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Review Article

Effect of Black Soldier Fly Larva Meal on Broiler Chicken Production, Meat Quality, and It's Physiological Properties: A Meta-Analysis

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Abstract

This study assessed the effect of feeding black soldier fly larva (BSFL) on broiler chicken production, meat quality, and physiological characteristics using a meta-analysis. Out of the 1878 articles that were reviewed from five databases (PubMed, Scopus, Science Direct, Cochrane Library, and ProQuest), 21 papers were selected for meta-analysis using the specific keywords "black soldier fly, *Hermetia illucens*, broiler chickens, growth performance" and the PRISMA flow diagram. The R Studio software version 4.4.2 (Posit PBC, USA) was employed to analyze the meta-analysis and compute the standard mean difference (SMD), 95% confidence interval (CI), and random effects. The current meta-analysis demonstrated that providing BSFL meals for broiler chickens had a favorable impact on weight gain, feed efficiency, carcass percentage, breast and thigh muscles, cooking loss, drip loss, meat color lightness, and yellowness. On the other hand, favorable effects were also reported on erythrocytes, packed cell volume, mean corpuscular volume, heterophil: lymphocyte (H/L) ratio, serum glucose, alanine transaminase, aspartate aminotransferase, gamma-glutamyl transferase, blood urea nitrogen, creatinine, uric acid, calcium, phosphorus, magnesium, and iron. It has been demonstrated using a meta-analysis study that the BSFL meal is beneficial for weight gain, feed efficiency, meat quality, and physiological properties in broiler chickens.

Keywords: Black soldier fly, Broiler chickens, Food production, Meat quality, Physiological properties

Introduction

Poultry products and consumption have been steadily increasing globally, as they are readily accessible and popular among consumers [1]. Currently, up to 58% of protein availability comes from animal-derived goods, whereas in the 1960s, the majority came from plant-derived products like wheat. A recent study concluded that Western consumption habits will continue to shift from red meat to white meat, despite the fact that European consumers' intake of animal-based protein has historically climbed gradually, with a particular spike in chicken consumption. As a result, animal products currently make up 30% of total caloric intake and are the main source of protein (28 g/person/day). Poultry meat has seen the largest increase in consumption among the various types of meat that are sold in markets. Furthermore, there is growing pressure to change European diets to include more environmentally friendly alternatives [2].

An investigation for more effective and substitute sources of protein for animal diets has been spurred by the expanding human population, climate change, and the shrinking quantity of land available for food crop

cultivation [3]. Edible insects have received attention as they natural source of food for omnivorous poultry species in the wild. The productivity and profit of the poultry industry can be improved by including insect components in poultry feed [4]. Fish meal and soybeans have been the primary sources of protein in chicken feed for decades. Small benthic forage fish, especially have grown increasingly rare as a result of ocean overfishing, and are used to make fish oil and fish meal. Additionally, there is a cap on the amount of land that can be utilized for soy cultivation [5]. This necessitates the use of economic, environmentally friendly, and highly nutritious alternative sources of protein that are also affordable and feasible to produce. In the natural habitat, poultry consume insects, which are regarded as a vital source of protein. Live insect feeding such as BSFL to chickens has already been approved in Europe [6]. Because of its exceptional capacity to ingest a wide range of organic detritus and its abundance of protein (up to 39-64%), BSFL has established a unique niche for itself in the insect-based diet [7].

Black soldier fly larva (BSFL) has been widely used as a component in animal diets because it has a more abundant amino acid composition, i.e. leucine (44.6 g/kg), lysine (38.8 g/kg) and valine (40.1 g/kg) and is a significant source of protein (37-63% DM) and fat (7-39% DM) compared to soybean meal, these three amino acid amounts are higher, and even the valine content is larger than that of fish meal [8,9]. In previous investigations on various poultry, including BSFL feeding on turkeys [10], laying hens [11], broiler chickens [12], Barbary partridge [13], and Muscovy ducks [14], reported positive impact on meat quality and the growth performance, and supplementing partially defatted BSFL meal at portions of 5% and 15% to their diets improve productive performance in broiler chicken [15]. Numerous studies investigated hepatic and renal function, hematological characteristics, blood proteins, glucose, lipids, electrolytes, growth performance, and meat quality [16-18]. Moreover, several meta-analysis-based systematic studies solely assessed the performance of chickens, excluding meat quality and physiological characteristics as a measure of the BSFL efficacy. Comprehensive studies using meta-analysis methods crucial to be conducted to combine data from various investigations on the beneficial effects of BSFL feed involving all investigated parameters on broiler production. Therefore, this study aimed to evaluate the impacts of feeding broiler chickens BSFL meals on their productivity, meat quality, and physiological characteristics utilizing a meta-analysis.

Material and Methods

Search Strategy and Selection of Studies

The authors involved conducted the pertinent literature screening, data extraction, data analysis, and visualization processes from June to December 2024 at Universitas Airlangga, Indonesia and Eskişehir Osmangazi University, Türkiye. The Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) flow diagram *(http://www.prisma-statement.org/)* was used in this inquiry to identify relevant papers. A methodical screening procedure was employed to find pertinent research from PubMed, Scopus, Science Direct, Cochrane Library, and ProQuest that reported the efficacy of BSFL meals in broiler chickens. The study question (P, population = broiler chickens; I, intervention = black soldier fly; C, comparison = control; and O, outcomes = meat quality and growth performance) was developed using the PICO algorithm *(Table 1)*. The search phrases "broiler chickens, meat quality, growth performance, *Hermetia illucens*, black soldier fly" were the most pertinent. The reference search included all studies indexed by the database and was not limited by study year. The MeSH term's inclusion of all pertinent and thorough keywords was confirmed. The following was the sample search algorithm used in the databases: #1 "chicken [MeSH Terms] OR broiler [Title/Abstract] OR broilers [Title/Abstract] OR broiler chickens [Title/Abstract]"; #2 "Black Soldier Fly [MeSH Terms] OR *Hermetia illucens* [Title/Abstract] OR maggot [Title/Abstract]"; #3 "meat quality [MeSH Terms] AND production performance [MeSH Terms] OR growth performance [Title/Abstract]".

Eligibility Criteria

Articles were selected based on the following eligibility specifications: full-text format and open-access documents,

Table 1. *Searching strategy based on the PICO methods*

PICO Items	PICO	Keywords
Problems, patients, population	Broiler chickens	((broiler[MeSH Terms]) OR (chicken[Title/Abstract])) OR (broiler chicken[Title/Abstract])
Intervention	Black soldier fly	((Black Soldier Fly[MeSH Terms]) OR (*Hermetia illucens*[Title/Abstract])) OR (maggot[Title/Abstract])
Comparison, control	Control	"Control groups"[MeSH Terms]
Outcomes	Primary outcome: meat quality, production performance Secondary outcomes: growth performance	((meat quality[MeSH Terms]) AND (production performance[MeSH Terms])) OR (growth performance[Title/Abstract])

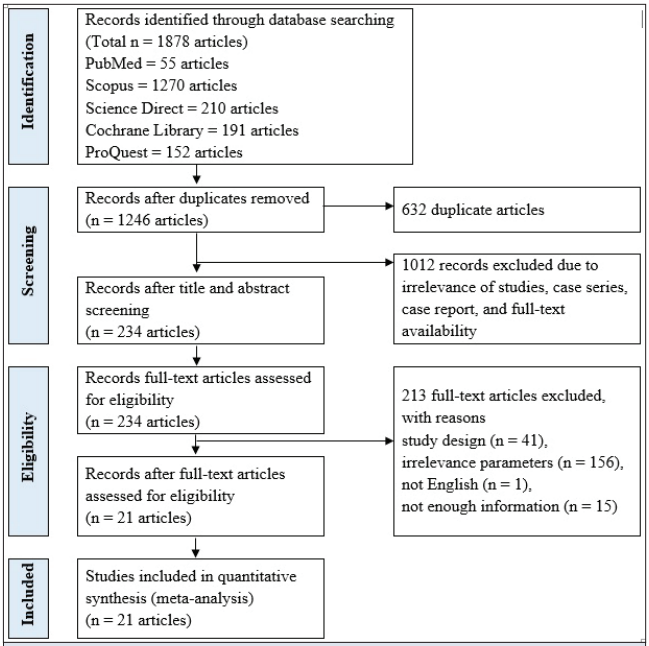

Fig 1. PRISMA flow diagram of the study selection process

in vivo investigations, randomized experimental studies, use of BSFL meals for feeding, original research papers written in English, and comprehensive disclosure of parameters. Exclusion criteria included duplicate studies, irrelevant studies, full-text not available, irrelevant types of studies (case series, case report), non-English language, and insufficient data. The PRISMA guidelines were adhered to in order to obtain references that were pertinent to the current study *(Fig. 1)*.

Data Extraction

The following data was presented once the data extraction process from the retrieved studies were completed: study reference, study year, country, broiler chicken breeds, BSFL ratio as the amount of BSFL mixed with the concentrates, breeding period, and total samples. Comprehensive data on the results were also extracted, such as the meat quality (slaughter weight, carcass yield, visceral organ weight, cooking loss, drip loss, shear force, meat color, and pH ultimate); hematological traits; serum proteins, glucose, and lipids; serum liver enzymes; renal physiology; and electrolyte levels; and chicken productivity (initial and final body weight, weight gain, feed intake, and feed efficiency).

Statistical Analysis

The "Meta package version 8.0-1" and "Metafor package version 4.6-0" in R Studio software version 4.4.2 (Posit PBC, USA) were used for statistical analysis and pooled cumulative meta-analysis. Following a pairwise meta-analysis of the data between the control group and the BSFL meals, the 95% CI, random effect, and standard mean difference (SMD) were calculated. Chi-squared tests (χ^2) were utilized to determine the heterogeneity during the investigation. The presence of considerable heterogeneity was determined based on a P-value <0.05 and an I^2 value >50%. The information displayed in the forest plot was verified and accurately depicted in the tables. The Egger's test was utilized to determine publication bias by considering the z-score and P-value <0.05.

Results

Identification and Selection of Studies

A total of 1878 articles have been identified from advanced searches across five electronic databases (PubMed supplied 55 articles, Scopus 1270 articles, Science Direct 210 articles, Cochrane Library 191 articles, and ProQuest 152 articles). Out of these, 1012 were eliminated due to the case reports, unavailability of pertinent research, and the complete text of the study, while 632 were duplicates. There were 234 full-text publications left after titles and abstracts were screened. Out of these, 213 papers were considered unsuitable for this analysis due to reasons including technical parameters, unsuitable study designs, non-English language, and inadequate data. In the end, 21 publications that could be evaluated were incorporated in meta-analysis *(Fig. 1)*.

Characteristics of The Included Studies

From the 21 incorporated studies, a total of 4676 broiler chicken samples representing the various BSFL feed treatment ratios were reviewed. Those studies were published in the period 2016-2024. A total of twelve studies reported from Europe, one reported from North America, four reported from Africa, and four reported from Asia were among the eleven countries whose data were published. Of the 4676 broiler chickens evaluated, 120 were Arbor Acres breed, 1382 Cobb 500, 280 New Lohmann strain MB 202, 2198 Ross 308, 480 Ross 708, and 216 Turkeys *(Table 2)*.

Growth Performance

According to the current meta-analysis, feeding BSFL meals improved weight gain (SMD = 0.29; P<0.001) and feed efficiency (SMD = 0.14; P = 0.01). On the other hand, feed intake was not significantly affected by providing BSFL meal (SMD = -0.26; P<0.001) *(Table 3)*.

Meat Quality

According to a meat quality assessment, feeding BSFL improved the carcass percentage (SMD = 0.76; P<0.001), breast muscle (SMD = 0.61; P<0.001), thigh muscle (SMD = 0.19; P = 0.19), total muscles (SMD = 5.15; P<0.001), cooking loss (SMD = 1.45; P<0.001), drip loss (SMD = 0.67; P<0.001), meat color lightness (SMD = 0.76; P<0.001), and yellowness (SMD = 0.66; P<0.001), respectively *(Table 4)*.

