This confirms the success of this surgical technique in inducing infertility [14, 15].

Microscopic examination of sperm individual motility of semen revealed a decrease of sperm percentage during the few weeks after operation, ultimately reaching complete stop and disappearance by the third and fourth weeks. This loss of sperm motility is a direct consequence of the sperm transport disruption. This data was also provided by [13].

Post vasectomy, the levels of testosterone in bucks were estimated and were found to be at zero group level (prior vasectomy); the vasectomy did not influence the secreted testosterone and sexual desire during the first month post vasectomy as a result of unaffected of interstitial cells and tissue by occluded passages [14]. However, by the second month, a notable decline in testosterone hormone was observed. This reduction may be explained by the scrotal pain resulting from semen accumulation within the epididymal duct and its enlargement [16].

This condition is similar to the chronic post-vasectomy pain syndrome described in human, which can be treated in this state by suppuration of the scrotum and administration of non-steroidal anti-inflammatory drugs (NSAIDs) as the first line of treatment. In the event of the treatment failure, caudal epididymectomy may be required [17,18]. By the third month post-vasectomy, the hormonal concentration rebounded to the higher levels, likely due to two factors: first, the reduction of severe scrotal pain with the transformation of acute scrotal pain into chronic scrotal pain, resulting from rupture of the epididymal and vas deferens ducts proximal to the ligature site, and exudation of semen into the surrounding tissue and formation of the sperm granulomas [19,20,21]. Second, the proliferation of Leydig cells within the expanded interstitial tissue, which secretes testosterone at the expense of the degraded seminiferous tubules [15,22]. However, during the fourth month, testosterone values declined as interstitial tissue shrank and a reduction of Leydig cell quantities, which are endocrine units of the testosterone hormone [23].

Parenchymal tests in the first month post vasectomy showed extensive destruction of the seminiferous epithelium and its cells (Spermatogenesis and Sertoli cells) undergoing apoptosis. These structures were separated from the germinal layer and pushed toward their lumen, caused due to the elevation of intraluminal pressure be-

cause of the vas deferens ligature. Although the interstitial tissue increased in size, and has shown proliferation of Leydig cells [24]. By the second month, the tubules were separated and shrank toward the central lumen, nearly all cells were apoptotic [22]. Decreased interstitial tissue size and Leydig cells accounted for the long-term vasectomy impact on the modulation of macrophage pathways, which regulate testicular immuno-endocrine functions [25]. During the third month, the number of Leydig cells increased again, similar to levels observed in the first month due to expansion of interstitial tissue and proliferation of Leydig cells. However, by the fourth month, interstitial tissue size retracted, and Leydig cell populations reduced, rendering the structures useless and exposed these cells for apoptosis [26,27].

In conclusion, the findings from this study demonstrated the suitability of using the males for teasers only during the first month. Libido declined during the second month, due to scrotal pains; thus, animals at this stage should be rested or considered for caudal epididymis removal surgery. These pains decreased during the third month to transform from the acute to chronic pain, which improved the sexual desire. By the fourth month, libido weakened again, likely due to Leydig cell degeneration and corresponding with low levels of testosterone.

Materials and Methods

Ethical Approval

All experimental procedures were conducted at the Faculty of Veterinary Medicine farm, University of Kufa, under the care and supervision of a veterinarian. Also the study was approved by the Scientific Ethic Committee, Faculty of Veterinary Medicine, University of Kufa (Approval No: 2117).

Experimental Animals

The study was conducted on 12 adult local cross breed Bucks aged between 1-2 years, and weighting 30-45 kg. The animals were acclimatized and observed at the Faculty of Veterinary Medicine, University of Kufa, from February 2023 to June 2023. All bucks were maintained under uniform nutritional conditions throughout the study. The feeding regimen consisted of 25% concentrated food (50% corn, barley, with 50% bran, soybean and supplement) and 75% hay, with free access to salt blocks and water, since 2 weeks before study until experimental end. All Bucks applied for Vasectomy operation were divided into 4 groups based on the period of post-operation: the first group continuing for one-month post-vasectomy, the second group for two months, the third group for three months and the fourth group until four months. The experimental timeline extended March 2023 to June 2023.

Vasectomy Procedure

The scrotal coats of each buck was clipped, shaved, and cleansed with soap followed by a 70% alcohol antiseptic solution. Sedation was achieved with Xylazine 2% (Alfasan, Woerden, Holland) administered intramuscularly at 0.2 mg/kg body weight (B.W). Local infiltration anesthesia was provided using lidocaine 2% (Rotexmedica, Trittau, Germany) at a dose of 4 mg/Kg B.W administered subcutaneously. The caudal aspect of the para-median scrotal skin was incised about 2 cm at the scrotal neck level on both sides. Also, the underlying tunica vaginalis was incised. The ductus deferentes were elevated with curved forceps and were double ligated by absorbable synthetic suture material (#0), spaced 1 cm apart, and 0.5 cm segment between the ligatures were excised. The ligated ends were returned to their ligature ends. The incised skin was sutured by a simple interrupted suture pattern using non-absorbable synthetic suture material (#1) monofilament polyamide. Another ductus difference was made in a similar manner, following the method described by [5].

Sexual Assessment

Ejaculate were collected from each buck using artificial vagina into a warmed glass tube at 37°C prior to vasectomy to evaluate the sexual efficacy. Sexual arousal time was measured from the duration of exposure of the female to the adult male until the mating moment and is recorded in minutes. Ejaculate volume was documented using a graduated glass tube (ml). Sperm motility was evaluated on the warmed slide (37°C) under electronic microscope (×400). The percentage of progressive sperm movement, was estimated visually on a 0-100% scale [6]. These investigations were performed on each Buck before the operation as a normal grade (considered the 0 or control period), and subsequently at 4, 7, 10, 14, and 21 days post-operation.

Testosterone Assessment

Blood samples (5 ml) were collected using from the jugular vein of each buck in the morning hours. Samples were placed in sterile transport tubes to isolate serum, and kept at -20 °C until analysis. Serum testosterone levels was measured using an ELISA kits (Goat Testosterone ELISA Kit "HSD17B3 elisa kit" from MyBioSource.com Germany) with Infitek Mpr-H200bc Elisa Machine Microplate Reader with 96/48 -Well Plate [7]. Blood samples were collected at (0) period as a normal grade (control) prior to operation, and at 1, 2, 3, and 4 months post-operation.

Histopathological Assessment

For histological evaluation, testicular biopsies (1cm3) were obtained after castration at 1, 2, 3 and 4 months post-operation, for 1, 2, 3 and 4 groups, respectively. Biopsies were fixed in 10% neutral buffered formalin for 72 hours, dehydrated, cleared in xylene and embedded in paraffin wax using routine methods. Sections of 5–6 µm thickness were cut, de-waxed, cleared in xylene, rehydrated and stained with Hematoxylin and Eosin for light microscope examination. Leydig cells were counted following the method described by [8], the number of Leydig cells among the three seminiferous tubules. A total of 20 areas for each section (slide) were examined, and these areas were distributed four in each corner and the last four in the centre of the slide.

Statistical Analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, version 25). All data were expressed as Mean ± Standard Error (M ± SE), and differences

among the groups of animals were compared using one-way Analysis of Variance (ANOVA). The least significant difference (LSD) was used to significantly compare between means for simplest and limited the samples. The level $P < 0.05$ is considered to be significant.

Authors' Contributions

Hussein K and Dhurgham H participated in the study of conception and design. Hussein K: Acquisition of data. Dhurgham H: Analysis and interpretation of data. Hussein K: Drafted of the manuscript. Both authors critically revised the manuscript for important intellectual content and approved the final manuscript.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript..

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RESEARCH ARTICLE

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The parasitic *isopod Anilocra physodes* (Cymothoidae; Crustacea) on its fish host from the Atlantic coast of Morocco

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ABSTRACT

This study investigated the parasitism of three species of fish species, Pagellus bogaraveo, (family Sparidae), Sardina pilchardus (family Clupeidae) and Umbrina canariensis (family Sciaenidae), collected from the Atlantic coast of Morocco. A total of 5419 fish specimens were examined. Among them, 3600 fish individuals of Sardina pilchardus, 920 of Umbrina canariensis and 899 of Pagellus bogaraveo, were found to carry 328 isopod parasites of the species Anilocra physodes (family Cymothoidae). Most of these ectoparasitic isopods were primarily located on the body surface or within the gill chambers of their hosts. Parasitism was quantified using various parasitological indices (prevalence, intensity which is the mean number of parasites per infected host and abundance) calculated for each fish species. The results showed that infestation rates by A. physodes were relatively low and did not exceed prevalence of 11.11%. The mean infestation intensity is ranged between 1 and 2 parasites per infected fish, while, abundance values remain below 0.14 parasites per examined fish across all host species. These findings indicate that infestation levels are strongly associated with the host species. Overall, this study provides valuable insights into the effects of this ectoparasite crustacea on fish health, emphasizing the need for fisheries management strategies and marine conservation in the region.

Keywords

Cymothoid isopods; ectoparasites; Anilocra physodes; parasitic indices; parasitism;

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Number of Pages: 9

Abbreviations

A. physodes: Anilocra physodes

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Introduction

Morocco benefits from having two maritime coastlines of approximately 3500km, including more than 500km along the Mediterranean and nearly 3,000km along the Atlantic Ocean. Cymothoid isopods belong to a group of highly host-specific parasites known to infect wide range of fish species worldwide, mainly in marine waters but also in freshwater [1,2]. There are 43 established [2]. Members are hematophagous protandrous hermaphrodites [3], their life cycle involves only one host (holoxenic cycle) [4]. These parasites can attach externally to the body surface, envelop in a pouch, or burrow inside the flesh, buccal cavity, or gill chamber [5,6]. They parasitize numerous species across various fish families, leading to substantial economic losses to fisheries and aquaculture industry [7]. These isopods cause various levels of damage to their hosts, ranging from minor tissue damage at the binding site to differential mortality [8]. Like most isopods, cymothoids are considered to feed principally on host blood, but they may also consume subcutaneous tissues, mucus, and epithelial tissues [4]. Infested fish often display symptoms such as slow growth rates, tissue damage, anemia and ultimately death [7]. Secondary bacterial or fungal inflammation and contamination have often been found around wounds, inspired by these ectoparasites, and may further facilitate the infection of other pathogenic microorganisms [9,10]. In some field studies, isopod-infested fish have been found to suffer from decreased condition index and growth [11,12], altered reproductive capacity and shortened lifespan [12]. However, cymothoid infestation cannot be generalized and standardized treatments cannot be performed for all fish, due to the biological variability of the host and parasites including differences in size, age, life stage, sex and behavior [1,13]. Generally, studies carried out in the field of parasitology are always based on parasitological indices. These indices give us a clear idea about the state of infestation in the studied populations and even provide information on the degree of parasite-host affinity (high infestation rates would explain the affinity of a parasite to its host).

Little information is known about the fish infestation status by Anilocra physodes, their distribution, host specificity, and ecological impacts along the Atlantic coast of Morocco. This region supports diverse fisheries critical to local economies, yet no comprehensive studies have assessed how A. physodes infestations affect commercially important fish species.

In this study, the main objectives were, to study the infestation rate, and characterize the prevalence and parasitism intensity of Anilocra physodes (Cymothoidae; Isopoda) on fish species hosted on the coasts of Morocco. This research will provide valuable insights into the prevalence and intensity A. physodes in Moroccan waters and contribute to the development of sustainable management strategies to mitigate parasite impacts on fish populations.

Although Anilocra physodes is a common and widely distributed species, many aspects of its ecology and biology, such as its impact on specific host traits, environmental conditions on the parasite dynamics and diversity of this ectoparasite have rarely been studied.

Result

Examination of the gills and external surfaces of the collected fish revealed a total of 328 parasites belonging to the family Cymothoidae, identified as Anilocra physodes (Figure 1). Among these, 38 individuals were found on Umbrina canariensis, 44 individuals on Pagellus bogaraveo and a high number, 246 individuals, on Sardina pilchardus. The evaluation of parasitological indices revealed that infestation rates by Anilocra physodes were generally low, with a maximum prevalence of 11.11% (Figure 2). The mean intensity of infestation was also very low, ranged between 1 and 2 parasites per infested fish, while the abundance values remained below 0.14 parasites per examined fish across all the host species.

1. Enumeration of collected parasites

The results revealed that the highest number of parasites was recorded in Sardina pilchardus during November on the South Atlantic coast, with 21 parasites collected. Conversely, the lowest number (3 individuals) was recorded during March on the North Atlantic coast. For Umbrina canariensis infestations were observed in January, June and July on the North Atlantic coast, with three ectoparasites collected during each of these months. In the case of Pagellus bogaraveo, infestations occurred in October on the South Atlantic coast, with six ectoparasites collected. Overall, the number of parasites collected from Sardina pilchardus was higher on the North Atlantic coast compared to the South Atlantic coast. The opposite trend was observed for Pagellus bogaraveo, which exhibited higher infestation levels on the South Atlantic coast compared to the North Atlantic coast. For Umbrina canariensis, infestation levels were similar between the two study areas.

2. Distribution of parasitological indices

2.1. By host species

The host-parasite relationship constitutes a biological entity that is expressed by parasite specificity

Figure 1.
Anilocra physodes (Linnaeus, 1758) were collected in this study. (a) Female. (b) Male. (c) Immature stage 3. (d) Gravid female, lateral view. (e) Male, lateral view. (f) Immature stage 3, lateral view. (g) Non-gravid female, lateral view

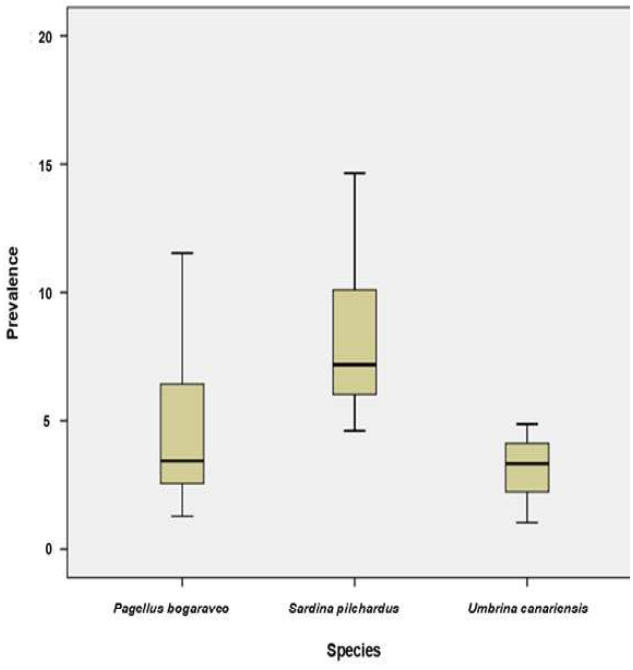

Figure 2.
Prevalence of parasite effects on fish species. The samples were carried out for 12 months: the data were recorded for a total of 3 samples per species per location, repeated 3 times. The boxplot represents averages and error lines of one standard deviation *** indicates the significant differences at $p < 0.01$ based on the SNK test.

[14]. According to Lumbery [15], specificity refers to the number of host species a parasite can infect and serves as a measure of the strength of the relationship between a parasite and its host. Data analysis revealed that infestation rates were globally low, with prevalence values not exceeding 11.11%. The highest prevalence was observed in Sardina pilchardus and Pagellus bogaraveo (p = 11.11%) followed by Umbrina canariensis (p = 8.33%) (Figure 2). In terms of parasitic load, Sardina pilchardus exhibited the highest mean infestation intensity. On the other hand, the abundance values do not exceed 0.14 parasites/fish examined in Sardina pilchardus and remain below 0.13 parasites/fish examined in the other species.

2.2. According to geographical distribution

On the southern Atlantic coast, Sardina pilchardus and Pagellus bogaraveo exhibited the highest infestation rates by Anilocra Physodes (p = 11.11%) during November, January and August, respectively. In contrast, Umbrina canariensis, showed its highest prevalence values on the Southern Atlantic coast as well (p = 9.09%) during July. Regarding mean intensity, results indicated that parasite load values (ranging from 1 to 2 parasites per infested fish), and abundance were very low and slightly higher the northern Atlantic coast compare to the southern Atlantic coast during the study period.

Discussion

The present study aimed to identify the parasitism intensity of Anilocra Physodes affecting three species of fish species, Pagellus bogaraveo, (family Sparidae), Sardina pilchardus (family Clupeidae) and Umbrina canariensis (family Sciaenidae) through the infestation rate, the prevalence and the mean infestation intensity.

This study highlights significant variations in the infestation rates across different host fish species, with Sardina pilchardus supporting the highest infestation, followed by Pagellus bogaraveo and Umbrina canariensis.

In general, isopods parasitize a wide range of fish species worldwide, causing significant economic losses [16]. The species Anilocra physodes has been reported in the Atlantic Ocean, Mediterranean Sea, Black Sea and Adriatic Sea [17–22]. This euryxene parasite has a wide host range. In Tunisia, infestations are predominantly recorded in the Sparidae family (D. annularis, S. cantharus, Dentex vulgaris, Pagrus auriga, B. boops) and in the Pomatomidae (Pomatomus saltator) [23,24]. In France, it has also been observed mainly on Sparidae and the Maenidae [19].

Infestations by cymothoid isopods such as Anilocra physodes can have significant biological and ecological consequences for host fish, affecting growth, reproduction, and overall health. As an ectoparasite, A. physodes attaches to the body surface of fish, often on the head or flank, and feeds on blood and tissue fluids, leading to chronic stress and energy depletion in the host [4]. Sasal et al., [25], reported that Acanthocephaloides propinquus, a parasite of Marine fish, have negatively effects on females reproduction level by reducing the gonadosomatic index and egg production. Similarly, Fogelman [26] suggest that Anilocra apogonae negatively affects host growth and reproduction. Ecologically, such impacts on individual fish can translate into population-level consequences, especially in heavily infested populations, potentially altering community structure and predator-prey dynamics. Therefore, A. physodes infestations represent not only a parasitic burden to individual fish but also a potential threat to the health and sustainability of fish populations in natural ecosystems.

Our study suggest a relatively high degree of host specificity for A. physodes along the Moroccan Atlantic coast. The parasite was recorded on three fish species belonging to different families: Pagellus bogaraveo (Sparidae), Sardina pilchardus (Clupeidae), and Umbrina canariensis (Sciaenidae). Brusca [27] noted that some species of Cymothoidae prefer a host based on their ecological characteristics than on taxonomic identity.

In the present study, Sardina pilchardus hosted the largest number of parasite (N = 246), while Pagellus bogaraveo harbored only 44 parasite and Umbrina canariensis harbored only 38. According to Zender and Kesting [28], the eutrophic state of an environment can increases the parasite infestation level. However, the prevalence of infested fish can vary over time and space [1]. In our study, we found high parasitization of Anilocra physodes on Sardina pilchardus, followed by Pagellus bogaraveo, and Umbrina canariensis respectively (Figure 2). The statistically analy-

Table 1.
Impact of ectoparasite Anilocra physodes on host species shown by one-way ANOVA test. Significant values are highlighted in bold: P < 0.05; **$p < 0.01$; ***$p < 0.001$.