Table 2. *Characteristics of the studies*

Study Reference	Country	Breeds	BSFL Ratio (%)	Age (days)	Samples (n)
Aprianto et al.[12]	Indonesia	New Lohmann strain MB 202	0; 1; 2; 3	1-35	280
Attia et al.[22]	Saudi Arabia	Arbor Acres	SBM; FM; BSFL; BSFP	1-42	120
Beller et al.[20]	Germany	Cobb 500	0; 7.5; 15	1-35	72
Dabbou et al.[19]	Italy	Ross 308	0; 5; 10; 15	1-35	256
Dabbou et al.[24]	Italy	Ross 308	0; 1.6; 4; 1.8	1-33	200
Elangovan et al.[23]	India	Cobb 500	0; 50	1-21	90
Fruci et al.[38]	Canada	Ross 708	0; 12.5; 25; 50; 100	1-35	480
Kierończyk et al.[33]	Poland	Ross 308	0; 3; 6; 9	1-35	400
Mat et al.[16]	Malaysia	Cobb 500	0; 4; 8; 12	1-42	360
Mohammed et al.[29]	Ghana	Cobb 500	0; 4	1-49	32
Murawska et al.[17]	Poland	Ross 308	0; 50; 75; 100	1-42	384
Mutisya et al.[30]	Kenya	Cobb 500	0; 25; 50; 75	1-42	120
Oddon et al.[35]	Italy	Ross 308	C; HI; TM	1-38	180
Onsongo et al.[39]	Kenya	Cobb 500	0; 11; 37.2; 55.5	1-49	288
Pieterse et al.[15]	South Africa	Cobb 500	0; 5; 10; 15	1-31	320
Schäfer et al.[31]	Germany	Cobb 500	0; 2.5; 5	1-35	100
Schiavone et al.[25]	Italy	Ross 308	0; 50; 100	1-35	150
Schiavone et al.[36]	Italy	Ross 308	0; 50; 100	1-48	120
Schiavone et al.[18]	Italy	Ross 308	0; 5; 10; 15	1-35	256
Seyedalmoosavi et al.[40]	Germany	Ross 308	0; 10; 20; 30	1-42	252
Sypniewski et al.[10]	Poland	Turkeys	0; 50; 100	7-35	216

Table 3. *Efficacy of BSFL meals on production performance of broiler chickens*

Parameter	n	Random Effect			Heterogeneity			Egger's Test	
		SMD	95% CI	P-value	Chi^2	I^2	P-value	z	P-value
Initial body weight (g)	1359	0.02	-0.10 - 0.14	0.74	596.97	98%	< 0.001	2.3432	0.0191
Final body weight (g)	1767	-0.24	-0.35 - -0.12	< 0.001	1109.65	99%	< 0.001	0.9560	0.3391
Weight gain (g)	2001	0.29	0.17 - 0.41	< 0.001	1730.28	99%	< 0.001	-0.4462	0.6555
Feed intake (g/bird/day)	1875	-0.26	-0.39 - -0.13	< 0.001	1846.72	99%	< 0.001	-0.7626	0.4457
Feed efficiency	2169	0.14	0.03 - 0.25	0.01	1701.72	99%	< 0.001	N/A	N/A

n = total samples, SMD = standard mean difference, 95% CI = 95% confidence intervals, I^2 = the primary index for reporting heterogeneity, z = significance tests for the weighted average effect size, N/A = not applicable

Hematological Profile, Electrolytes, Liver and Renal Physiology

Based on hematological traits evaluation, the BSFL meals were revealed to have a favorable effect on erythrocytes (SMD = 1.10; P<0.001), PCV (SMD = 1.67; P<0.001), MCV (SMD = 1.41; P<0.001), H/L ratio (SMD = 0.31; P = 0.005) *(Table 5)*, and serum glucose (SMD = 0.30; P = 0.03). In contrast, we reported that BSFL meals did not have a favorable effect on triglycerides (SMD = -0.72; P<0.001) and cholesterol (SMD = -0.65; P<0.001) *(Table 6)*. Concurrently, this meta-analysis study investigated the favorable effects of BSFL meals on various physiological indicators related to the liver, kidneys, and electrolyte balance. These yielded favorable findings on the following parameters: ALT (SMD = 0.20; P = 0.02), AST (SMD = 0.24; P = 0.003), GGT (SMD = 0.09; P = 0.36) *(Table 6)*, blood urea nitrogen (SMD = 0.49; P = 0.004), creatinine (SMD = 0.07; P = 0.48), uric acid (SMD = 0.83; P<0.001) *(Table 7)*, calcium (SMD = 1.64; P<0.001), phosphorus (SMD = 0.78; P<0.001), magnesium (SMD = 0.44; P<0.001), and iron (SMD = 0.19; P = 0.07) *(Table 8)*, respectively.

Publication Bias

The current meta-analysis revealed an accurate publication bias assessment using Egger's test, as several parameters

Table 4. *Efficacy of BSFL meals on meat quality and carcass trait of broiler chickens*

Parameter	n	Random Effect			Heterogeneity			Egger's Test	
		SMD	95% CI	P-value	Chi^2	I^2	P-value	z	P-value
Slaughter weight (g)	1084	-0.76	-0.92 - -0.59	< 0.001	952.04	99%	< 0.001	-0.8334	0.4046
Carcass yield (g)	1112	-0.18	-0.33 - -0.02	0.02	879.52	99%	< 0.001	0.1425	0.8866
Carcass percentage (%)	1020	0.76	0.61 - 0.90	< 0.001	468.04	98%	< 0.001	3.7575	0.0002
Breast yield (g)	764	0.61	0.40 - 0.81	< 0.001	735.26	99%	< 0.001	1.3603	0.1737
Breast percentage (%)	860	-0.81	-1.01 - -0.61	< 0.001	856.13	99%	< 0.001	-3.0604	0.0022
Thigh yield (g)	572	0.19	-0.10 - 0.48	0.19	743.49	100%	< 0.001	1.9006	0.0574
Thigh percentage (%)	860	0.98	0.82 - 1.14	< 0.001	391.87	98%	< 0.001	-0.8671	0.3859
Muscles (%)	352	5.15	4.89 - 5.60	< 0.001	44.31	98%	< 0.001	N/A	N/A
Abdominal fat (g)	556	-2.88	-3.21 - -2.56	< 0.001	481.94	99%	< 0.001	1.7928	0.0730
Abdominal fat (%)	680	-1.06	-1.33 - -0.79	< 0.001	846.53	100%	< 0.001	N/A	N/A
Liver (g)	638	0.64	0.42 - 0.86	< 0.001	585.30	99%	< 0.001	-0.5610	0.5748
Liver (%)	744	-1.14	-1.31 - -0.96	< 0.001	325.07	98%	< 0.001	0.4124	0.6800
Heart (g)	548	0.77	0.56 - 0.98	< 0.001	308.12	99%	< 0.001	1.4430	0.1490
Heart (%)	744	-0.63	-0.81 - -0.45	< 0.001	481.20	99%	< 0.001	N/A	N/A
Spleen (g)	446	-0.29	-0.48 - -0.09	0.003	50.92	92%	< 0.001	2.2077	0.0273
Spleen (%)	552	-0.18	-0.38 - 0.01	0.07	301.30	99%	< 0.001	N/A	N/A
Bursa fabricius (g)	170	-0.23	-0.57 - 0.12	0.19	75.39	99%	< 0.001	N/A	N/A
Bursa fabricius (%)	492	-1.09	-1.34 - -0.85	< 0.001	387.82	99%	< 0.001	N/A	N/A
Gizzard (g)	314	-0.43	-0.66 - -0.20	0.0003	29.02	93%	< 0.001	0.2941	0.7686
Gizzard (%)	616	-1.12	-1.32 - -0.93	< 0.001	269.37	99%	< 0.001	N/A	N/A
Cooking loss (%)	620	1.45	1.23 - 1.67	< 0.001	423.11	99%	< 0.001	0.9313	0.3517
Drip loss 24 h (%)	780	0.67	0.51 - 0.83	< 0.001	267.09	99%	< 0.001	0.8542	0.3930
Shear force (kg/cm^2)	460	-0.25	-0.47 - -0.04	0.02	247.68	99%	< 0.001	-4.4825	< 0.001
Meat color lightness (L*)	1020	0.76	0.59 - 0.93	< 0.001	869.75	99%	< 0.001	-0.1746	0.8614
Meat color redness (a*)	1020	-1.06	-1.21 - -0.92	< 0.001	289.18	98%	< 0.001	-0.4460	0.6556
Meat color yellowness (b*)	1020	0.66	0.46 - 0.87	< 0.001	1228.72	100%	< 0.001	0.2375	0.8123
pH 60 min	360	-0.35	-0.64 - -0.05	0.02	351.69	100%	< 0.001	N/A	N/A
pH 24 h	880	-0.55	-0.72 - -0.37	< 0.001	684.97	99%	< 0.001	N/A	N/A

n = total samples, SMD = standard mean difference, 95% CI = 95% confidence intervals, I^2 = the primary index for reporting heterogeneity, z = significance tests for the weighted average effect size, N/A = not applicable

Table 5. *Efficacy of BSFL meals on hematological traits of broiler chickens*

Parameter	n	Random Effect			Heterogeneity			Egger's Test	
		SMD	95% CI	P-value	Chi^2	I^2	P-value	z	P-value
Erythrocytes (x $10^6/\mu L$)	700	1.10	0.90 - 1.30	< 0.001	505.72	99%	< 0.001	-0.2203	0.8257
Leukocytes (x $10^3/\mu L$)	640	-0.29	-0.47 - -0.11	0.001	311.60	98%	< 0.001	-0.4568	0.6478
Hemoglobin (g/dL)	272	-1.45	-1.79 - -1.12	< 0.001	177.84	99%	< 0.001	3.2698	0.0011
PCV (%)	92	1.67	0.98 - 2.35	< 0.001	84.58	99%	< 0.001	0.9603	0.3369
MCV (μm^3)	272	1.41	1.04 - 1.77	< 0.001	243.20	99%	< 0.001	N/A	N/A
MCH (pg/dL)	272	-1.47	-1.86 - -1.08	< 0.001	280.57	99%	< 0.001	2.1084	0.0350
MCHC (%)	272	-1.29	-1.69 - -0.88	< 0.001	309.92	99%	< 0.001	N/A	N/A
H/L ratio	428	0.31	0.09 - 0.52	0.005	180.47	98%	< 0.001	0.8246	0.4096

n = total samples, SMD = standard mean difference, 95% CI = 95% confidence intervals, I^2 = the primary index for reporting heterogeneity, z = significance tests for the weighted average effect size, N/A = not applicable
PCV = packed cell volume, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration

Table 6. *Efficacy of BSFL meals on serum proteins, glucose, lipids, and liver enzymes of broiler chickens*

Parameter	n	Random Effect			Heterogeneity			Egger's test	
		SMD	95% CI	P-value	Chi²	I²	P-value	z	P-value
Total protein (g/dL)	990	-0.75	-0.90 - -0.60	< 0.001	548.55	99%	< 0.001	2.5507	0.0108
Albumin (g/dL)	630	-1.13	-1.35 - -0.92	< 0.001	482.63	99%	< 0.001	0.8934	0.3716
Glucose (mg/dL)	284	0.30	0.03 - 0.56	0.03	116.61	99%	< 0.001	N/A	N/A
Triglyceride (mg/dL)	872	-0.72	-0.90 - -0.55	< 0.001	631.13	99%	< 0.001	1.1622	0.2452
Cholesterol (mg/dL)	872	-0.65	-0.81 - -0.48	< 0.001	545.37	99%	< 0.001	-1.9403	0.0523
HDL (mg/dL)	564	-1.86	-2.12 - -1.61	< 0.001	433.38	99%	< 0.001	-0.3318	0.7400
LDL (mg/dL)	564	-0.28	-0.47 - -0.08	0.006	307.94	99%	< 0.001	N/A	N/A
ALT (IU/L)	784	0.20	0.04 - 0.37	0.02	426.61	99%	< 0.001	7.6141	< 0.001
AST (IU/L)	732	0.24	0.08 - 0.40	0.003	222.15	97%	< 0.001	-0.8821	0.3777
ALP (IU/L)	160	-1.10	-1.43 - -0.77	< 0.001	1.71	42%	0.19	N/A	N/A
GGT (IU/L)	460	0.09	-0.10 - 0.29	0.36	108.94	97%	< 0.001	5.1303	< 0.001

n = total samples, SMD = standard mean difference, 95% CI = 95% confidence intervals, I² = the primary index for reporting heterogeneity, z = significance tests for the weighted average effect size, N/A = not applicable
HDL = high-density lipoprotein, LDL = low-density lipoprotein, ALT = alanine transaminase, AST = aspartate aminotransferase, ALP = alkaline phosphatase, GGT = gamma-glutamyl transferase

Table 7. *Efficacy of BSFL meals on renal physiology of broiler chickens*

Parameter	n	Random Effect			Heterogeneity			Egger's test	
		SMD	95% CI	P-value	Chi²	I²	P-value	z	P-value
Blood urea nitrogen (mg/dL)	368	0.49	0.16 - 0.82	0.004	130.26	100%	< 0.001	-1.2092	0.2266
Creatinine (mg/dL)	468	0.07	-0.13 - 0.27	0.48	169.25	98%	< 0.001	-3.7100	0.0002
Uric acid (mg/dL)	528	0.83	0.64 - 1.01	< 0.001	61.12	93%	< 0.001	N/A	N/A

n= total samples, SMD= standard mean difference, 95% CI= 95% confidence intervals, I²= the primary index for reporting heterogeneity, z= significance tests for the weighted average effect size, N/A= not applicable

Table 8. *Efficacy of BSFL meals on electrolyte levels of broiler chickens*

Parameter	n	Random Effect			Heterogeneity			Egger's test	
		SMD	95% CI	P-value	Chi²	I²	P-value	z	P-value
Calcium (mg/dL)	408	1.64	1.34 - 1.95	< 0.001	353.99	99%	< 0.001	4.5363	< 0.001
Phosphorus (mg/dL)	588	0.78	0.54 - 1.02	< 0.001	568.42	99%	< 0.001	1.8924	0.0584
Magnesium (mEq/L)	588	0.44	0.27 - 0.62	< 0.001	106.59	96%	< 0.001	N/A	N/A
Iron (µg/dL)	408	0.19	-0.02 - 0.40	0.07	115.88	97%	< 0.001	1.2430	0.2139

n = total samples, SMD = standard mean difference, 95% CI = 95% confidence intervals, I² = the primary index for reporting heterogeneity, z = significance tests for the weighted average effect size, N/A = not applicable

were reported P-values <0.05. Meanwhile, the parameters of weight gain, feed intake, slaughter weight, carcass yield, meat color, hematological traits, serum proteins and lipids did not represent publication bias with P-value >0.05 (*Table 3*, *Table 4*, *Table 5*, *Table 6*, *Table 7*, *Table 8*).