		Sum of squares	Average of squares	F	Signification
Prevalence	Inter- groups	228,043	45,609	4,432	·001***
	Intra-groups	2160,979	10,290		
	Total	2389,022			
Intensity	Inter- groups	·037	,007	5,456	,000***
	Intra-groups	,286	,001		
	Total	,323			

sis revealed that the prevalence of parasite species was significantly greater within the intergroups (F = 4.432 and p = 0.001), and the intensity also differed significantly (F = 5.456 and p = 0.0001, Table 1). In Morocco, Dollfus and Trilles [29], provided some figures on the intensity of Cymothoidae and reported values that varied between 1 and 2 parasites per fish, without specifying the number of fish specimens examined. The results obtained from our parasite load assessment are in accordance with those of Dollfus and Trilles [35], which who reported that the intensity of infestation is very low, ranged between 1 and 2 parasites per infested fish. According to Ternengo et al. [30], emphasized that each fish species harbors a characteristic parasitic fauna and unique infestation dynamics. Isopod dispersion and abundance are influenced by many environmental factors such as salinity, predators, water temperature, light intensity, and food availability [31]. In our study, the three host species sampled presented abundance values of less than 0.14 parasites per fish examined. The study of parasitism according to the geographical distribution of the fishes examined confirmed that: in Sardina pilchardus the highest rate of infestation was recorded on the North coast. In contrast it is more important in the South Atlantic in the host species Pagellus bogaraveo. In the host Umbrina canariensis there was an equality between the two study areas. This may be related to and due to anthropogenic pressure, which can affect the immune system of fish hosts and host-parasite interactions. Zharikova [32], reported that environmental pollution, the infestation of fish by parasites decreases and that the emergence of dominant species may reflect adaptive response of the parasite. Results from the multivariate linear model test (Table 2), confirmed that infestation levels of ectoparasite Anilocra physodes were strongly influenced by host species (p < 0.0001). Comparatively, there was no significant association between time and locality (p = 0.121 and I = 0.230) and (p = 0.828 and I = 0.833) respectively. Interaction between time-species locality were also not significant (Table 2). The causes of these variations may be due, to the influence of multiple factors related to biogeography, the environment, ethology, the immune system, the presence of other parasites, genetics, and the age of the host [33]. The results of the distribution of parasite indices of Anilocra physodes across host species show an inequality in the face of parasitism toward the latter; they vary from one host to another and from one marine area to another along the Moroccan coasts.

Table 2.
Results of the multivariate approach linear model test comparing the prevalence and intensity of Anilocra physodes in the three fish species according to factor month, species and locality. Significant values are highlighted in bold, p < 0.05; **p < 0.01; ***p < 0.001.

Source of variation	Dependent variable	F value	P value
Month	Prevalence	1,546	,121
	Intensity	1,301	,230
Species	Prevalence	11,598	,000***
	Intensity	14,254	,000***
Locality	Prevalence	,047	,828
	Intensity	,045	,833
Month * Species	Prevalence	,917	,574
	Intensity	1,153	,300
Month * Locality	Prevalence	1,101	,365
	Intensity	1,348	,204
Species * Locality	Prevalence	,047	,954
	Intensity	,551	,577
Month * Species * Locality	Prevalence	1,267	,204
	Intensity	1,354	,147

Materials and Methods

Study site

The present study was conducted in 2022 along the Atlantic coast of Morocco. The study area is subdivided into two zones, as follows: zone 1, the Moroccan North Atlantic coast from Tanger to North West to Essaouira, and zone 2: from Agadir to South West to Dakhla corresponds to the Moroccan South Atlantic coast (Figure 3).

Fish collection

A total of 5419 marine fish specimens belonging to three fish species infested by Anilocra physodes (Linnaeus, 1758) (Figure 1) were examined. The species composition included Sardina pilchardus (3600 individuals), Umbrina canariensis (920 individu-

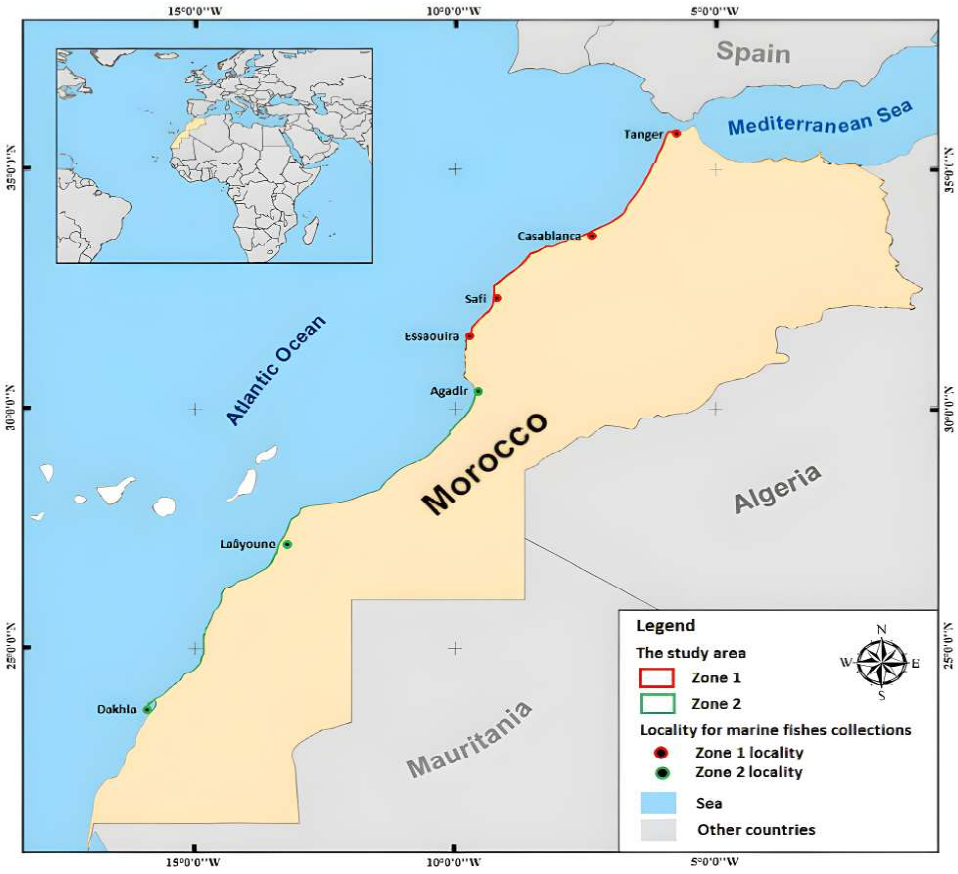

Figure 3.
Map showing the sampling areas. The study area is subdivided into too zones, which are, zone 1: the Moroccan North Atlantic coast from Tangier to Essaouira (indicated by red circles), and zone 2: the Moroccan South Atlantic coast from Agadir to Dakhla.

als) and Pagellus bogaraveo (899 individuals). Fish samples were obtained with the aid of fishers from commercial catches and individual fishermen from January 2022 to December 2022. This one-year sampling period allowed for the evaluation of any seasonal variations in infestation prevalence. Sampling sites were selected based on the previous frequent records indicating isopod infestations reported in these areas. Samplings were performed twice a month with three replicate samples per site, in order to enhance temporal resolution and statistical reliability. The prevalence (number of infested hosts/total number of examined hosts ×100), mean intensity (total number of parasites collected/total number of infested hosts), and abundance (total number of parasites collected/ total number of examined hosts) of the ectoparasite during the entire study period (12 months), were calculated according to Margolis et al. [34] and Bush et al. [35]. These parameters define the level of parasitic infestation.

Treatment and identification of parasites

After collection, isopod parasites were detached from the their host fish and immediately fixed in 70% ethanol. The sampling locality, date and host fish were noted. In the laboratory, specimens were identified, using stereoscopic microscopes in collaboration with specialists and reference materials. Species identification followed standard taxonomic keys and descriptions from previous works [19,36–38]. Host nomenclature and fish taxonomy are performed according to the guide of identification of the marine resources of Morocco [39], and the FishBase database [40–42]. All laboratory analyses were conducted at the Laboratory of Agricultural Production Improvement, Biotechnology and Environment, Faculty of Sciences, Mohammed First University.

Statistical analyses

All raw data obtained from field and laboratory examinations were entered into Microsoft Excel. The analyses were performed in triplicate with the corresponding standard deviations of our results for each month (for each parameter: 3 determinations × 3 samples = 9). One-way ANOVA and Tukey's test revealed significant differences between the sources of variation. Significant differences between the 12-month infestation results were determined via the t-student test. The threshold for a significant difference was set at 5% threshold ($p < 0.05$). All statistical analyses were analyzed using IBM SPSS Statistics (version 21 software program).

Conclusions

This study highlights the significant impact of the parasitic isopod Anilocra physodes on fish host from the Atlantic coast, Morocco. The results demonstrate that the prevalence and intensity of infestation vary notably among host species and geographic localities. Infestation levels were found to be associated with host fish species studied, suggesting that probably intrinsic characteristics of each host species play a major role in determining susceptibility to parasitism.

Among the examined species, Sardina pilchardus exhibited the highest infestation rate by A. physodes followed by Pagellus bogaraveo and Umbrina canariensis. However, overall infestation rates were relatively low, with prevalence not exceeding 11.11%. The mean intensity of infestation, show a very low average infestation intensity, ranging between 1 and 2 parasites per infested fish, while, abundance values remain below 0.14 parasites per examined fish across all host species. The data obtained in this study contribute valuable insight into the parasitic infections dynamics of Anilocra physodes on the Moroccan coast in particular and worldwide in general.

These findings stress the need for further studies on defense mechanisms in host species, environmental influences on parasitism and better understand the ecology and biology of this ectoparasite to improve fisheries management strategies and marine conservation.

Authors' Contributions

M.B., A.H., Y.M., K.E.B., K.C conceived and planned the experiments. M.B, A.H., Y.M., K.E.B. and K.C carried out the experiments. M.B. contributed to sample preparation. M.B., and A.H. contributed to the interpretation of the results. M.B. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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Managmental Studies of Different Liquid Feeding Regimes for Nili-Ravi Buffalo Calves

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ABSTRACT

A liquid feeding trial was carried out at the Dairy Animals Training and Research Centre, UVAS Ravi Campus, Pattoki, to explore effective methods for rearing Nili-Ravi buffalo calves using alternative feeding sources. Twenty-four (n=24) buffalo calves were divided into three groups, with eight calves in each group, and fed for 90 days. The feeding regimes included Whole Milk (WM), Milk Replacer-Vegetable Protein (MR-VP), and Milk Replacer-Milk Protein (MR-MP), following a Completely Randomized Design. Data on daily dry matter intake, weekly weight gain, feed efficiency, fecal score and linear body measurements were collected and analyzed. Mean daily dry matter intake (DMI) was significantly ($p < 0.001$) higher (991.89 ± 331.41gm) in calves fed WM and lowest in calves fed MR-VP (833.07 ± 297.64 gm). Mean daily weight gain for WM, MR-VP and MR-MP groups was 227.18 ± 88.04, 135.34 ± 47.17, 189.72 ± 86.99gm, respectively. Feed efficiency was highest (0.23 ± 0.08) in calves fed MR-VP, compared to calves raised on WM and MR-MP. Daily fecal score were 1.75 ± 0.10, 1.70 ± 0.16 and 1.58 ±0.16 for MR-VP, MR-MP, and WM groups, respectively, showing no significant difference among treatments ($p > 0.05$). Body measurement (height at withers, body length and heart girth) differed significantly among treatments ($p < 0.05$). The findings suggest the potential of milk replacer–vegetable protein source as a cost effective alternate for whole milk in resource limited systems.

Keywords

Dry matter intake, linear body measurements, milk replacer, Nili-Ravi buffalo calves, weight gain, whole milk

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Number of Tables: 2
Number of References:: 20
Number of Pages: 5

Abbreviations

CP: Crude Protein
WM: whole milk
MR-VP: Milk replacer with vegetable protein
MR-MP: Milk replacer with milk protein
DM: Dry Matter
DMI: Dry Matter Intake

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The livestock sub-sector is a crucial component of Pakistan's economy, providing essential animal-based products such as milk, meat, and eggs. Among livestock species, the buffalo, often referred to as the "Black Gold of Pakistan," plays a critical role in milk and meat production. In 2023–2024, Pakistan's buffalo population was estimated at 46.3 million, producing approximately 41.9 million tons of milk annually, of which only 5% is used for nourishing calves [1]. Modern dairy farming employs a variety of feeding and management practices, including milk replacers, calf starters, and concentrate mixtures, to optimize general productivity [2,3].

Buffaloes are renowned for their naturally high milk fat content (6-8.5%), and nearly 99% of the global buffalo population (205 million) resides in Asia [4]. Buffalo milk due to its higher quality, is preferred over cow milk in South Asia and commands a higher price in the market [5,6].

Economic challenges often prevent the provision of sufficient whole milk to newborn calves. In many cases, calves are slaughtered at an early age to reserve milk for human consumption [7], resulting in high early mortality and poor growth in calves [8]. Given the increasing rising cost and demand for milk, exclusive whole milk feeding is economically impractical. Therefore, employing alternative feeding regimes can enhance both beef production and general development of replacement female calves, addressing these challenges viably [9].

Milk replacer is preferred and desirable when the price of whole milk is high, as it formulated to mimic the nutrient composition and digestibility of natural milk. They are free from pathogens such as salmonella, and can be medicated to enhance calf health. Additionally, they reduce the rate of scour incidence in calves and enhances their nutritional intake by supplementing essential vitamins such as A, D, E, and B complex. Whole milk and milk replacer (20-22% CP and 15-20% fat) are early feeding regimens for dairy calves. Calves raised with a diet containing 20% crude protein (CP) have shown superior weight gain and improved feed efficiency [10].

During the early stages of life, calves depend entirely on milk or similar liquid feeds, gradually transitioning to solid feed. Since newborn calves cannot digest hard solid feeds efficiently, making liquid feeds, such as whole milk or milk replacers, are essential for their early growth, development and nutrition [11].

To address challenges such as slow growth and high early mortality rates in calves, techniques like substituting whole milk with milk replacers and early weaning need to be explored more. These approaches have been broadly implemented in modern dairy systems for breeds such as Holstein Friesian and Jersey cattle [8]. However, similar data or studies focusing on buffalo calves are limited, highlighting the need for further research in this area.

Considering the significance of alternative feeding programs for cost-effective raising of calves, a study was conducted to evaluate the impact of different feeding regimes, including whole milk and milk replacer, on the growth performance of Nili-Ravi buffalo calves. The study focused on key parameters such as weight gain, dry matter intake, body measurements, and fecal scores to evaluate the effectiveness of these feeding strategies in enhancing growth rate and feed efficiency.

The study was conducted at the Dairy Animals Training and Research Centre, UVAS Ravi Campus, Pattoki, to evaluate alternative feeding regimes for Nili-Ravi buffalo calves. Twenty-four (n=24) calves, aged 3-4 days, were randomly assigned to three dietary treatments (n = 8 per group). The groups were raised on either whole milk (WM), milk replacer with vegetable protein (MR-VP), or milk replacer with milk protein (MR-MP), for over a 90-day period. Liquid diets were administered at 10% of each calf's body weight, and calves had free-choice access to calf starter. Data were collected on parameters such as daily dry matter intake, weekly weight gain, feed efficiency, linear body measurements, and fecal scores, with feed intake measured on a dry matter basis.

The average initial body weight of each calf was noted at the start of the experiment. Thereafter, the weight of each calf was weighed at weekly intervals to monitor growth throughout the study period. Feed efficiency was calculated using the weekly feed consumption and weekly weight gain of calves by dividing weight gain (kg) from feed intake (DM basis). Fecal score of each calf was noted on daily basis. Fecal scoring, which shows the digestive health of calves, was evaluated on a scale from 1 to 5. Feces that retained their shape upon falling to the ground were scored as 1, while semi-formed, pasty feces were given a score of 2, loose feces that remained on top of the bedding were scored as 3, watery feces that seeped through the bedding were relegated a score of 4, and watery feces containing blood received a score of 5. Additionally, the body length (cm) of each calf was measured from the pin bone to the point of the shoulder. Heart girth (cm) was measured as the chest circumference behind forelegs. Body length (cm) was taken as length from the withers to the platform surface. Data were analyzed under a Completely Randomized Design (CRD) using Analysis of Variance (ANOVA), and means were compared via the Least Significant Difference (LSD) Test [12].

Mean daily dry matter intake was significantly among treatments ($p < 0.001$). Calves fed WM con-

sumed the most (991.89 ± 331.41gm), while those fed milk replacer containing vegetable protein recorded the lowest intake (833.07 ± 297.64gm) (Table 1). The findings of the present study indicated higher DMI on milk replacer-milk protein source than milk replacer-vegetable source. Conversely, some studies reported no significant difference in DMI in Holstein Friesian calves fed on different sources of milk replacers during pre-weaning period [13,14]. Moallem et al. [15] reported higher DMI in Israeli Holstein heifer calves reared on milk replacer as compared to the whole milk during pre-weaning period, which this finding is in contrast with the results of the present study.

Mean daily weight gain varied significantly among groups ($p < 0.05$). Calves fed WM gained 227.18 ± 88.04 g/day, compared to 135.34 ± 47.17 g/day for MR-VP and 189.72 ± 86.99 g/day for MR-MP (Table 1). Least Significant Difference (LSD) analysis revealed a significant difference between the calves fed WM and MR-VP ($p < 0.05$), but it was non-significant between MR-MP and MR-VP ($p > 0.05$). Some studies also reporting higher weight gain in Sahiwal and Holstein calves fed whole milk compare to calves fed milk replacer [8,16-18].

Feed efficiency for each calf was recorded on weekly basis allocated to different treatments. Feed efficiency was numerically highest (0.23 ± 0.08) in WM (control) followed by MR-MP and MR-VP, but the difference were Statistically non-significant ($p > 0.05$). Donovan et al. [13] reported similar finding and recorded a non-significant difference for feed efficiency in calves raised under different feeding regimes ($p > 0.05$). The findings of Bhatti et al. [8] also consistent with the present study, who reported better feed efficiency in calves raised on whole milk compared to calves raised on milk replacer.

The daily fecal score were 1.58 ± 0.16 in WM, 1.75 ± 0.10 in MR-VP and 1.70 ± 0.16 in MR-MP. Statistically fecal score data indicated a significant difference between the calves on WM and MR diets ($P<0.05$). Hill et al. [19] and Donovan et al. [13] reported no differences in fecal scores of calves on WM and milk replacer diets, which is not in line with the result of the present study.