Discussion

In this meta-analysis study, BSFL inclusion in feeding compared with basal feed as a control, and the findings demonstrated that BSFL was effective in terms of weight increase and feed efficiency. In a previous investigation, feeding BSFL at a ratio of 2:3 with basal diet improved the weight gain and feed efficiency of the chickens during the starting phase, contributing to improved growth performance [17]. According to other studies, utilizing BSFL at a ratio of 1:5 with basal diet improved average daily weight growth as well as final weight [12,15,18-20]. The recommended daily feed intake was stated to be highly elevated during the early period. The late period of broiler chicken growth was characterized by optimal growth, as indicated by adequate feeding efficiency and body weight measures. Due to the growth and development progress

of broilers at an accelerated rate during the initial stage, it is thought to be the most crucial phase in the production process [21].

The nutrient composition of BSFL meal to rear chickens was found to be adequate and it can be regarded as a rich source of digestible amino acids and energy, resulting in a nutritious feed product for poultry. In investigations conducted to replace soybean meal with BSFL as a source of protein, BSFL showed favorable findings concerning growth performance [22]. Because of its higher protein concentration and preferable amino acid properties than many vegetable proteins, BSFL may be a desirable feed ingredient. However, the large substrate composition variations also affect its nutritional properties. A previous study reported a wide range in nutrient composition, including crude proteins (33-55%), calcium (2.4-5.8%), lysine (1.9-2.7%), and methionine (0.5-0.8%), because these larvae were raised on a variety of substrates, thereby such as organic material, vegetables and fruit wastes, chicken manure, and the pantry garbage [23].

Previous research linked increased feed efficiency to the benefits of diets high in medium-chain fatty acids (MCFA), which improve digestion and nutrient absorption. The conflicting results show that the chitin in BSFL, which is indigestible by monogastric animals and may reduce the digestibility of proteins, could be the reason why the feed efficiency has decreased. Because of this, the final weight of broiler chickens falls as the amount of BSFL in their diet rises, potentially endangering the output of intensive rearing techniques [24]. As a source of saturated fatty acid (SFAs), the BSFL mostly consisted of lauric acid (52.6%), myristic acid (8.54%), and palmitic acid (10.9%), and collectively contributed to 72% of the total fatty acid methyl ester (FAME). Lauric acid is one of the medium-chain fatty acids (MCFA), which are especially advantageous as dietary supplements and are widely recognized for their antimicrobial properties through disruption of cellular membranes [18]. It was reported that BSFL was composed of comparatively rare polyunsaturated fatty acids (PUFAs), which were mostly reflected by α-linoleic acid (7.8% of PUFAs) and linoleic acid (89.9% of PUFAs) [25]. The substantial fatty acid content of BSFL generally degrades the fat composition of the meat, which is visible as contemporary consumers shift concerning more nutritious meat and meat-related products. This is undoubtedly an area that continues to demand investigation. To determine whether the fatty acid profile of BSFL fat may be improved through substrate composition, a study should be done as the fatty acid properties of BSFL fat may fluctuate significantly depending on the rearing substrate applied [26,27].

In the previous study, diets including 10% and 15% BSFL meal instead of soybean meal and soybean oil did not have an adverse effect on the weight of breast muscles or their percentage of the overall weight of the growing broiler carcass [15,18,19]. Despite certain outliers that demonstrated a linear effect, such as C12:0, C15:0, and C18-PUFAs, the increasing BSFL fat in chicken diets primarily reveals a linear response in terms of summarised fatty acids and a quadratic response in the case of individual SFAs and unsaturated fatty acid (UFAs) [28]. It is notable to highlight that the significant amount of BSFL fat (60 and 90 g/kg) in broiler diets led to a beneficial rise in the content of PUFA in the breast meat, especially as a result of increased linolenic acid (C18:2). In contrast, oleic acid decreased monounsaturated fatty acid (MUFA) levels (C18:1). In the end, the higher UFA level and lower SFAs point to a positive impact for consumers [29]. A dose-dependent decrease in BSFL fat in the hens' meals, however, should not be linked to the elevated linolenic acid concentration. It should be highlighted that although feed intake did not rise in birds fed more BSFL fat, FAs in tissues might be synthesised. Furthermore, it is widely known that elevated PUFAs reduce 9-desaturase activity, which in turn inhibits MUFA production in the liver [30]. However, another study found that the proportion of pectoral muscles increased while the proportion of quadricep muscles drastically decreased in broiler-fed dietary regimens with 5% BSFL, a significantly lower proportion of BSFL meal [31].

This study demonstrated that proteins' water-holding capacity (WHC) increases as they shift beyond their isoelectric point because they absorb excess water [32]. Since well-hydrated meat absorbs light more intensely and has a different pH value in different muscle parts, an increase in WHC can therefore have an impact on the color of the meat. In the present study reported a slight correlation between the aforementioned associations and drip loss independently. Nonetheless, the broilers fed BSFL meal had an elevated L* (lightness) contribution and b* (yellowness). The carcasses of broiler chickens fed BSFL meal had slightly higher levels of L*, b* and a* (redness). Reduced soybean meal gluten levels in meals containing insect meal may lead to lower yellowness and redness due to the buildup of pigments from the BSFL meal [17,18,33].

The physiological parameters reviewed in this study demonstrated that the animals' health state was unaffected by the BSFL diet. Furthermore, routine blood parameters measured for this investigation fell within the standard physiological range. The hematology profile of broiler chickens and turkeys fed BSFL did not alter significantly [10,18]. A prior study found that serum samples containing BSFL fat had lower levels of high density lipoprotein cholesterol (HDL-C) and total cholesterol. In contrast, BSFL fat had no effect on triglycerides, uric acid, alanine aminotransferase (ALT), or aspartate aminotransferase (AST) [34]. This outcome can be explicated through BSFL's high lauric acid

concentration. An increase in HDL content was linked to increased ApoA1 secretion brought on by MCFA use. The main protein component of HDL particles is apolipoprotein A1 (ApoA1). HDL-C development is significantly influenced by ApoA-I. 70% of ApoA-I is produced in the liver, making it the main organ responsible for both its synthesis and excretion. An increase in HDL levels in the blood can result from elevated ApoA-I levels [31]. When compared to the control group, the erythrocyte level and H/L ratio of the chickens fed BSFL showed a favorable effect [35]. On the other hand, leukocyte counts were within normal limits, suggesting that the broiler chickens' immune systems were unaffected by the feeding regimen [24]. Elevated serum AST, ALT, and gamma-glutamyl transferase (GGT) activities serve as indications of liver necrosis and are typically linked to liver injury. The finding that AST or ALT activity was unaffected implies that BSFL feeding may not be harmful to the health of the liver or the hepatopancreas [22]. Mean creatinine levels did not exhibit any negative effects, suggesting that BSFL meal had a comparable impact on renal physiology [19,36,37]. When the broiler chickens and mirror carps were fed BSFL meal instead of soybean meal, their haematological characteristics were unaffected, thereby, confirming the nutritional sufficiency of BSFL. The increased phosphorus bioavailability in BSFL meal may cause of the elevated blood phosphorus concentrations detected in chickens fed with BSFL diet [19,25]. Similarly, supplementing broilers with BSFL raised their blood albumin and total protein levels. Since MCFA are more ketogenic than long-chain fatty acids (LCFA), the body can produce more proteins. Furthermore, because MCFA offers a sufficient energy source, less protein is used as an energy source, which raises the concentration of protein [38]. The conclusions drawn from the earlier studies are placed in context by the wide range of results found in the previously described studies. This heterogeneity may also be related to the composition of the BSFL meal that was utilized, which can be influenced by the life stage of the insect (adult, larva, or pupa), the substrate used for insect rearing, the defatting process, and the growth season during which the chickens were fed.

Conclusion

Out of 1878 studies, 21 studies covering the years 2016-2024 on the nutritional benefits of BSFL meal for broiler chickens were reviewed using meta-analysis. The study's findings demonstrated that feeding broiler chickens BSFL meals improved the chickens' weight gain, feed efficiency, carcass percentage, muscles in the breast and thighs, cooking loss, drip loss, and the lightness and yellowness of their meat. Meanwhile, favorable results were also reported for serum glucose, erythrocytes, liver and renal physiology, and electrolyte properties.

Declarations

Availability of Data and Materials: All the generated data are included in the manuscript.

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Research Article

Anticoccidial Effects of *Trachyspermum ammi* Essential Oil Against Caecal Coccidiosis in Broiler Chickens

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Abstract

In present study the anticoccidial activity of essential oil of *Trachyspermum ammi* (seeds) was evaluated by *in vivo* methods. For *in vivo* trial, total of 72 (day-old) broiler chicks were randomly divided into six groups A, B, C, D, E and F (each group having 12 chicks). At one week of age groups A, B, and C were treated with essential oil of *Trachyspermum ammi* (seeds) at concentration 1%, 2%, and 3% by supplementing in feed for three consecutive days. Group D served as positive control (infected and medicated with Toltrazuril treatment), group E served as negative control group (infected and non-medicated) and group F as normal (non-infected and non-medicated) control group. All groups except F were infected orally with 50.000 sporulated oocysts of mixed *Eimeria* species. Anticoccidial potential of essential oil was determined by different parameters such as mortality rate, fecal score, oocyst per gram of feces, weight gain, feed conversion ratio. Furthermore, hematological and serum chemistry profile was also evaluated. Hence treatment of *Trachyspermum ammi* (seeds) essential oil at dose rate 1%, 2% and 3% supplementation produces anticoccidial effects in terms of enhanced feed conversion ratio, decreased fecal and OPG count and improved weight gain in infected chickens. These treatment of essential oil showed to put positive effects also on hematological parameters like hemoglobin level, packed cell volume, red blood cells and white blood cells count and also put positive response towards serum biochemistry.

Keywords: *Trachyspermum ammi*, Essential oil, Coccidiosis, Chicken

Introduction

Poultry meat is one of the significant sources of proteins, fats, natural and inorganic constituent's which require to our daily life. The poultry business is increasing day by day however, coccidiosis is major parasitic disease effecting poultry industry overall and causing huge financial loss all over the world [1,2]. In poultry industry coccidiosis is one of the major intestinal parasitic disease of chicken caused by *Eimeria* species. The disease is caused by *Eimeria* protozoa having various species that harm the digestive system of avian host which eventually cause bloody diarrhea, decline feed consumption and reduce weight gain [3]. Anticoccidial drugs are used to control coccidiosis in chickens administered through water and feed, this approach is the main pillar in coccidiosis

control program. Different classes of anticoccidials have been used previously. Chemical drugs like Amprolium, Clopidol, Halofuginone that are extensively used against coccidiosis. Nonetheless, the exorbitant utilization of anticoccidial drugs have brought emergence of resistance in *Eimeria* species in different countries including Pakistan [4]. Moreover, the constant utilization of anticoccidial drugs prompted the poisonous consequences for birds and residual impacts of these medications in poultry items [5]. Furthermore, immunization is also an effective approach in controlling avian coccidiosis. However, immunization may not be successful due to geological varieties of *Eimeria* strain [6]. Among recent approaches, alternative plant drive compounds have shown promising results against coccidiosis.

The plants belonging to family *Asteraceae, Lamiaceae* and *Apiaceae* are well known to possess antiparasitic properties. The different plant components and their essential oils frequently studied due to having positive response in controlling avian Coccidiosis [7]. *Trachyspermum ammi* that is also known as "Ajwain" is well-known medicinal plant that have therapeutic activities. Therefore, keeping in view the anticoccidial potential of various botanicals and their essential oils in the light of previous reports, the current study was planned to evaluate the anticoccidial potential of essential oil of *Trachyspermum ammi* (seeds). The specific goals of this study was: To determine the *in vivo* anticoccidial potential of essential oil of *Trachyspermum ammi* (seeds) in coccidiosis infected broiler chickens.

Material and Methods

Ethical Statement

This research was conducted with the ethical approval of ethics committee on 20/2/18 NO. DGS/3333-36

Trachyspermum ammi Essential Oil

Trachyspermum ammi essential oil was procured from the National® company from Faisalabad Pakistan that was 100% pure form. The essential oil was stored at 4ºC for proper usage in coccidiosis infected groups to monitor the efficiency of essential oil against naturally infected broiler chickens from coccidiosis.

Parasite Collection and Preservation

Poultry guts were collected from different sale points in Faisalabad. Positive guts cecal material were collected in 2.5% potassium dichromate solution. The parasite oocysts were recovered by sedimentation technique and different *Eimeria* species were identified under microscope. To ensure the purity of oocysts washed to remove debris in sodium hypochlorite solution. The washed oocysts were kept in potassium dichromate solution of 2.5% in incubator. To ensure the regular supply of oxygen proper string of solutions were maintained after every four hours. The standard temperature (35-39ºC) and humidity (60-80%) were maintained in the incubator for proper sporulation of coccidian oocysts [8]. After 48 to 72 h overall sporulation was noticed under light microscope at 40X and sporulated oocysts were separated and counted by McMaster technique. Then challenged dose (50.000/bird) of sporulated oocysts were prepared in order to induce coccidiosis in broiler chickens [9].