Calves fed WM had the highest mean height at wither (0.13 ± 0.03 cm), followed by calves fed MR-MP (0.12 ± 0.03 cm) and calves fed MR-VP (0.08 ± 0.03 cm) (Table 2). The BL and heart girth in calves fed on WM, MR-VP and MR-MP were 0.13 ± 0.03, 0.07 ± 0.02 and 0.12±0.03 cm, 0.22 ± 0.06, 0.13 ± 0.06 and 0.20 ± 0.06 cm, respectively. Body measurements regarding height at withers, body length and heart girth indicated a significant difference between WM and MR-VP diets ($p < 0.05$). Similarly, significantly ($p < 0.05$) higher wither height, body length and heart girth were also reported in calves raised on WM compared to calves raised on milk replacer [17,20].

The feeding trial assessed the impact of Whole Milk, Milk Replacer-Vegetable Protein, and Milk Replacer-Milk Protein on the growth performance of Nili-Ravi buffalo calves over a 90-day period. Results showed higher dry matter intake and weight gain in WM-fed calves, while MR-VP demonstrated superior feed efficiency. The study showed promising results, and suggests milk replacers as cost-effective alternatives whole milk in resource limited systems.

Table 1.
Mean (±SD) daily dry matter intake (DMI) weight gain, feed efficiency and fecal score in Nili-Ravi buffalo calves fed on different liquid feeding regimes

Treatments	DMI (gms)	Weight Gain (gms)	Feed Efficiency	Fecal Score
Whole Milk (WM)	991.89 ± 331.41^{a}	227.18 ± 88.04^{a}	0.23 ± 0.08^{a}	1.58 ± 0.16^{b}
Milk Replacer (MR-VP)	833.07 ± 297.64^{c}	135.34 ± 47.17^{b}	0.16 ± 0.05^{a}	1.75 ± 0.10^{a}
Milk Replacer (MR-MP)	884.35 ± 505.45^{b}	189.72 ± 86.99^{ab}	0.21 ± 0.08^{a}	1.70 ± 0.16^{ab}

a,b,c Means with different superscripts within same column are significantly different ($p < 0.05$)

Ethical Statement

The required ethics committee report for the

Table 2.
Means (±SD) of height at wither (HAW), body length (BL) and heart girth (HG) in Nili-Ravi buffalo calves on weekly basis fed on different liquid feeding regimes

Treatments	HAW (cm)	BL (cm)	HG (cm)
Whole Milk (WM)	0.13 ± 0.03^{a}	0.13 ± 0.03^{a}	0.22 ± 0.06^{a}
Milk Replacer (MR-VP)	0.08 ± 0.03^{b}	0.07 ± 0.02^{b}	0.13 ± 0.06^{b}
Milk Replacer (MR-MP)	0.12 ± 0.03^{a}	0.12 ± 0.03^{a}	0.20 ± 0.06^{a}

a,b Means with different superscripts within same column are significant($p < 0.05$).

study was obtained from Dairy Animals Training and Research Centre, UVAS Ravi Campus, Pattoki.

Availability of Data and Materials

The data presented in this study are available on request from the corresponding author Ray Adil Quddus.

Authors' Contributions

RAQ conceived and planned the experiments. RAQ also contributed to conceptualization, methodology and writing-original draft preparation. AYK contributed to validation, writing-review and data curation. MM helped in validation, formating and editing.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript..

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Abstract in Persian

شاخص های رشد، پارامترهای ایمنی غیراختصاصی، پروفایل پروتئین مخاط پوست و مقاومت به قارچ ساپرولگنیا پارازتیکا (*Saprolegnia parasitica*) در ماهی قزل آلای رنگین کمان تغذیه شده با پونه (*Mentha longifolia*)

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چکیده

در مطالعه حاضر، اثر عصاره هیدروالکلی پونه (Mentha longifolia) بر شاخص‌های رشد، پارامترهای خونی، پاسخ‌های ایمنی سرم و الگوی پروتئین موکوس پوست در ماهی قزل‌آلای رنگین‌کمان بررسی شد. ۳۶۰ ماهی با میانگین وزن (۱۰.۱۷ ± ۰.۱۸ گرم) پس از سازگاری با محیط در ۱۲ مخزن (چهار گروه در سه تکرار) با تراکم ۳۰ ماهی در هر تانک نگهداری شدند. عصاره هیدروالکلی پونه به میزان ۱، ۲ و ۳ گرم بر کیلوگرم به جیره پایه اضافه شد. پس از ۶۰ روز غذادهی با جیره حاوی عصاره پونه، ماهی ها در معرض قارچ ساپرولگنیا پارازتیکا (Saprolegnia parasitica) قرار گرفتند. نتایج نشان داد که تمام ماهیان تغذیه شده پونه افزایش قابل توجهی در سرعت رشد ویژه و افزایش وزن و کاهش معنی داری در ضریب تبدیل غذایی در مقایسه با تیمار شاهد نشان دادند. پس از ۶۰ روز تغذیه و ۱۵ روز پس از قرار گرفتن در معرض ساپرولگنیا پارازیتیکا، تعداد گلبول های قرمز و سفید، فعالیت انفجار تنفسی، پروتئین کل و آلبومین به طور معنی داری نسبت به گروه کنترل افزایش یافت. ماهیان تیمارهای تغذیه شده با عصاره پونه، افزایش قابل توجهی در میزان بقا در مقایسه با گروه کنترل پس از چالش با قارچ نشان داند. بر اساس نتایج، استفاده از عصاره هیدروالکلی پونه به میزان ۱ گرم به ازای هر کیلوگرم غذا در ماهی قزل آلای رنگین کمان برای بهبود شاخص های رشد و ایمنی و همچنین افزایش مقاومت ماهی در برابر تهاجم قارچ ساپرولگنیا پارازتیکا توصیه می شود.

واژگان کلیدی

پونه، رشد، ایمنی، پروتئین موکوس، ساپرولگنیا پازتیکا، قزل آلای رنگین کمان

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اثر مکمل ویتامین D بر سطح سرمی آن و پروفایل متابولیسم انرژی در میش های کبوده شیراز

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چکیده

این مطالعه با هدف بررسی اثرات مکمل ویتامین D بر سطح سرمی این ویتامین و پروفایل متابولیک میش‌های نژاد کبوده شیراز در دوره آبستنی انجام شد. ۶۰ میش سالم به سه گروه تقسیم شدند: گروه ۱ (کنترل)، گروه ۲ که در زمان تلقیح ۱۰،۰۰۰ IU ویتامین D به صورت داخل عضلانی دریافت کردند، و گروه ۳ که در میانه دوره آبستنی ۱۰۰۰۰ واحد مکمل ویتامین D دریافت کردند. خونگیری در چهار مرحله انجام گرفت: زمان تلقیح، اواسط آبستنی، اواخر آبستنی و پس از زایمان. سطح ویتامین D در گروه کنترل (گروه ۱) طی آبستنی به‌طور قابل‌توجهی کاهش یافت. در مقابل، چنین کاهشی در دو گروه دریافت کننده مکمل رخ نداد، به نحوی که بالاترین غلظت ویتامین D در اواخر آبستنی و پس از زایمان در گروه ۳ مشاهده شد. از نظر تعادل انرژی، غلظت β-هیدروکسی‌بوتیرات (BHBA) و اسید چرب غیر استریفیه (NEFA) در گروه دریافت کننده مکمل در میانه آبستنی کمترین میزان را نشان داد. علاوه بر این، در گروه ۳، سطح انسولین در مراحل پایانی آبستنی افزایش یافت، در حالی که غلظت کلسیم و فسفر سرم در این گروه در مقایسه با سایر گروه‌ها بالاتر بود و پس از زایمان به حداکثر سطح خود رسید. هر سه گروه افزایش تری‌گلیسرید و کلسترول را تجربه کردند، اما گروه ۳ پس از زایمان بالاترین سطح تری‌گلیسرید را داشت. همچنین، سطح پروتئین سرم و آلبومین در گروه ۳ پس از زایمان به‌طور قابل‌توجهی بالاتر بود که نشان‌دهنده وضعیت تغذیه‌ای بهتر و سنتز پروتئین بود. یافته‌های این مطالعه نشان می‌دهد که تجویز ویتامین D در میانه آبستنی می‌تواند سلامت متابولیک و تعادل انرژی میش‌های کبوده شیراز را بهبود بخشد و پیامدهای مثبتی برای سلامت میش و بره داشته باشد.

واژگان کلیدی

پونه، رشد، ایمنی، پروتئین موکوس، ساپرولگنیا پازتیکا، قزل آلای رنگین کمان

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Abstract in Persian

ارزیابی پروتئین فیبری ویروس سندرم کاهش تخم مرغ به عنوان کاندیدای واکسن: آنالیز بیوانفورماتیکی، بیان، خالص سازی و پایداری آن

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چکیده

ویروس سندرم افت تولید تخم مرغ (EDSV)، یک آدنوویروس پرندگان، که باعث کاهش شدید تولید و کیفیت تخم مرغ در مرغ‌های آلوده می‌شود و در نتیجه تأثیرات اقتصادی قابل توجهی بر بخش طیور دارد. بر اساس مطالعات قبلی، پروتئین فیبر EDSV می‌تواند کاندیدایی برای واکسن زیر واحد علیه EDSV باشد. تحقیق حاضر بر بیان، خالص‌سازی و ارزیابی پایداری حرارتی پروتئین فیبر نوترکیب به عنوان واکسن علیه EDSV تمرکز دارد. در یک رویکرد in silico، ما ساختار پروتئین فیبر را بررسی کردیم و آن را در Escherichia coli (E. coli) به عنوان کاندیدای واکسن بیان کردیم. ما همچنین پایداری حرارتی پروتئین بیان شده را ارزیابی کردیم. پروتئین عمدتاً به صورت پروتئین‌های تریمری محلول در E. coli بیان شد و سطح بیان پس از خالص‌سازی با میل ترکیبی نیکل ۱۵ میلی‌گرم در لیتر بود. تجزیه و تحلیل ساختاری با استفاده از ابزارهای ایمونولوژیکی و بیوانفورماتیک نشان داد که پروتئین نوترکیب ساختار تریمری طبیعی را حفظ می‌کند. بر اساس ارزیابی پایداری حرارتی بر روی این پروتئین نوترکیب، این پروتئین پایداری حرارتی خوبی را نشان داد که می‌تواند کاندیدای خوبی برای واکسن علیه EDSV باشد.

واژگان کلیدی

پروتئین فیبر، خالص سازی، درون رایانه ای، سندرم افت تولید تخم مرغ، واکسن

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Guide for authors

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Conflict of interest and author agreement form (can be downloaded from the IJVST website)

Guide for Authors

For further information, please contact the Editorial Office:
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PREPARATION OF MANUSCRIPT

Manuscripts submitted to IJVST should neither be published previously nor be under consideration for publication in another journal. The main article types are as follows:

Research Articles should contain Title page, Abstract, Keywords, List of Abbreviations, Introduction, Results, Discussion, Materials and methods, References, and Figure legends. Tables and figures should be appended as individual files.

Review Articles should contain Title page, Abstract, Keywords, List of Abbreviations, Introduction, appropriate sections dependeing to the subject, Conclusions and future directions. Tables and figures should be appended as individual files. The review article should provide an update on recent advances in a particular field. Authors wishing to submit review articles should contact the Editor with an outline of the proposed paper prior to submission.

Case Reports should include Title page, Abstract, Keywords, List of Abbreviations, Introduction, Case Presentation, Results and Discussion, and References. Case reports should not exceed 2000 words (excluding the references) and should include no more than two tables or figures. Tables and figures should be appended as individual files.

Short Communications should not exceed 2000 words (excluding the references) and include no more than two tables or figures. They should include Title page, Abstract, Keywords, List of Abbreviations, the text summarizing results with no other divisions, and References. Tables and figures should be appended as individual files.

Submission Process

Manuscripts for IJVST should be submitted online at https://ijvst.um.ac.ir/ .

The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting author must ensure that all eligible co-authors have been included in the tittle page and that they have all read and approved the submitted version of the manuscript.

To submit your manuscript, register and log in (https://ijvst.um.ac.ir/contacts?_action=loginForm). All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

Reviewer Suggestions: During the submission process, please suggest three potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last three years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may suggest reviewers from among the authors that you frequently cite in your paper. For detailed information regarding the qualifications and responsibilities of the reviewers, please visit Review Guide.

Ethics: Authors must state that the protocol for the research project has been approved by the Ethics Committee of the institution within which the work was undertaken. Authors are responsible for animal welfare and all statements made in their work.

Accepted file format

Authors are encouraged to use the Microsoft Word template to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. The total amount of data for all files must not exceed 120 MB. If this is a problem, please contact the Editorial Office ijvst@um.ac.ir. Accepted file formats are:

Microsoft Word: Manuscripts prepared in Microsoft Word must be written in English, with Abstract in both English and Persian (where applicable), typewritten in MS Word program, double-spaced, in 12-point "Times New Roman" font on A4 paper size. Authors are requested to reserve margins of 2.5 cm all around the pages. Manuscript should also have line numbers. All pages of the manuscripts should also be enumerated.Templates can be downloaded from the following links:

Template MS Word file for Title page (https://ijvst.um.ac.ir/page_3.html).
Template MS Word file for Original articles (https://ijvst.um.ac.ir/page_3.html).
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Template MS Word file for Tables (https://ijvst.um.ac.ir/page_3.html).
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Tables: Please submit tables as individual files and editable text and not as images. Place all table notes below the table body. Each table should have a title which is followed by explanation of results shown in the table. Use of vertical rules must be avoided. Tables should be self-explanatory, and clearly arranged. Tables should provide easier understanding and not duplicate information already included in the text or figures. Each table should be typewritten with double spacing on a separate file and numbered in order of citation in the text with Arabic numerals. Each table should have a concise heading that makes it comprehensible without reference to the text of the article. Explain any non-standard abbreviations in a footnote to the table.

Figures: Figures must be submitted in individual files (format: TIFF, Dimensions: Width: 789 – 2250 pixels at 300 dpi Height maximum: 2625 pixels at 300 dpi, Resolution: 300 – 600 dpi, file size: less than

10 MB, Text within figures: Arial or Symbol font only in 8-12 point). The text and other labels should be placed in the figure as un-compressed layers. Each figure should have a title which is followed by explanation of results shown in the figure. Figures should be numbered in order of citation in the text with Arabic numerals. If a published figure is used, the publisher's permission needs to be presented to the office, and the figure should be referenced in its legend.

diagrams : For the use of bar diagrams the following publication should be consulted:
Weissgerber TL, Milic NM, Winham SJ, Garovic VD. Beyond bar and line graphs: time for a new data presentation paradigm. PLoS Biol. 2015 Apr22;13(4):e1002128. The bar diagrams should be provided in color and in a well-designed and professional format. Please do not use different shades of gray. The axes of diagrams should have titles and units. Also, the source file of the image (Excel etc.) should be provided for typesetting.Illustrations should be numbered as cited in the sequential order in the text, with a legend at the end of the manuscript. Color photographs are accepted at no extra charge. The editors and publisher reserve the right to reject illustrations or figures based upon poor quality of submitted materials.

Title Page information

Full Title Page should include title (concise and informative), author(s) (including the complete name, department affiliation, and institution), running head (condensed title) (≤ 50 characters, including spaces), name and address of the authors to whom correspondence and reprint requests should be addressed, Acknowledgements, Author contributions, and Conflict of interest.

Acknowledgements: Personal acknowledgement, sources of financial support, contributions and helps of other researchers and everything that does not justify authorship should be mentioned in this section, if required.

Author contributions: Authors are required to include a statement to specify the contributions of each author. The statement describes the tasks of individual authors referred to by their initials. Listed below is an example of author contributions statement:
" Conceived and designed the experiments: HD, SS. Performed the experiments: SS. Analyzed the data: HD, SS, MMM, ARB.
Research space and equipment: HD, MMM, ARB. Contributed reagents/materials/analysis tools: HD. wrote the paper: SS, HD."
Conflict of interest: All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. If there are no conflicts of interest then please state 'The authors declare that there is no conflict of interest'. This form can be downloaded from the IJVST website.

Abstract: Abstract (in English and Persian) no more than 250 words should contain the purpose of the study, findings and the conclusion made on the basis of the findings. Authors who are not native Persian speakers may submit their manuscript with an abstract in English only. Abbreviations and

reference citations may not be used in the abstracts.

Keywords: For indexing purposes, each submitted manuscript should include three to seven keywords, following the abstract and preferably chosen from the Medical Subject Headings (MESH). Keywords should express the precise content of the manuscript.

Abbreviations: Define abbreviations that are not standard in this field in a list to be placed on the tittle page. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the tittle page. Ensure consistency of abbreviations throughout the article.

Main Text

Introduction: Introduction should be as concise as possible, and clearly explain the main objective and hypothesis of the investigation.

Results: Results indicate the results of an original research in a clear and logical sequence. Do not repeat data that are already covered in tables and illustrations. In manuscripts describing more than one animal, all animals should be assigned a case number.

Discussion: Discussion should include the answer to the question proposed in the introduction and emphasize the new and important aspects of the study and the conclusions that follow from them. It could include the implication, application, or speculation of the findings and their limitations, relate the observations to other relevant studies, and links the conclusions with the goals of the study. Recommendations, when appropriate, may be included.

Materials and Methods: Materials and methods should be described in sufficient details to allow other researchers to reproduce the results. Specify any statistical computer programs used .The methods of data collection and use of statistical analysis will be checked by the referees and if necessary, a statistician. Drugs and therapeutic agents, reagents, softwares and equipments should be given in the format: name (trade name, manufacturer name, city, country), e.g. Statview 5 (SAS Institute, Inc., Cary, NC, USA).

Animals: All animal experiments should comply with the ARRIVE guidelines and the authors should clearly indicate in the manuscript the ethical code of the study.

Gene names: The standard gene names, as provided by HGNC should be used. Gene names must be italicized. If the case of mammalian species and if gene names refer to rodent species, they must be upper case; if they refer to non-rodent species they must be written in capitals. If they refer to other species, they must written lower case. Protein names are written in capitals and are not italicized. As an example:

Mouse beta actin gene: Actb

Bovine beta actin gene: ACTB

Chicken beta actin gene: actb

Beta actin protein: ACTB

Quantitative PCR: If the quantitative PCR method has been used, the related section in Materials and Methods and Results must be written following the reference:

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr;55(4):611-22.

Protocol for DNA/RNA extraction, including quantification and determination of purity.

Reverse transcription (if used): amount of RNA, concentration of all reagents: primers conce ntration (either random primers or oligonucleotides), reverse transcriptase and master mix components.

qPCR: sequence of forward and reverse primers, probes, amplicon size, accession number of Genebank;

thermocycler parameters (i.e. denaturation, annealing and extension steps, number of cycles, melting curves);

validation of PCR products; non-template controls for reverse transcription and qPCR should be included in all reactions; and

Data analysis: details for the quantitative or relative analysis.