Birds Management

Day old 72 chicks were purchased from Big Bird Company® in Faisalabad Pakistan. The birds were reared under standard conditions. All chicks were given offered anticoccidial free feed. The first two weeks starter ration was given and next followed to finisher ration till seven weeks of age. The chicks were vaccinated for infectious bronchitis disease, Newcastle disease and infectious bursal disease according to standard vaccination schedule [10]. The temperature was maintained 90-95ºC (32.2 to 35ºC) in first week and maintained 75ºC (23.8ºC) at fourth week. The proper light intensity was maintained during 24 h till 42 days of age.

Experimental Design

At day 7th the all chicks were be divided into six equal groups A, B, C, D, E and F every one including 12 chicks. At 7th day all groups excluding F were orally immunized sporulated oocysts. The first three groups (A, B, C) were treated with the essential oil of *T. ammi* at concentrations of 1%, 2% and 3% respectively. Group D served as infected medicated (IM) and was treated with rational anticoccidial drug Toltrazuril. Group E served as infected non-medicated (INM) and Group F served as normal control group (non-infected, non-medicated) (NINM). The medication continued for three consecutive days with essential oils.

Evaluated Parameters

Following anticoccidial and growth performance parameters were evaluated in study.

Mortality Rate

In this parameter the death rate of chickens was determined. The death of chicken due to environmental stress, nutrition, disease outbreak or any other factor was recorded. The mortality rate was calculated by the overall number of dead birds/ live birds.

Fecal Score

The fecal score of birds from each group were monitored to estimate the disease intensity. Hence the standard fecal score chart was followed to estimate fecal score in chickens. From day 3rd to day 7th of post inoculation fecal score was estimated chart from 1 to 5 numbers in ascending order as described [10].

Oocyst per gram of feces (OPG)

McMaster technique was used to evaluate the number of oocysts per gram of feces. The OPG was performed on the 7th and 14th day of post infection. In this procedure 3 g feces were mixed in 42 mL saturated sodium chloride solution. After sieving the solution was poured in beaker. The 0.3 mL feces sample was poured in each chamber of Master. The McMaster chamber was kept undisturbed for 2 to 3 min. Then oocysts were examined in the chamber microscopically

Feed Conversion Ratio

The feed conversion ratio was determined by following formula

Feed conversion ratio = Average quantity feed consumed (gm)/Average weight gain (gm)

Weight Gain of Chicks

The average weekly weight gain of each group was recorded. The average weight gain was mentioned according to feed consumption. To access the disease Burdon, the average weight gain was calculated in each group in order to identify the medicinal response in controlling disease outbreak.

Hematology and Serum Chemistry

All blood parameters were checked after 35th day infection. Hematological parameters like red blood cells count (RBC), white blood cells (WBC) and hemoglobin (Hb) were estimated by standard protocols. The counting of RBC and WBC were done with the help of Sahlis apparatus. The hematological parameters like hemoglobin determination, monitoring of packed cell volume (PCV), counting of erythrocyte and leukocyte were done according to standard protocol [11]. Serum chemistry was also analyzed by using manufactured Kits.

Statistical Analysis

Different Parameters were statistically analyzed by ANOVA, SAS statistical analysis software (SAS, 2004) and data was considered significant at P≤0.05.

Results

The results of different parameters were discussed to evaluate the potential of plant driven essential oil of *Trachyspermum ammi* at concentration of 1%, 2% and 3% in feed. The mortality rate of different group is given in *Table 1*. *T. ammi* essential oil reduced mortality rate in infected birds and maximum survival rate was observed in birds treated with 3% of *T. ammi* essential oil which was significantly different to (P<0.05) infected group.

Table 2 shows that mean fecal score of *T. ammi* essential oil treated groups was significantly different to (P<0.05)

Table 1. *Mortality rate in in different treatment groups*

Treatment Groups	Mortality Days Post Infection					Total Mortality	Mortality %Age	Survival %Age
	3rd	4th	5th	6th	7th			
T. ammi 1%	1	-	-	-	–	1	0.083	0.92
T. ammi 2%	-	1		-	1	2	0.17	0.83
T. ammi 3%	–		1-	-	-	1	0.083	0.92
IM		-	1	-		1	0.083	0.92
INM	-	1		1	–	2	0.17	0.83
NINM	–	-	-	-	-	-	0	100

IM: *Infected medicated,* ***INM:*** *Infected non medicated,* ***NINM:*** *Non infected non medicated*

Table 2. *Mean fecal score in different treated groups*

Treatment Groups	Fecal Score				
	3rd Day	4th Day	5th Day	6th Day	7th Day
T. ammi 1%	-	3.67 ± 0.33^{AB}	3.00 ± 0.00^{A}	2.67 ± 0.33^{A}	-
T. ammi 2%	-	3.00 ± 0.00^{B}	2.33 ± 0.33^{AB}	2.00 ± 0.00^{AB}	-
T. ammi 3%	-	1.67 ± 0.33^{C}	1.33 ± 0.33^{BC}	1.67 ± 0.33^{AB}	-
IM	-	1.33 ± 0.33^{C}	1.00 ± 0.00^{CD}	1.33 ± 0.33^{B}	-
INM	-	4.33 ± 0.33^{A}	3.33 ± 0.33^{A}	2.67 ± 0.33^{A}	-
NINM	-	0.00 ± 0.00^{D}	0.00 ± 0.00^{D}	0.00 ± 0.00^{C}	-

Means sharing similar letter in a column are statistically non-significant (P>0.05)
IM: *Infected medicated,* ***INM:*** *Infected non medicated,* ***NINM:*** *Non infected non medicated*

Table 3. *Mean oocyst per gram of feces (OPG) in different treated groups*

Treatment	OPG (x 10^3 g-1) at Day 7th	OPG (x 10^3 g-1) at Day 14th
T. ammi 1%	77.37 ± 1.02^{B}	50.85 ± 2.01^{B}
T. ammi 2%	70.40 ± 0.61^{BC}	45.87 ± 0.87^{BC}
T. ammi 3%	65.75 ± 1.02^{C}	39.44 ± 1.17^{C}
IM	64.22 ± 1.01^{C}	39.45 ± 1.58^{C}
INM	95.70 ± 3.41^{A}	75.76 ± 2.52^{A}
NINM	0.00 ± 0.00^{D}	0.00 ± 0.00^{D}

Means sharing similar letter in a column are statistically non-significant (P>0.05)
IM: *Infected medicated,* ***INM:*** *Infected non medicated,* ***NINM:*** *Non infected non medicated*

Table 4. *Feed conversion ratio (FCR) in different treated groups*

Treatment Groups	FCR
T. ammi 1%	2.41
T. ammi 2%	2.32
T. ammi 3%	2.21
IM	2.22
INM	2.61
NINM	2.20

IM: *Infected medicated,* ***INM:*** *Infected non medicated,* ***NINM:*** *Non infected non medicated*

Table 5. *Mean weight gain (weekly basis) in different treated groups*

Treatment	WG (g) 1st WK PI	WG (g) 2nd WK PI	WG (g) 3rd WK PI	Total WG (g)
T. ammi 1%	207.33 ± 4.67^{C}	241.33 ± 5.36^{B}	366.00 ± 3.79^{A}	356.00 ± 5.20^{C}
T. ammi 2%	267.67 ± 4.63^{B}	262.00 ± 5.20^{AB}	351.33 ± 5.49^{A}	378.00 ± 4.93^{BC}
T. ammi 3%	296.00 ± 2.08^{A}	274.00 ± 2.65^{A}	354.00 ± 4.00^{A}	387.67 ± 4.63^{AB}
IM	301.67 ± 5.49^{A}	279.33 ± 5.49^{A}	358.00 ± 4.36^{A}	403.33 ± 2.03^{A}
INM	201.00 ± 5.51^{C}	197.67 ± 2.91^{C}	273.00 ± 2.08^{B}	292.33 ± 5.55^{D}
NINM	285.00 ± 3.21^{AB}	278.00 ± 4.62^{A}	347.67 ± 4.33^{A}	386.00 ± 5.51^{AB}

Means sharing similar letter in a column are statistically non-significant (P>0.05)
WG (g): *Weight in grams,* ***WKPI:*** *Week after Post infection*
IM: *Infected medicated,* ***INM:*** *Infected non medicated,* ***NINM:*** *Non infected non medicated*

Table 6. *Mean Heamatological values in different treated Groups*

Treatment	PCV%	Hb g/dL	RBC 10*6/µL	WBC 10*3/µL
T. ammi 1%	27.63 ± 0.20^{AB}	12.83 ± 0.09^{A}	4.04 ± 0.04^{A}	24.84 ± 0.10^{B}
T. ammi 2%	28.23 ± 0.17^{A}	11.80 ± 0.10^{B}	3.28 ± 0.04^{C}	25.67 ± 0.41^{B}
T. ammi 3%	28.42 ± 0.27^{A}	12.77 ± 0.19^{A}	3.66 ± 0.01^{B}	22.16 ± 0.10^{C}
IM	27.25 ± 0.19^{B}	11.33 ± 0.17^{B}	2.82 ± 0.06^{D}	22.53 ± 0.17^{C}
INM	19.20 ± 0.10^{C}	11.17 ± 0.12^{B}	1.87 ± 0.04^{E}	34.00 ± 0.58^{A}
NINM	27.55 ± 0.23^{AB}	7.34 ± 0.10^{C}	3.36 ± 0.04^{C}	21.33 ± 0.33^{C}

Means sharing similar letter in a column are statistically non-significant (P>0.05)
IM: *Infected medicated,* ***INM:*** *Infected non medicated,* ***NINM:*** *Non infected non medicated*

Table 7. *Mean serum enzymes values in different treated groups*

Treatment	ALT	AST	LDH	Urea	Creatinine
T. ammi 1%	10.23±0.15^{B}	174.37±2.99^{B}	478.00±6.08^{B}	5.32±0.10^{B}	0.16±0.01^{B}
T. ammi 2%	9.87±0.11B^{C}	168.00±4.36^{B}	462.00±7.23^{B}	5.50±0.14^{B}	0.15±0.01^{B}
T. ammi 3%	9.29±0.14B^{C}	174.00±3.51^{B}	468.00±9.50^{B}	5.42±0.11^{B}	0.16±0.00^{B}
IM	9.61±0.25B^{C}	181.67±2.73^{B}	477.00±5.51^{B}	5.29±0.10^{B}	0.17±0.01^{B}
INM	23.67±0.44^{A}	277.00±4.36^{A}	891.33±5.81^{A}	19.68±0.30^{A}	0.57±0.01^{A}
NINM	8.92±0.03^{C}	184.00±2.65^{B}	468.42±9.03^{B}	5.37±0.18^{B}	0.16±0.01^{B}

Means sharing similar letters in a column are statistically non-significant (P>0.05).
IM: *Infected medicated,* ***INM:*** *Infected non medicated,* ***NINM:*** *Non infected non medicated*

infected group. Mean fecal score was reduced in *T. ammi* essential oil treated groups at different days of post *Eimeria* infection in birds.

Table 3 shows that mean Oocyst per gram (OPG) of *T. ammi* essential oil of treated group was significantly different to (P<0.05) infected group. OPG was significantly reduced and minimum OPG was observed groups treated with 3% of *T. ammi* essential oil.

The *Table 4* clearly showed that essential oil treated groups exhibited improved feed conversion ratio (FCR) in chickens infected with mixed *Eimeria* species. Groups treated with *T. ammi* essential oil shown to improve FCR and better FCR was observed at higher dose of *T. ammi* essential oil.

T. ammi essential oil treated groups also improved mean weight gain as compared to infected group (P<0.05) *Table 5*. *Table 5* shows that weight gain was significantly improved in birds treated with *T. ammi* essential oil in dose dependent manner.

Table 6 shows that mean hematological parameters of natural oil treated group were improved was significantly different (P<0.05) from infected group. Mean PCV, HB, RBCs and WBCs values were improved which shows positive impact on hematological parameters in phase of infection.

Similarly, *T. ammi* essential oil improved serum chemistry parameters (ALT, AST, Urea, Creatinine) in infected birds which shows that it has no toxic effects on birds and these values which were significantly different (P<0.05) from infected group as shown in *Table 7*.

Discussion

Recent investigations about treating the coccidiosis showed that using herbal medicine having antioxidant compounds like flavonoids and phenols are batter choice to avoid the resistance [7]. Furthermore, botanical driven essential oils redirect the consideration of numerous analysts toward poultry industry for their helpful potential and therapeutic effects. There are a few reports that demonstrated the positive effect of natural compounds like essential oils for use them against different diseases and role in improvement of parameters like intestinal performance and feed conversion ratio in animals [12-14]. The *Trachyspermum ammi* and *Anethum graveolens* are both popular plants having medicinal values in controlling different infectious diseases and hence found in all over the world. The different studies proved that *T. ammi* (seeds) is an excellent remedy for the gastrointestinal problems including antimicrobial, antiviral, antifungal and antioxidant potential [15,16].

The *T. ammi* plant belong the *Apiaceae* family. The *T. ammi* is one of the most traditional medicinal plants known due to its wide therapeutic properties like antifungal, antibacterial, antihypertensive, antitussive, antioxidant, antispasmodic, bronchodilation, antifilarial, antiseptic, and anthelmintic potential [17]. The essential constituents found in the *T. ammi* seed are thymol and carvacol as well other fractions including p-cymene, g-terpiene and β-pinene [18]. The scientist proved that therapeutic and antiparasitic potential of essential oil of *T. ammi* is due to the presence of essential component like thyumolm and carvacol [19]. The essential oils are more effective as compare to synthetic drugs and are less toxic, no drug residues in poultry meat and can be used as multipurpose against various infectious disease like coccidiosis [20].

In an experimental trial the ethanolic extract of *T. ammi* was utilized to show the inhibitory action on different enzymes like lactate dehydrogenase that is the regulatory key of a parasitic nematode *H. contortus*. The ethanolic extract found to oppose the enzyme activity in favour of worm *H. contortus* in fovour to inhibit to the energy metabolic pathway [21]. Therefore, it was concluded that the prohibition of LDH enzyme, inhibition of protein synthesis and ATP prohibition potential of essential oil put a positive behavior toward the anthelmintic potential of essential oil of *T. ammi* oil. Hence the inhibition of LDH enzyme in maintenance of *H. contortus* proved that *T. ammi* has a main role to restrict the development of the worm [22]. The active constituents present in the essential

oil of *T. ammi* are thymol and carvacol have been used in different therapeutic potential to monitor the various infectious diseases of viral, bacterial, parasitic and to boost up immune potential of the host. The *T. ammi* essential oil components have the potential to fight against the various infectious agents of parasitic origin including filarial worms like *Setaria digitate*. Hence it was concluded that the methanolic extract of *T. ammi* having the therapeutic potential against the filarial worms [23,24].

The current study was planned by keeping in view the antiprotozoal potential of essential oil of *T. Ammi* in broiler chicken preciously infected from coccidiosis. The *in vivo* potential was measured in terms of improved weight gain, effective feed conversion ratio, decrease oocyst per gram of feces, reduced bloody diarrhea and better feed consumption ratio. The *T. ammi* treatment results in favorable anticoccidial potential in terms of substantial efficacy ($P<0.05$) from diseased non medicinal group and comparable to diseased medicinal group (G, D). The intake of essential oil of *T. ammi* favored a positive response toward the hematological values. The *T. ammi* having positive response in measuring serum biochemical values like AST, ALT, urea creatinine, LDH proved to be hepatoprotective and nephroprotective effect [25,26]

Hence, this study concludes that the supplementation of essential oil in broiler feed has given similar results as in the previous studies [27-29]. In this experimental trial the supplementation of essential oil of *T. ammi* was given to the broiler chickens infected from coccidiosis at a concentration of 1%, 2% and 3% in feed. The intake of essential oil of 3% in feed showed comparable results to treated groups with Toltrazuril medicine in controlling coccidiosis in terms of increased feed intake, improve weight gain, maintenance of intestinal gut health, decreased bloody diarrhea, decreased fecal scoring, and improved lesion scoring. The *T. ammi* treatment also put positive response in controlling coccidiosis in terms of hematological monitoring and serum biochemical values (ALT, AST Urea and Creatinine). Hence it was demonstrated that the treatment of coccidiosis with the essential oils of *T. ammi* oil is a likeable approach for the future prospective in poultry industry. Therefore the *T. ammi* essential oil 3% in feed can be used as best alternative for controlling coccidiosis disease in broiler chickens [30].

Declarations

Availability of Data and Materials: Data will be offered by the author (R. Fayyaz) on demand.

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Author Contributions: MRF intended the study; KH, AA, SS, SI, AR, SAR, MUW, RZA, HS, MK, WAH, MMM helped in methodology, work plan, and statistical analysis.

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Research Article

Inhibitory Effect of Immune Checkpoint Cytotoxic T-Lymphocyte-Associated Antigen-4 Inhibitor Combined with Curcumin on Tumor Growth in Mice with Lung Cancer

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Abstract

We aimed to investigate whether immune checkpoint cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) inhibitor plus curcumin can inhibit tumor growth in mice with lung cancer. A mouse model of lung cancer was established by injecting Lewis lung cancer cells. The mice were allocated to an anti-CTLA-4 group, a curcumin group, an anti-CTLA-4+curcumin group, and a Model group in random (n=10). The tumor inhibition rate and metastasis inhibition rate were calculated. Hematoxylin-eosin staining was performed on tumor tissues for their pathological changes. The apoptosis rate was measured by TUNEL assay. In comparison with the Model group, the tumor volume, tumor mass, number of lung metastatic nodules, and the protein and relative mRNA expressions of Bcl-2 in tumor tissues significantly decreased in anti-CTLA-4, curcumin, and anti-CTLA-4+curcumin groups, while the tumor inhibition rate, metastasis inhibition rate, apoptosis rate of tumor cells, and Caspase-3 and Bax contents in tumor tissues increased (P<0.0001). CTLA-4 inhibitor plus curcumin can inhibit tumor growth in mice with lung cancer, which may be associated with the promotion of lung cancer cell apoptosis by regulating the Caspase-3/Bcl-2/Bax signaling pathway.

Keywords: Curcumin, Tumor growth, Inhibitor, Lung cancer

Introduction

As a leading frequently occurring malignancy in clinical practice, lung carcinoma is dominated by NSCLC, taking a proportion of nearly 85% [1]. Based on a statistical report in 2020 [2], there are about 2.207 million confirmed cases and about 1.796 million death cases of pulmonary carcinoma each year. Surgery and radiotherapy have been commonly adopted for treating this carcinoma over the past few years, but the survival rate of patients within 5 years is still below 15% due to adverse reactions such as chemotherapy resistance. Hence, exploiting efficacious therapeutic drugs is of particular importance for lung cancer for ameliorating the prognosis of patients [3].

Recently, immunotherapy has demonstrated significant survival benefits among patients with malignant solid tumors, such as malignant melanoma and NSCLC. As a novel anti-tumor drug for immunotherapy, immune checkpoint inhibitors (ICIs) have been widely studied [4]. Currently, the most representative ICIs in clinical practice range from PD-1 inhibitors, PD-L1 inhibitors to CTLA-4 inhibitors. Of note, CTLA-4 inhibitor can be applied in dual-immunotherapy, which greatly enriches the modes of tumor immunotherapy [5,6]. Specifically, the combination of nivolumab with anti-CTLA-4 demonstrates better therapeutic efficacy than chemotherapy alone on advanced NSCLC tumors [7].

Curcumin, a diketone compound extracted from Chinese herbal medicine, is supported to obstruct the malignant phenotypes of cancer cells and demonstrates optimal efficacy in preclinical as well as clinical investigations [8]. Previous research has illuminated that curcumin can facilitate apoptosis in liver cancer cells, coupled with descended Bcl-2 content, as well as ascended Bax and Caspase-3 contents [9]. Intriguingly, Endo et al. have

corroborated that curcumin can stimulate apoptosis in lung cancer [10]. Nevertheless, the therapeutic impacts of CTLA-4 inhibitor plus curcumin on lung cancer cell apoptotic level remain vague.

Collectively, this paper was conceived to plumb the efficacy of CTLA-4 inhibitor plus curcumin on the tumor growth in lung cancer mice models, which might preliminarily underscore the potential of CTLA-4 inhibitor plus curcumin as a prospective candidate for lung cancer relieve.

Materials and Methods

Ethical Approval

This study has been approved by the ethic committee of our university (No. JSNJM2022104), date: 13th/5/2022, and great efforts have been made to minimize the animals' suffering.

Animals

SPF-grade BALB/c mice sourced from Chengdu Dossy Experimental Animals Co., Ltd. (China) [18-22 g, n=40, male, animal license No. SCCK (Sichuan) 2020-030]. All mice were adaptively raised for one week in temperature-controlled (23-25°C) cages with 12/12 h light-dark cycle, together with relative humidity of 50-60%, allowed for freely drinking water and eating fixed food.

Reagents and Apparatus

Mouse Lewis lung cancer cell lines were purchased from ATCC (USA), curcumin (purity: 98%) was purchased from Zhengzhou Linuo Biotechnology Co., Ltd. (China). Anti-CTLA-4 was offered by Shanghai Yihui Biotechnology Co., Ltd. (China). Antibodies against Caspase-3, Bcl-2, GAPDH, and Bax sourced from Proteintech (USA). TUNEL was provided by Shanghai Beyotime Biotechnology Co., Ltd. (China). A microplate reader (Model: PT-3502C) was bought from Beijing Putian Xinqiao Technology Co., Ltd. (China). An optical microscope was provided by Shenzhen OSWare Medical Instrument Co., Ltd. (China).

Tumor Model and Experimental Grouping

Mouse Lewis lung cancer cells were subjected to 10 min of 3000 r/min centrifugation, collection and preparation into a suspension. The resulting suspension was inoculated into the armpit of the right forelimb of mice at $1x10^6$ cells/mL (0.2 mL/mouse) for seven consecutive days. Modeling was considered successful if a soybean-sized tissue mass could be obviously palpated at the inoculation site. Then a Model group, an anti-CTLA-4 group, a curcumin group, and an anti-CTLA-4+curcumin group were established for random assignment of forty successfully modeled mice, each of which contained 10 mice. As for the anti-CTLA-4 group, anti-CTLA-4 was injected once every two days at 0.5 mg/kg into the mice *via* the tail vein for four times. In the curcumin group, the mice received intraperitoneal injection of 0.5 mL/mouse curcumin suspension once daily for eight days in a row. Mice in anti-CTLA-4+curcumin group received 0.5 mg/kg anti-CTLA-4 administration via the tail vein and intraperitoneal injection of 0.5 mL curcumin suspension. An equal volume of normal saline was intraperitoneally injected for eight days in the Model group.

Detection of Tumor Inhibition Rate

Twenty-four h post the final administration, the mice underwent euthanasia and were subjected to dissection, and the tumor was harvested to measure its maximum diameter *a* plus short diameter *b*. Then the tumor volume was calculated: $V=0.52a\times b2$, the tumor mass was weighed and the tumor inhibition rate was finally calculated.

Detection of Lung Metastasis Inhibition Rate

A portion of tumor tissues was frozen by liquid nitrogen rapidly for preservation in a -80°C refrigerator. The remaining tumor tissues underwent fixation using 4% paraformaldehyde solution. Then the two lungs were harvested, fixed with Buoin's solution, and washed 24 h later. Lung metastases were quantified under a light microscope, and the lung metastasis inhibition rate was finally calculated.

Hematoxylin-Eosin (HE) Staining for Pathological Changes in Tumor Tissues

The paraformaldehyde solution (4%)-fixed tumor tissues were taken out, treated by paraffin embedding, sliced into sections (thickness: 5 μm) by a microtome, and attached to glass slides, followed by deparaffinization and hydration. Finally, HE staining and observation using the microscope were performed on the sections.

TUNEL Assay for Detecting Apoptosis Rate

The resulting sections in 1.6 were subjected to dehydration, added dropwise with proteinase K (100 μL) solution and left to stand. 30 min later, the sections were added dropwise with TUNEL solution (50 μL) and left to stand away from light. 1 h later, stop buffer was supplemented for reaction termination. After 15 min, the sections were added dropwise with converter-POD solution (50 μL), left to stand away from light, and added with DAB solution (100 μL) for 10-min reaction 30 min later, followed by hematoxylin counterstaining and dehydration. Finally, the sections were transparentized with xylene and mounted with neutral resin, followed by microscope observation. Apoptosis index (%) = (number of apoptotic cells/total number of tumor cells) ×100%.

Table 1. Tumor growth and metastasis in mice (X±s, n=10)

Group	Tumor Volume (cm^3)	Tumor Mass (g)	Tumor Inhibition Rate ($\times10^{-2}$)	Number of Lung Metastatic Nodules	Metastasis Inhibition Rate ($\times10^{-2}$)
Model	6.85±0.72	6.48±0.67	0	15.21±1.67	0
Anti-CTLA-4	5.71±0.60*	5.39±0.56*	19.86	9.33±1.02*	31.54
Curcumin	5.63±0.59*	5.21±0.55*	20.07*	9.06±1.00*	32.67*
Anti-CTLA-4 + curcumin	4.26±0.45*#	3.46±0.48*#	41.21*#	5.23±0.55*#	65.18*#
F	31.45	48.53		132.2	
P	<0.0001	<0.0001		<0.0001	

** P<0.05 vs. Model group, # P<0.05 vs. Anti-CTLA-4 group and Curcumin group*

Western Blotting

The tumor tissues stored in the refrigerator at -80°C in 1.5 were taken out, added with 100 μL of pre-prepared lysate mixture, mixed well in a tissue homogenizer, and subjected to 15 min of 15.000 r/min centrifugation at 4°C. An EP tube was employed to acquire the supernatant, and BCA approach was adopted for concentration quantification. Next, protein samples (50 μg) were denatured *via* boiling, segregated utilizing SDS-PAGE, and translocated to PVDF membranes. Then 5% skim milk was added for 1-h membrane blocking, TBST solution was used for membrane washing, and primary antibodies (1:500) were supplemented for 4°C overnight membrane incubation. Subsequently, the membrane underwent 2 h of conventional incubation again under HRP-labeled secondary antibodies (1:1000). Next, the sample was added dropwise with ECL solution for development. Finally, images were acquired using a camera and the protein bands were analyzed for the gray value using Image Lab.