Use of antibodies: Authors must show that the antibodies are validated and their specificity sis confirmed.

References: Must be up-to-dated and limited to those that are necessary. Lists of references should be given in numerical order in the text, and in the reference list. Please use Vancouver style. To download the Vancouver Style follow the link in the IJVST website which could be used in the Endnote software.

Example piece of text and reference list :
An unhealthy diet, obesity and physical inactivity play a role in the onset of type 2 diabetes, but it has been shown that increased physical activity substantially reduces the risk [1], and participation in regular physical activity is one of the major recommendation of the evidence based guidelines for the primary prevention of diseases [2]. According to the 2004-05 National Health Survey, more than half a million Australians (3.5% of the population) have diabetes mellitus which had been medically diagnosed and most of these people have the Type 2 condition [3]. Gestational diabetes is also on the increase, rising steadily between 2000-01 and 2005-06 [4]. Approximately two thirds of those with diabetes have been prescribed medication [3], but it is of concern that a recent review of the literature found that many people do not take their medication as prescribed [5]. Many patients also self monitor the disease by measuring their blood glucose levels with a glucose meter but Song and Lipman [6] have concerns about how well this is managed.

References for the above example:
1. Hull J, Forton J, Thompson A. Paediatric respiratory medicine. Oxford: Oxford University Press; 2015.
2. Eckerman AK, Dowd T, Chong E, Nixon L, Gray R, Johnson S. Binan goonj: bridging cultures in

Aboriginal health. 3rd ed. Chatswood, NSW: Elsevier Australia; 2010.
3. Johnson C, Anderson SR, Dallimore J, Winser S, Warrell D, Imray C, et al. Oxford handbook of expedition and wilderness medicine. Oxford: Oxford University Press; 2015.
4. McLatchie GR, Borley NR, Chikwe J, editors. Oxford handbook of clinical surgery. Oxford: Oxford University Press; 2013.
5. Petitti DB, Crooks VC, Buckwalter JG, Chiu V. Blood pressure levels before dementia. Arch Neurol. 2005 Jan;62(1):112-6. Doi: 10.1001/archneur.62.1.112 .
6. Liaw S, Hasan I, Wade, V, Canalese R, Kelaher M, Lau P, et al. Improving cultural respect to improve Aboriginal health in general practice: a multi-perspective pragmatic study. Aust Fam Physician. 2015;44(6):387-92.Doi: 10.1001/archneur.62.1.112 .
Use of Italics

Gene symbols, Latin terms (i.e. in vivo, in vitro, ex vivo, in utero, in situ, and etc.) and species scientific names (using the binomial nomenclature), should be typed in italics, while the first letter of the genus name must be capitalized (i.e. Homo sapiens).

Copyright

PUBLICATION ETHICS

Iranian Journal of Veterinary Science and Technology is a member of the Committee on Publication Ethics (COPE), best practice guidelines for dealing with ethical issues in journal publishing and adopts the COPE guidelines. The journal members (editor, editorial board and the journal manager) have agreed to meet the purposes and objectives of the Journal.

Ethical guidelines for authors:

- Manuscripts must be submitted with the understanding that they have not been previously published and are not currently under consideration by another journal.
- Authors are expected to submit manuscripts with enough detail and references to enable others to replicate the work.
-Authors may be requested to provide the original data from their study for editorial review and should be ready to make the data publicly available if feasible.

Publication Ethics

-The corresponding author is responsible for ensuring that all co-authors have approved the manuscript prior to submission.

- Only individuals who meet the authorship criteria should be listed as authors in the manuscript, as they are expected to take public responsibility for the content. The "Conflict of interest declaration and author agreement form" must be signed and completed by all authors. This statement and signatures certifies that all authors have seen and approved the manuscript being submitted. Also, the authors by signing this form warrant that the article is the Authors' original work, that the article has not received prior publication and is not under consideration for publication elsewhere, and that the corresponding author shall bear full responsibility for the submission.

Before submission, all authors are required to review the Article Submission Checklist.

- Authors should disclose any conflicts of interest that might be perceived as influencing the results or their interpretation in the manuscript at the earliest stage possible. This can be done by uploading the Conflicts of Interest Form along with the manuscript submission.

-The authors are responsible for ensuring that the submitted manuscript is a complete and original work, free from any form of plagiarism. All authors are advised to use plagiarism prevention software to check for similarities.

-Authors are required to identify in their manuscript if their work involves chemicals, procedures, or equipment that have any inherent unusual hazards.

- All researchers should have a written and signed informed consent form from whom voluntarily participate in their researches. This signed form shows obviously the consent of the subject to participate. All steps of the experiment were carried out based on the Guidelines for Animal Care at Ferdowsi University of Mashhad in Iran that are approved by the Committee of Biological Ethics, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Iran (https://ethics.research.ac.ir/docs/pages/Guideline-En.pdf). The experiments that are carried outside the university should have a written and signed informed consent form related to ethic protocols of university or institute that they are carried.

- If the decision is 'Needs Revision,' authors are expected to respond systematically and promptly to the reviewers' comments, addressing point by point, and revising their manuscript accordingly. The revised manuscript should then be submitted to the journal within the given deadline.

- Authors are requested to clearly identify who financially supported the research and/or preparation of the manuscript and briefly describe the role of the founder/ sponsor in any part of the work at the end of their manuscript under "Acknowledgements" section.

-It is a condition for submission of a manuscript that the authors permit editing of the paper for readability.

- All authors agree to allow the corresponding author to serve as the correspondent with the Journal's editorial office, to review the edited manuscript and proof.

-Under open access license, authors retain ownership of the copyright for their content, but allow anyone to download, reuse, re-print, modify, distribute, and/or copy the content as long as the original authors and source are cited properly.

- When author(s) discover(s) a significant error or inaccuracy in his/her own published work, it is the author's obligation to promptly notify the Journal editor or publisher to retract or correct the

manuscript.

- All authors must know that the submitted manuscripts under review with the IJVST are subject to screening, using Plagiarism Prevention Software. Plagiarism is a serious violation of publication ethics and in all its forms constitutes unethical publishing behavior and is unacceptable.

- Editors and members of editorial board as authors should be excluded from publication decisions when they are authors or have contributed to a manuscript.

-The artificial intelligence (AI) tools such as ChatGPT or Large Language Models cannot meet the requirements for authorship. Authors who use AI tools in the writing of a manuscript, production of images or graphical elements of the paper, or in the collection and analysis of data, must be transparent in disclosing in the Materials and Methods (or similar section) of the paper how the AI tool was used and which tool was used. Authors are fully responsible for the content of their manuscript, even those parts produced by an AI tool, and are thus liable for any breach of publication ethics(Authorship and AI tools).

Ethical guidelines for Peer reviewers

- Reviewers are expected to provide insightful comments that assist the editors in making a decision about whether or not to publish the submitted manuscript.

- Reviewers are expected to maintain the confidentiality of the manuscripts they are invited to review.

-Reviewers are expected to disclose any conflicts of interest they have with the authors, companies, or institutions associated with the manuscripts they are invited to review. If a conflict of interest exists, reviewers should immediately notify the Editor-in-Chief, decline the invitation to review, and suggest alternative reviewers.

- If reviewers feel unqualified to review an assigned manuscript or are unable to provide a timely review, they should inform the Editor-in-Chief and excuse themselves from the review process. If they know of any other expert reviewers, they may suggest them to the Editor-in-Chief through the dedicated email/comments section in the Reviewer Dashboard.

- Reviewers are expected to maintain the confidentiality of the manuscripts they review and not discuss any information from the manuscript with anyone other than the Editor-in-Chief, unless they have obtained explicit permission to do so. This also applies to invited reviewers who decline the review invitation.

- Reviewers are obligated to treat the manuscripts they receive for peer review as confidential and must not use any information obtained through this process for personal gain.

-Reviewers are expected to provide technical, professional, and objective comments on the manuscripts they are invited to review.

-Reviewers are expected to avoid personal biases in their comments and judgments, and express their views clearly with supporting arguments that assist the author in improving the manuscript.

-Reviewers are expected to identify any relevant published work that has not been cited by the authors. If a statement has been previously reported elsewhere, it should be accompanied by the appropriate citation.

-Reviewers should also bring to the attention of the Editor-in-Chief any significant similarity

or overlap between the manuscript under consideration and any other publications of which they are personally aware.

The process has been explained in the section "Peer Review Process".

Ethical guidelines for Editor

-The editors should evaluate submitted manuscripts to determine if they fall within the scope of the journal. Additionally, the editors should recommend expert reviewers based on their integrated recognition of specialized reviewers.

-The Editor-in-Chief is responsible for deciding whether to accept or reject submitted manuscripts for the journal. This decision takes into consideration several factors, such as the judgment of the editorial board members, the validation of the work in question, its significance to researchers and readers, as well as any feedback from reviewers. Furthermore, the decision must also comply with legal requirements regarding libel, copyright infringement, and plagiarism, which are currently in force. The Editor-in-Chief works closely with other editors and reviewers to ensure that all submissions are fairly evaluated.

-The editors ought to uphold the anonymity of both reviewers and authors.

-The editors should disclose any potential conflicts of interest and make efforts to avoid them. If such circumstances arise, they are expected to delegate the handling of the manuscript to another member of the editorial board.