RT-qPCR

The tumor tissues were lysed on ice into a suspension, and lysed with TRIzol reagent. By reference to the kit instructions, total RNA obtained from tissues or cells was converted into cDNA through reverse transcription. In accordance with the PCR kit instructions, a 10 μL reaction system was prepared (5 μL of 2×SYBR Mix, 0.4 μL of cDNA, forward and reverse primers in a single volume of 0.4 μL, 0.4 μL of reference dye Rhodamine X, and 3.4 μL of DEPC-treated water), and quantitative RT-PCR was implemented. With the internal reference determined as GAPDH, 2-△△CT method was adopted for the calculation of gene expression level. The primers used were as follows:
Caspase-3 F: 5'-GGTGGCATCTCCTGTGATTGTG-3',
R: 5'-CAGGAGCTTCTGATCTGG-3'.
Bcl-2 F: 5'-CGGGAGATCGTGATGAAGTAC-3',
R: 5'-CTCAGGCTGGAAGGAGAAGA-3'.
Bax F: 5'-GCTACAGGGTTTCATCCAGGGT-3',
R: 5'-TGTTGTCCAGTTCATCGC-3'.
GAPDH F: 5'-CAAGGAGTAAGAAACCCTGGA-3',
R: 5'-CCCTGTTGTTATGGGGTCTGG-3'.

Statistical Analysis

GraphPad Prism 8.0 for statistics was employed. The format of mean ± standard deviation (X±s) was applied to present the measurement data. The normally distributed data underwent one-way ANOVA and comparison *via* the LSD-*t* test between two groups. The abnormally distributed data were subjected to the Kruskal-Wallis rank sum test. A difference of statistical significance was denoted with P lowering than 0.05.

Results

Tumor Growth and Metastasis in Mice

Relative to the Model group, the tumor volume, tumor mass and number of lung metastatic nodules were smaller in mice received the treatment of anti-CTLA-4 or/and curcumin, while the tumor inhibition rate and metastasis inhibition rate were significantly higher (P<0.0001). The anti-CTLA-4+curcumin group had significantly smaller tumor volume, tumor mass and number of lung metastatic nodules, and significantly higher tumor inhibition rate and metastasis inhibition rate than the anti-CTLA-4 plus curcumin groups (P<0.0001) *(Table 1)*.

Pathological Changes in Tumor Tissues

The lung cancer cells had an intact structure, and exhibited tumor cell morphology, without obvious necrosis, in the Model group. In the anti-CTLA-4, curcumin and anti-CTLA-4+curcumin groups, a lot of necrotic tumor cells were observed, most of the cells showed karyopyknosis or karyorrhexis, but karyokinesis was rare, they were arranged irregularly, and the intercellular space around the necrotic region increased and was distributed unevenly, especially in the anti-CTLA-4+curcumin group *(Fig. 1)*.

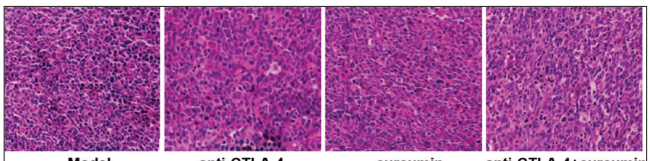

Fig 1. HE staining images of tumor tissues (×200)

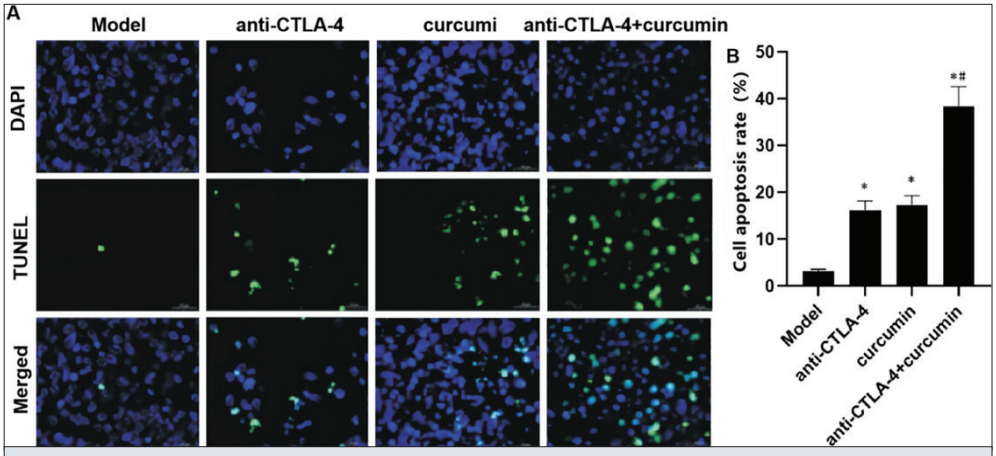

Fig 2. Tumor cell apoptosis rate. A: TUNEL assay for tumor cell apoptosis. B: The quantitative analysis of cell apoptosis. *P<0.05 *vs.* Model group, #P<0.05 *vs.* Anti-CTLA-4 group and Curcumin group

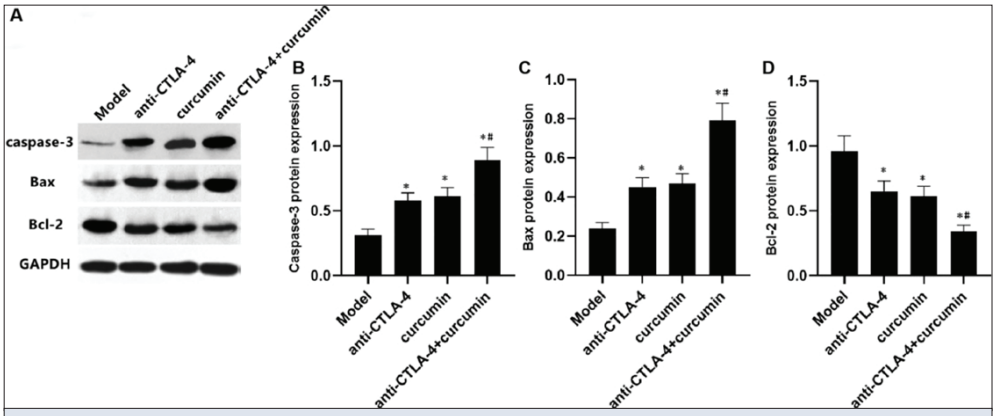

Fig 3. Protein contents in Caspase-3, Bcl-2 and Bax in tumor tissues. A: Protein bands of Caspase-3, Bcl-2 and Bax. B-D: Analysis of Caspase-3, Bax and Bcl-2 protein contents in tumor tissues. *P<0.05 *vs.* Model group, #P<0.05 *vs.* Anti-CTLA-4 group and Curcumin group

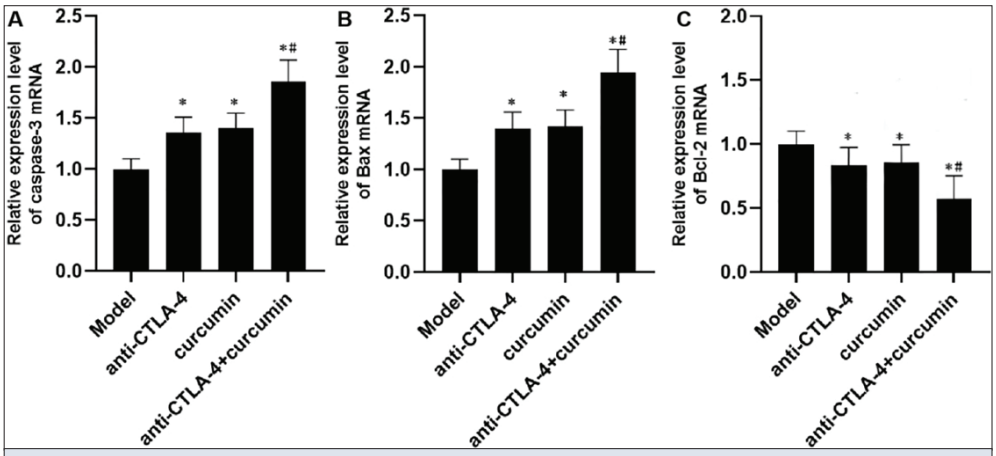

Fig 4. mRNA expressions of Caspase-3, Bcl-2 and Bax. A: Caspase-3 mRNA expression in tumor tissues. B: Bax mRNA expression in tumor tissues. C: Bcl-2 mRNA expression in tumor tissues. *P<0.05 *vs.* Model group, #P<0.05 *vs.* Anti-CTLA-4 group and Curcumin group

Tumor Cell Apoptosis Rate

Relative to the Model group, apoptosis in mice treated with anti-CTLA-4 or curcumin was conspicuously enhanced. Noticeably, the combination of anti-CTLA-4 and curcumin demonstrated more promotive impacts than anti-CTLA-4 or curcumin treatment alone (P<0.0001) *(Fig. 2)*.

Protein Expressions of Caspase-3, Bcl-2 and Bax in Tumor Tissues

Compared with the Model group, the anti-CTLA-4, curcumin and anti-CTLA-4+curcumin groups had significantly increased Caspase-3 plus Bax contents and a significantly reduced Bcl-2 content in tumor tissues

(P<0.0001). In the anti-CTLA-4+curcumin group, Caspase-3 and Bax contents were markedly ascended, whereas Bcl-2 descended significantly at the protein expression level in tumor tissues by contrast to the anti-CTLA-4 and curcumin groups (P<0.0001) *(Fig. 3)*.

mRNA Expression of Caspase-3, Bcl-2 and Bax in Tumor Tissues

The mRNA expressions of Caspase-3 plus Bax were distinctly increased, but that of Bcl-2 in tumor tissues significantly declined following the administration of anti-CTLA-4 or/and curcumin relative to the Model group (P<0.0001). Moreover, an enhancement in mRNA expressions of Caspase-3 and Bax together with a decline in mRNA expression of Bcl-2 in tumor tissues could be inspected in anti-CTLA-4+curcumin group relative with the anti-CTLA-4 and curcumin groups (P<0.0001) *(Fig. 4)*.

Discussion

Pulmonary carcinoma ranks among the malignant tumors featured by the highest incidence and death rates in China. Due to its high degree of malignancy and difficulty in early diagnosis, most patients have been in the mid-late stage or developed metastasis at the time of diagnosis, resulting in ineffective surgical treatment, a poor prognosis and an unsatisfactory overall survival rate [11]. Therefore, searching for effective treatment means is urgently needed so far.

Immune checkpoints are an immunoregulatory factor that can regulate the immune response by maintaining autoimmune homeostasis [12]. Under normal circumstances, tumor cells can cause disorders of antitumor immune response by inactivating some immune checkpoints, thus contributing to tumor growth and proliferation. ICIs can promote immune cell activity by suppressing immune checkpoints and inhibit tumor cells by activating autoimmune responses [13]. CTLA-4 is an immune checkpoint commonly used in recent years. As a transmembrane protein, CTLA-4 presents widespread expressions in activated T cells and conjugates with B7 ligand to suppress T lymphocyte activation, thus preventing T cells from attacking tumor cells [14]. It can be seen that blocking the CTLA-4 pathway can achieve an antitumor immune response by promoting immune cell proliferation. The ability of CTLA-4 inhibitor to inhibit the proliferation of childhood acute lymphoblastic leukemia cells, induce apoptosis, and hinder the growth of $ALDH^+$ stem-like tumor cells has been verified [15]. Curcumin, a phenolic pigment, possesses many advantages such as low toxicity, a variety of sources and low price, which can exert a good antitumor effect. It has been proved that curcumin inhibits various tumor cells from the aspect of propagation and movement [16]. Nevertheless, the effect of CTLA-4 inhibitor plus curcumin on tumor growth in mice with lung cancer remains unclear.

Lung carcinoma animal models have important significance for human studies on this disease, and appropriate animal models are particularly essential for research on the pathogenesis and diagnosis of this cancer as well as screening of drugs [17]. Heterotopic subcutaneous transplantation characterized by simple operation and easy observation of tumor formation has been widely used in clinic. In this study, Lewis lung cancer cell suspension was subcutaneously injected to establish the mouse models of lung cancer. It was uncovered that the tumor volume, tumor mass and number of lung metastatic nodules significantly decreased, while both tumor inhibition rate and metastasis inhibition rate significantly increased in the anti-CTLA-4, curcumin and anti-CTLA-4+curcumin groups. As observed by HE staining, there were a lot of necrotic tumor cells, and most of the cells showed karyopyknosis or karyorrhexis, but karyokinesis was rare in the above three groups, suggesting that CTLA-4 inhibitor or curcumin can inhibit tumor growth and metastasis in mice with lung cancer, and CTLA-4 inhibitor plus curcumin have a more significant effect.

Cell death can be classified into physiological and pathological death according to its cause, and apoptosis functions as a common cell death mode [18]. Apoptosis is an active and programmed death process that can maintain healthy development and metabolism by eliminating damaged cells in time [19]. A study has shown that apoptosis may be affected by the relative balance between cellular pro-apoptotic and anti-apoptotic proteins [20], among which Bcl-2 and Bax have been widely studied as an antitumor and pro-tumor gene, respectively. Both Bcl-2 and Bax belong to the Bcl-2 family, in which activated Bcl-2 can bind to Bax to form a heterodimer, thus inhibiting Bax activity and apoptosis. In addition, activated Bax can bind to Bax to form a homodimer, thus activating Caspase-3. Then activated Caspase-3 induces apoptosis by initiating a caspase cascade [21]. Research suggests that activating the Bcl-2/Bax signaling pathway serves as one of the possible mechanisms of inducing apoptosis in HepG2 cells [22]. Wen et al.[23] also confirmed that apoptosis of breast cancer cells was enhanced by up-regulation of Caspase-3 plus Bax protein expressions as well as inhibition of Bcl-2 protein expression. Our results presented that anti-CTLA-4 or curcumin treatment could evidently facilitate cell apoptosis, coupled with declined Bcl-2 content as well as enhanced Bax and Caspase-3 contents. Noticeably, the combination of anti-CTLA-4 and curcumin exhibited better therapeutic effects than anti-CTLA-4 or curcumin alone.

Nevertheless, this study has limitations. This study focused on the Bcl-2/Bax/Caspase-3 axis as a representative

apoptotic signaling pathway. However, the apoptosis cascade is highly complex and involves multiple other regulators and signaling modules, such as cytochrome c release, Apaf-1-mediated apoptosome formation, caspase-9 activation, and extrinsic death receptor pathways. These components were not comprehensively examined in the current work. Further investigations are warranted to explore these additional mechanisms and their potential crosstalk in the regulation of apoptosis, which may enrich our understanding of the cellular response to apoptotic stimuli.

In conclusion, CTLA-4 inhibitor plus curcumin can obstruct tumor growth in mice with lung cancer, the protective mechanism of which possible is related to the regulation of the Caspase-3/Bcl-2/Bax signaling pathway for promoting lung cancer cell apoptosis.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author (T. Lv) on reasonable request.

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Declaration of Generative Artificial Intelligence: The article, tables and figures were not written by AI and AI-assisted technologies.

Authors Contributions: W.T. designed the study and drafted the paper; H.W. performed and analyzed the experiments; T.L. supervised the study and significantly revised the paper. W.T. and H.W. contributed equally to this study.

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Research Article

Effects of Different Culture Media of Lactic Acid Bacteria on Performance, Carcass Yield, Blood Parameters, and Natural Antibodies in Broiler Chickens

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Abstract

An experiment was conducted to evaluate the effect of various culture media used to provide nutrients for lactic acid bacteria (LAB) on performance, carcass yield, blood parameters, natural antibody titers, and lipid and protein metabolism namely plasma triglycerides, total protein, albumin, and urea in broiler chickens. A total of 400 one-day-old Cobb broiler chicks was allocated to four treatments**:** a control feed (no probiotics), feed supplemented with 2% probiotic powder, which had been cultured in one of the following media: (1) 100% cow milk (CM), (2) a mixture of 50% cow milk and 50% soybean milk (SM), or (3) a combination of 50% cow milk, 25% soybean milk, and 25% mung bean milk (MM), reared for 28 days of experimental period. The results showed that, birds fed SM exhibited higher body weight, body weight gain, feed intake, and better feed conversion ratio than other groups, as well as higher carcass percentages, lower abdominal fat, and lower plasma and meat cholesterol. They also showed significantly lower plasma triglyceride, total protein, and albumin levels ($P<0.01$) and reduced IgY and IgM antibody titers binding keyhole limpet hemocyanin (KLH). Nonetheless, all treatments demonstrated an increasing immune response over time. These data indicate that a 50:50 cow milk and soybean milk probiotic culture could enhance broiler performance, carcass yield, plasma lipid metabolism, blood metabolite profiles (such as protein, albumin, and urea) and immune status.

Keywords: Antibody titers, Broiler, Carcass yield, Immunoglobulin Y, Lipid metabolism, Probiotic

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Introduction

It is known that using antibiotics in poultry production, both as immunomodulators and as growth promoter leads to bacterial resistance against antibiotics, which can also be transferred to humans [1,2]. Therefore, identifying effective alternatives to antibiotics is of growing importance in veterinary research and poultry production systems. One of the common alternatives is the use of probiotics; The growing global interest in probiotics is not only reflected in the expanding market size, but also in the sharp rise in academic publications over the past decade [3].

Probiotics are live microorganisms which when supplemented in adequate amounts confer a beneficial health effect on the host [1,4]. Most microorganisms that can perform such activities are lactic acid bacteria (LAB), such as *Bifidobacteria* and *Bacillus* strains [1]. Originally, probiotics were used for human and animal microbiota composition and environment modification, which can improve their health [5]. Currently, probiotics aim to result in multiple benefits, from nutrient absorption enhancement [6] to immunomodulatory effects [7,8]. Probiotics have been widely recognized for their positive impact on poultry immune status, health, and performance [9]. While other alternatives such as organic acids, enzymes, and phytogenics have also been explored for similar benefits [10] their practical application in commercial settings can be limited by factors such as cost, formulation complexity, or regulatory hurdles.

In the context of probiotic cultivation, ensuring the viability and metabolic activity of LAB is of paramount importance [11]. A conducive culture medium is integral to this, playing a pivotal role in imparting essential nutrients that foster the growth and metabolic vitality of the LAB [11,12]. Carbohydrate-rich solutions traditionally act as the milieu in which LAB exhibit optimal growth [12]. Despite its ubiquity, cow's milk as a LAB culture medium presents challenges, primarily owing to its limited proteolytic activity which often inhibits maximal LAB proliferation, thus prompting the exploration of alternative culture media [13]. Recent scientific explorations have opened avenues beyond traditional dairy media. A novel approach involves employing functional foods for direct LAB inoculation, positing a significant departure from conventional methodologies [14].

Extending this line of inquiry, our current research evaluates the potential of leguminous substrates, specifically soybean *(Glycine max)* and mung bean *(Vigna radiata),* as culture media of LAB. These legumes were selected due to their rich content of oligosaccharides, dietary fiber, and bioactive compounds and isoflavones and phytosterols and α-linolenic acid, which have been associated with hypocholesterolemic, anti-inflammatory, and immunomodulatory effects [15-17]. Soybean, in particular, has been shown to support microbial fermentation by enhancing the growth and metabolism of probiotic strains such as *Bifidobacterium animalis*, through the activity of its digested peptides and their regulatory effects on metabolic pathways like glycolysis and pyruvate metabolism [18]. Likewise, mung bean milk (MBM) has demonstrated strong potential as a LAB fermentation medium. Wu et al.[19] optimized fermentation conditions for *Lactobacillus plantarum* B1-6 in mung bean meal, achieving a high LAB count of 8.96 log CFU/mL, and noted significant protein hydrolysis (up to 64%) and ACE-inhibitory activity (67.5%), indicating health-promoting properties relevant to gut and metabolic health. Further studies have shown that *Lactococcus lactis* fermentation of mung bean milk improves antioxidant capacity, protein solubility, and sensory properties, while reducing anti-nutritional factors [20].

Altogether, these attributes make soybean and mung bean ideal candidates for probiotic media development, providing a sustainable, plant-based alternative to dairy substrates for LAB cultivation with potential added benefits for poultry health and performance. The academic discourse around plant-based yogurt alternatives, especially those derived from pulses, has witnessed a burgeoning interest. Despite being relatively nascent entrants in the commercial market, their physicochemical propensity to undergo gelation, mirroring the acid-induced gelation characteristic of dairy yogurts, is of particular interest to microbiologists [21-23]. The synergistic dynamics between specific thermal treatments and LAB strains, particularly those proficient in Exopolysaccharide (EPS) synthesis, offer intriguing prospects to enhance fermentation efficiency and optimize the textural attributes of the resultant product [24-26].

Prior to translating these microbiological findings to practical applications, it is imperative to quantitatively assess the viability of LAB cultivated in these alternative media using standard plate count methods (CFU/mL) and evaluating their acidification activity and survival rates over time under simulated gastrointestinal and storage conditions. Accurate enumeration of these viable microorganisms provides a critical metric for subsequent studies. In the current study, this foundational research was extended by administering probiotics to broiler chickens and evaluating not only growth performance but also health indicators, including blood profiles, lipid metabolism, and immunological responses, specifically, the natural antibody titer against the foreign chicken antigen keyhole limpet hemocyanin (KLH) [26].

Material and Methods

Ethical Statement

All procedures involving animals were conducted in accordance with ethical standards for the care and use of laboratory animals and were approved by the Ethical Committee of the Universitas Padjadjaran, Indonesia (approval number: 0718070998). The study adhered to national animal welfare regulations and followed the principles of the 3Rs (Replacement, Reduction, and Refinement) to minimize animal suffering. Birds were monitored daily for health and well-being, and all handling was performed by trained personnel to ensure humane treatment.

Probiotics Preparation

The preparation of the probiotics was based on the procedure described by Kumalasari et al.[27] and Nabila et al.[28]. The pasteurized cow milk used in this study was procured from a dairy farm cooperation (altitude: 1200 masl) in Bandung (KPSBU Lembang, Bandung, Indonesia), while the soybean and mung bean were obtained from the local commercial market (Bandung, Indonesia). The probiotics utilized came from yoghurt with a consortium of microbiota, which included 5% (v/v) of *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, and *Bifidobacterium* sp. [27,29]. This blend was introduced to the de Man, Rogosa and Sharpe (MRS) medium (Merck, Darmstadt, Germany) and left to incubate at 45°C for 14 h. Post incubation, the probiotics were mixed into different culture media: 100% cow milk (CM), a 50% cow milk and 50% soybean

milk mixture (SM), or a combination of 50% cow milk, 25% soybean milk, and 25% mung bean milk (MM), followed by thorough homogenization. The blend was then fermented at room temperature for 14 h. To create an encapsulation, the fermented blend was combined with food grade DE 10-12 maltodextrin 5%, skimmed milk, and pure distilled water at a 1:2 ratio to the overall solution volume. Maltodextrin acted as a protective layer against potential damage from external conditions like intense temperature fluctuations found in processes like spray drying. The probiotics were then converted into powder form through a spray drying method at 160°C input temperature and 65-70°C output temperature. Probiotic powders were freshly prepared weekly and added to the basal feed at a concentration of 2% (w/w). Feed analyses were conducted on final pooled samples representative of each treatment formulation. After this, a viability assessment was conducted, resulting in the microbiota's viability in the probiotic powder aligning with the standard lactic acid bacteria count for yogurt, 1.6×10^7 CFU/g, as mentioned in the Indonesian Standardization Body for yoghurt production [30].

Animals, Experimental Design, Housing and Rations

In a completely randomized design, 400 1-day-old Cobb broiler chicks used in this experiment were obtained from a commercial hatchery (initial weight = 43±2 g), vaccinated at day 1 for Newcastle, Marek's, and Infectious Bursal Disease, and randomly allocated to 4 experimental dietary treatments with 4 replicates. The experiment was conducted over a 28-day period, beginning on day 1 (the day of arrival and initial vaccination) and continuing until day 28. This duration was chosen based on the standard commercial grow-out period for Cobb broilers, which typically ranges between 28 to 35 days. The 4-week duration was deemed sufficient to evaluate early growth performance, blood parameters, and immune responses relevant to the probiotic supplementation effects. Each treatment consisted of 100 broilers. Each replicate was allocated into 5 floor pens so that there were 20 broilers per pen. The birds were raised on wood shavings litter (approximately 5-7 cm thickness) with maintained humidity (max. 60%). The heat was provided by an automatic temperature controller (Temptron 616, AgroLogic, Israel). Ambient temperature was maintained at 29±1°C at the starter phase and 25±1°C for the rest of the experiment. A 23L:1D lighting program was applied during the whole experiment. The basal diets were formulated according to Cobb-Vantress nutritional guidelines [29], and then supplemented with probiotic powders in accordance with the respective dietary treatment groups (see Probiotics Preparation section for details). During the whole study period, the chicks had ad libitum access to water and feed. The experimental diets were without any commercial growth promoter additives. The chemical composition of basal diet is shown in *(Table 1)* and the chemical composition of each treatment diet is shown in *(Table 2)*. The probiotics added to the basal feed were:

Table 1. *Composition of the basal diet used in this study*

Ingredients	Content (%)
Corn	57.30
Wheat bran	4.50
Soybean meal	27.00
Fish meal	7.60
Coconut oil	2.00
Meat bone meal	1.50
Methionine	0.10
Total	100

Table 2. *Chemical analysis of the treatment diet during the trial*

Item	CT	CM	SM	MM
Metabolize energy (ccal/kg)	3071.6	3129.47	3103.46	3106.10
Dry matter (%)	91.49	93.27	93.23	93.65
Crude protein	21.40	21.75	21.64	21.68
Crude fiber	4.62	4.73	4.75	4.80
Ether extract	8.21	8.42	8.32	8.32
Lysine	1.29	0.65	0.65	0.65
Methionine	0.51	0.98	0.98	0.98
Methionine + Cysteine	0.65	0.65	0.65	0.65
Calcium	0.98	0.98	0.98	0.98
Phosphorus	0.66	0.65	0.65	0.65

CT: *control (no probiotics supplementation);* ***CM:*** *basal ration + 2% of probiotics cultured in cow milk (100%) medium;* ***SM:*** *basal ration + 2% of probiotics cultured in cow milk (50%) and soybean emulsion (50%) media;* ***MM:*** *basal ration + 2% of probiotics cultured in cow milk (50%), soybean emulsion (25%), and mungbean emulsion (25%) media*

CT: control basal feed (no probiotics supplementation)

CM: basal feed + 2% (w/w) of probiotic powder ($4.51x10^3$ CFU/g)/kg of feed cultured in 100% cow milk

SM: basal feed + 2% (w/w) of probiotic powder ($3.85x10^3$ CFU/g)/kg of feed cultured in 50% cow milk and 50% soybean milk

MM: basal feed + 2% (w/w) of probiotic powder ($7.09x10^2$ CFU/g)/kg of feed cultured in 50% cow, 25% soybean, and 25% mung bean milk.