-The editors, particularly the Editor-in-Chief, should demonstrate a willingness to investigate cases of plagiarism and fraudulent data. When ethical concerns are raised about a submitted manuscript or published paper, the editors will take appropriate measures in response. Any reported incidents of unethical publishing behavior will be thoroughly examined, even if they come to light years after publication.

-When dealing with cases of suspected misconduct, the Editor-in-Chief follows the COPE Flowcharts. If an investigation supports the ethical concern, the journal will publish a correction, retraction, expression of concern, or any other relevant note.

-The editors must not share any information about submitted manuscripts with anyone until they are published, as appropriate.

-The Editor-in-Chief and members of the editorial board will not use unpublished materials disclosed in a submitted paper for their own research purposes without obtaining explicit written consent from the author.

-Editors are expected to give fair consideration to all manuscripts submitted for publication, evaluating each on its own merits and without prejudice based on the author(s)' country, race, religion, nationality, sex, seniority or institutional affiliation. Decisions about editing and publishing are made solely based on the quality and relevance of the manuscript and are not influenced by external policies of governments or other agencies beyond the scope of this journal.

-The Editor-in-Chief has complete authority over the editorial content of the journal as well as the timing of its publication.

Ethical guidelines for Publisher

"Ferdowsi University of Mashhad press (FUM)" is promising to ensure that the decision on manuscript submissions is only made based on professional judgment and will not be affected by any commercial interests.

- FUM is committed to maintain the integrity of academic and research records.

- FUM is monitoring the ethics by Editor-in-Chief, Associate Editors, Editorial Board Members, Reviewers, Authors, and Readers.

- FUM, together with the Journal's editors, shall take reasonable steps to identify and prevent the publication of manuscripts where research misconduct has occurred, and under no circumstances encourage such misconduct or knowingly allow taking place.

- FUM is always checking the plagiarism and fraudulent data issues involving in the submitted manuscripts and willing to publish corrections, clarifications and retractions involving its publications as and when needed.

-FUM as the publisher supports the Journal for each published issue by paying a defined budget according to its published annual rank in the Portal of Scientific Journals of Iranian Ministry of Science, Research and Technology for costs including those pertaining to setup and maintenance of the publication infrastructure, routine operation of the Journal, processing of manuscripts through peer-reviews, editing, publishing, maintaining the scholarly record, and archiving.

Violation of Publication Ethics

The Editorial board of IJVST acknowledges that plagiarism is unacceptable in any of its forms:

Plagiarism:

Plagiarism is intentionally using someone else's ideas or other original material as if they are one's own. Copying even one sentence from someone else's manuscript, or even one of your own that has previously been published, without proper citation is considered by the JAM as plagiarism. All manuscripts under review or published with JAM are subject to screening using plagiarism prevention software (e.g. iThenticate). Thus, plagiarism is a serious violation of publication ethics.

Simultaneous Submission:

Care should be taken to ensure that the work has not been published elsewhere, in any language and is not simultaneously submitted to other journals.

Duplicate Publication:

Duplicate publication occurs when two or more articles, without full cross referencing, share essentially the same hypotheses, data, discussion points, and conclusions.

Redundant Publications:

Redundant publications involve the inappropriate division of study outcomes into several articles, most often consequent to the desire to plump academic vitae.

Data Fabrication:

Data fabrication means the researcher did not really carry out the study, but made up data or results and had recorded or reported the fabricated information. Data falsification means the researcher

did the experiment, but manipulated, changed, or omitted data or results from the research findings.

Citation Manipulation:

Citation Manipulation implies excessive citations in the submitted manuscript that do not contribute to the scholarly content of the article and have been included solely for the purpose of increasing citations to a given author's work, or to articles published in a particular journal. This leads to misrepresenting the importance of the specific work and journal in which it appears and is thus a form of scientific misconduct.

Improper Author Contribution or Attribution:

All listed authors must have made a significant scientific contribution to the research in the manuscript and approved all its claims. Do not forget to list everyone who made a significant scientific contribution, including students and laboratory technicians.

Handling Misconduct Cases

The Editorial board of IJVST takes the necessary measures to examine the incoming papers on their originality, reliability of contained information and correct use of citations.

-If any of the unethical publishing behavior is detected by the Journal Editorial board or by one of the reviewers, the first action is to inform the Editor-in-chief by supplying copies of the relevant material and a draft letter to the corresponding author asking for an explanation in a nonjudgmental manner.

- If the infraction is less severe, the Editor, upon the advice of the Committee on Publication Ethics, sends the author a letter of reprimand and reminds the JAM publication policies; if the manuscript has been published, the Editor may request the author to publish an apology in the journal to correct the record.

- If the author's explanation is unacceptable and it seems that serious unethical conduct has taken place, the matter is referred to the Publication Committee via Editorial board. After deliberation, the Committee will decide whether the case is sufficiently serious to warrant a ban on future submissions.

Post-Publication Discussions and Corrections

This journal allows debate post publication on journal's site, through "Send comment about this article" section to the editor up to one month before final publication. Our mechanisms for correcting, revising or retracting articles after publication depends on the content of the received comment and if the sent comments are useful and applicable for readers/authors, they will be showed under reference section of the articles pages.

Complaint Policy

If the authors disagree with the editorial decision on their manuscripts, they have a right to appeal. Authors who wish to appeal an editorial decision should contact the Editor-in-Chief of the Iranian Journal of Veterinary Science and Technology. In such cases the Editor-in-Chief will review the manuscript, the editorial and peer reviewers' comments and gives his/her decision for accepting or rejecting a manuscript. Editor-in-Chief may, if so required, send the manuscript to a new handling editor for a fresh editorial review and to new reviewer for further peer reviewing. In such case, the final decision

maker will be the Editorial board of the journal.

How to Make a Complaint

The procedure to make a complaint is quite simple. The complaint can be made by writing an e-mail to: ijvst@um.ac.ir. All complaints will be acknowledged within a week.

PEER REVIEW PROCESS

Iranian Journal of Veterinary Science and Technology peer reviews all submitted manuscripts with contents within the scope of the journal.

Initial assessment

The submitted manuscript will be subjected to a primary review by the editor or a member of the editorial board for suitability and relevance of the findings to the scope of the journal and quality of the science presented in the paper (sufficient originality, having a message that is important to the general field of Veterinary Medicine, quality of data, novelty, English language, and overall manuscript quality) within two weeks. If the paper is evaluated to be relevant to the scope of the journal and having enough scientific rigor and novelty, it will be sent for the next stage. Otherwise, those manuscripts which are evaluated as not-appropriate in the initial review will be rejected at this stage.

Initial screen

The initial screen will be performed by the editorial office for the structure and format of the manuscript.

Peer review (double-blind)

The manuscripts which are found to be appropriate after the initial screen will be sent for external review by experts in the related field. We have prepared a checklist for reviewers that summarizes their evaluation of the manuscript. The items in this checklist are:

1. TITLE is clear and adequate
2. ABSTRACT clearly presents objects, methods, and results.
3. INTRODUCTION well-structured and provides a rationale for the experiments described.
4. MATERIALS AND METHODS are sufficiently explained and is detailed enough to be reproduced.
5. RESULTS are clearly presented and supported by figures and tables.
6. DISCUSSION properly interprets the results and places the results into a larger research context, and contains all important references.
7. Conclusions are logically derived from the data presented.
8. English Language/style/grammar is clear, correct, and unambiguous.
9. Figures and tables are of good quality and well-designed and clearly illustrate the results of the study.
10. References are appropriate.
11. Regarding this article are you concerned about any issues relating to author misconduct such as plagiarism and unethical behavior.

12. Comments on the importance of the article.

Final Decision

Based on the reviewers' recommendations a final decision is made by the editor and if needed the help of a member of the editorial board (depending on the field of study). Decisions will include accept, minor revision, major revision with and without re-review, and reject. We aim to reach a final decision on each manuscript as soon as their review results are available.

Acknowledgement to Reviewers

IRANIAN JOURNAL OF VETERINARY SCIENCE AND TECHNOLOGY

The Editor-in- Chief would like to extend his sincere gratitude to the following reviewers for evaluating and assessing manuscripts of IJVST journal in 2025.

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Acknowledgement to Reviewers

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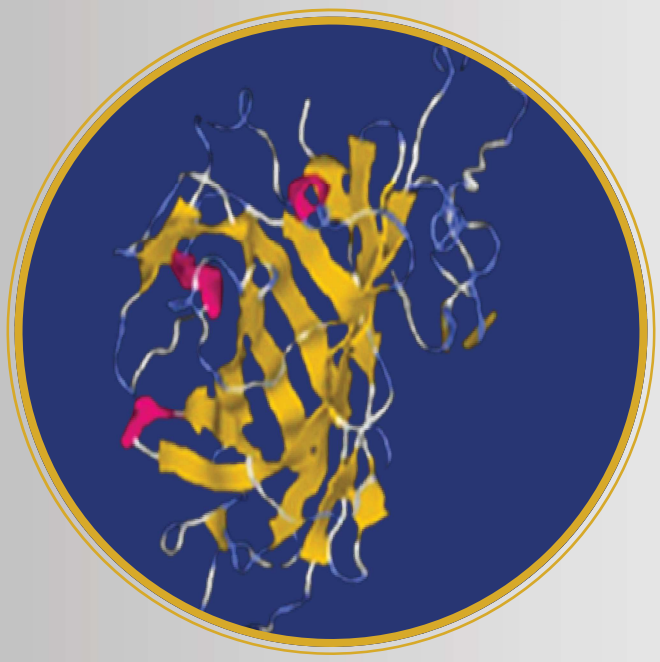

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