Performance parameters such as body weight gain, feed intake, feed conversion ratio, and mortality) were recorded each week from day 1 to day 28. Blood samples were collected on randomly selected birds within each treatment group at day 1, day 14, and day 28 (i.e., once

every two weeks). These samples were used to determine plasma metabolites and natural antibody titers, in accordance with the bi-weekly sampling schedule. Weight of bursa fabricius, abdominal fat, carcass percentages and meat samples were recorded and collected at the end of the treatment.

Blood and Meat Collection

Blood samples were collected from the pectoral vein at days 0, 14, and 28 during the early morning, with feed and water available ad libitum. Blood was collected in evacuated tubes (BD Vacutainer, Plymouth, UK) containing K2 EDTA (to obtain plasma) for plasma metabolites and natural antibody levels. Blood was collected in evacuated tubes containing K2 EDTA to obtain plasma, which was used for the analysis of plasma metabolites and natural antibody titers. EDTA plasma was selected due to its compatibility with the commercial assay kits and ELISA procedures used in this study. After collection, blood samples were kept on ice (maximum 2 h) to minimize cellular metabolism and preserve plasma integrity, until it centrifugated at 3000 x g (approximately 5000 rpm) for 15 min at room temperature. Plasma was decanted, aliquoted, and frozen at -20°C until analysis.

Meat collection has been described in our earlier study [27]. In brief, meat samples were taken at the end of the treatment period. A small portion of the chicken from the breast ± 5 g as a diced sample. Samples were kept frozen at -20°C until analysis.

Determinations of Blood Parameters

Determination of plasma triglycerides, total protein, cholesterol, urea, and albumin level was performed at the laboratory of Animal Physiology and Biochemistry, Universitas Padjadjaran (Bandung, West Java, Indonesia), using spectrophotometric analysis with commercial kits (Biolabo, Maizy, France) with the catalogue number of 80019, 80016, 80106, 80221, and 80002, respectively.

Determinations of Natural Antibodies (NAbs) Titers Against Keyhole Limpet Hemocyanin (KLH)

Determination of natural antibodies (Nabs) levels binding KLH were performed in the laboratory of Pharmacology and Biochemistry, Faculty of Pharmacy, Universitas Padjadjaran using an indirect enzyme-linked immunosorbent assay (ELISA) as described by Mayasari et al.[31]. Levels of NAb titers were determined by ELISA against KLH, a foreign antigen originating from *Megathura crenulata*, a keyhole limpet that lives off the coast of California, USA. This antigen was intended to reduce false positives in detecting NAb levels because the keyhole limpet is a gastropod, phylogenetically distant from avian proteins. Briefly, plates were coated with 4 µg/mL of KLH (Cat No. H8283, Sigma-Aldrich Merck, Darmstadt, Germany) in 100 µL/well. Natural antibodies of the IgY isotype were detected using a 1:20,000 dilution of Rabbit Anti-chicken IgY-heavy and light chain antibody conjugated to horse radish peroxidase (HRP) (Cat. No. A30-107A, Bethyl Laboratories, Montgomery, TX, USA), while levels of the IgM isotype were detected using a 1:20,000 dilution of Rabbit Anti-chicken IgM Antibody conjugated to HRP (Cat. No. A30-102P, Bethyl Laboratories, Montgomery, TX, USA). After washing the plates, a substrate containing tetramethylbenzidine (TMB) and 0.05% hydrogen peroxide was added and incubated at room temperature for 10 min. The enzymatic coloring reaction was stopped by adding 1.25 M sulfuric acid. Extinctions were measured with an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, Vermont, USA) at a wavelength of 450 nm. Levels of IgY and IgM antibodies binding KLH were expressed as titers being the log2 values of the dilutions that gave an extinction closest to 50 per cent of Emax, where Emax represents the highest mean extinction of a standard positive (pooled) plasma present on every microtiter plate [32].

Determination of Meat Cholesterol

Meat cholesterol analysis has been described in an earlier study by Kumalasari et al.[27]. In brief, meat cholesterols were determined using the enzymatic calorimetry test method Cholesterol Oxidase Phenylperoxidase Aminophenazone (CHOD-PAP). The meat sample was ground and weighed 1 g, then put into an empty tube. Three ml of ether solution was added, left for 24 hours, and homogenized. Centrifuge at 3000 rpm for 15 min. The supernatant was transferred into an Eppendorf tube. The blank cuvette was filled with 1000 µL of reagent and 10 µL of aquadest. The standard cuvette is filled with 1000 µL of reagent and 10 µL of standard. The assay cuvette (sample) is filled with 1000 µL of reagent and specimen as much as 10 µL. The blank absorbance value is read using a spectrophotometer wavelength of 500 nm [32].

Statistical Analysis

Data were analyzed by using one-way analysis of variance (ANOVA) in a general linear model (proc GLM) of SAS version 9.2 (Cary, NC, USA) to determine effects of different probiotics culture media at different time points. Body weight was analyzed on a weekly basis for 4 weeks (week 0, week 1, week 2, week 3, and week 4). Body weight gain, average daily gain, feed intake, and feed conversion ratio (FCR) were analyzed on a biweekly basis, starting on week 0 of the experimental period (week 0-2, week 2-4) and week 0-4). Moreover, blood parameters, natural antibody titers were also analyzed bi-weekly, starting from week 0 of the experimental period (week 0, week 2, and week 4). Carcass percentage, meat cholesterol, abdominal

fat, and weight of bursa Fabricius were analyzed at week 4. The effects of different probiotics culture media were considered significant at P≤0.05. The analyses were then continued with Duncan's multiple range test as multiple comparisons. The data were expressed as least square means (LSM) of the respective parameter with pooled standard error of means (SEM).

Data from blood parameters, natural antibodies, and lipid metabolism were analyzed using the MIXED procedure (PROC MIXED) in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). A repeated-measures mixed model was used to assess the fixed effects of treatment (T), time (week, W), and their interaction (TxW), with the individual replicate (pen) specified as a random effect. Covariance structures (autoregressive 1) were tested and selected based on the lowest Akaike Information Criterion (AIC). Least square means (LSM) were compared using Duncan's multiple range test, and significance was declared at P≤0.05.

Results

Probiotic Characteristic

Probiotic characteristic such as pH, lactic acid levels, total lactic acid bacteria and the total bacteria was not significant difference among treatments *(Table 3)*. However, MM had higher total lactic acid bacteria compared with CM and SM.

Performance and Carcass Yield

During the experiment, the mortality of birds was not exceeding 4% of the total population, therefore it was neglected in the statistical analysis. At the day 1, the probiotics in different culture media treatments were not yet administered, therefore it can be considered as default status *(Table 4)*. However, birds were already grouped in day 1. In day 1, there was a statistically significant difference in body weight, i.e. a lower body weight in the SM-fed group compared to other groups (P<0.0001). Though statistically significant, the actual mean difference in body weight was not exceeding 1.5 g, therefore it can still be considered homogenous.

Current study showed that body weight gain, average daily gain, feed intake, and final body weight were significantly higher in bird fed SM treatment compared with birds fed CT group *(Table 3)* especially from week 2 to week 4. The final body weight of birds in SM fed group was significantly higher compared to other groups, especially compared to the CM fed group (P<0.05). This is in line with the feed intake in the SM fed group which is significantly higher compared to other treatment groups. The lower FCR in the SM fed group also reflected this.

Blood Parameters and Immune Response

In the week 0, there was no significant difference in plasma metabolites, and plasma Nabs except in titers of IgM. This study showed there was significant difference in plasma metabolites after the administration of probiotics dietary treatments. *Table 5* showed that birds fed MM treatments had higher plasma total protein (P<0.01) compared with other treatments. Birds fed CT treatments had higher plasma triglycerides (P<0.1) compared with other treatments. Birds fed MM treatments had higher plasma total protein (P<0.001) compared with other treatments. There was significant difference in plasma metabolites between weeks *(Table 5)*.

There was no significant difference in plasma Nabs except in titers of IgM *(Table 6)*. At week 2, with respect to levels of plasma Nabs, the IgM anti-KLH titer tended to be higher in the SM fed group compared to the CM fed group. The plasma IgM titer decreased in the CT, CM, and SM fed birds from week 0 to 2, while, in contrast, the plasma IgM titer in MM fed birds increased in the same period. SM fed birds had higher carcass percentages with the lower abdominal fat percentage and lower cholesterol in plasma and meat compared with other treatments. Triglyceride, total protein, and albumin levels in plasma of SM fed birds were significantly lower compared to the other treatment groups (P<0.01). Moreover, SM fed birds had lower levels of IgY and IgM antibodies binding keyhole limpet hemocyanin (KLH) compared with the control group.

In week 4, plasma cholesterol levels in the SM and MM-fed groups were significantly lower compared with CT and CM groups (P<0.01), suggesting a low level of fat

Table 3. *Physicochemical properties and viable microbial counts of probiotic treatment in this study*

Item	Treatment			SEM	P-value
	CM	SM	MM		
pH	3.97	3.78	4.03	0.68	0.31
Lactic acid level (%)	0.68	0.74	0.73	0.09	0.19
Total lactic acid bacteria (CFU/g)	4.51×10^3	3.85×10^3	7.09×10^2	n.a[2]	n.a[2]
Total bacteria (CFU/g)	1.27×10^3	1.70×10^3	1.28×10^3	n.a[2]	n.a[2]

CM: *basal ration + 2% of probiotics cultured in cow milk (100%) medium;* ***SM:*** *basal ration + 2% of probiotics cultured in cow milk (50%) and soybean emulsion (50%) media;* ***MM:*** *basal ration + 2% of probiotics cultured in cow milk (50%), soybean emulsion (25%), and mungbean emulsion (25%) media; [2]n.a: not applicable*

Table 4. *Effects of different lactic acid bacteria culture media on performance*

Treatment	Body Weight Gain (g/head)			Average Daily Gain (g/head/day)			Feed Intake (g/head)			Feed Conversion Ratio			Final BW (g)	Mortality (%)
	0-2 wk	2-4 wk	0-4 wk	0-2 wk	2-4 wk	0-4 wk	0-2 wk	2-4 wk	0-4 wk	0-2 wk	2-4 wk	0-4 wk		
CT	325.6	922.3^{b}	1247.9^{b}	23.26	65.9^{b}	44.6^{b}	428.2	1258.1	1686.3^{b}	1.32	1.37^{b}	1.36^{b}	1294.4^{a}	3.63
CM	328.0	901.0^{c}	1229^{bc}	23.43	64.4^{c}	43.9^{bc}	427.6	1422.5	1850.1^{a}	1.30	1.58^{a}	1.51^{a}	1275.4^{bc}	0
SM	328.5	959.0^{a}	1287.4^{a}	23.46	68.5^{a}	46.0^{a}	431.7	1461.4	1893.0^{a}	1.31	1.52^{a}	1.47^{a}	1332.4^{a}	1.82
MM	327.8	889.6^{c}	1217.4^{c}	23.41	63.5^{c}	43.5^{c}	426.7	1395.4	1822.1^{a}	1.30	1.57^{a}	1.5^{a}	1263.3^{c}	0
SEM	1.62	4.51	4.91	0.12	0.32	0.18	1.76	20.97	20.58	0.003	0.024	0.017	4.5	-
P-value	0.93	<.001	<.001	0.93	<.001	<.001	0.77	0.0027	0.0018	0.2368	0.0048	0.0072	<.001	-

CT: *control (no probiotics supplementation);* ***CM:*** *basal ration + 2% of probiotics cultured in cow milk (100%);* ***SM:*** *basal ration + 2% of probiotics cultured in cow milk (50%) and soybean milk (50%);* ***MM:*** *basal ration + 2% of probiotics cultured in cow milk (50%), soybean emulsion (25%), and mungbean milk (25%); ** P<0.01; *** P<0.0001;* ^{a,b,c}*Means with different superscripts within the same column are different in accordance with respective significance levels*

Table 5. *Effects of different lactic acid bacteria culture media on blood parameters*

Item	Treatment				SEM	Week			SEM	P-values	
	CT	CM	SM	MM		0	2	4		T	W
Total protein (g/dL)	2.53^{b}	2.55^{c}	2.60^{b}	3.13^{a}	0.12	1.2717^{c}	2.9642^{b}	3.8734^{a}	0.10	0.0011	<.001
Albumin (g/dL)	5.62	5.26	4.95	5.39	0.18	4.24^{c}	5.77^{b}	5.91^{a}	0.15	0.0775	<.001
Blood urea nitrogen (mg/dL)	4.47	4.62	4.27	5.52	0.43	4.75^{b}	3.91^{c}	5.50^{a}	0.36	0.1826	0.0036
Triglyceride (mg/dL)	151.94^{a}	130.1^{b}	110.49^{d}	111.63^{c}	8.93	147.93^{c}	133.75^{b}	96.4385^{a}	7.53	0.0032	<.001

CT: *control (no probiotics supplementation);* ***CM:*** *basal ration + 2% of probiotics cultured in cow milk (100%) medium;* ***SM:*** *basal ration + 2% of probiotics cultured in cow milk (50%) and soybean emulsion (50%) media;* ***MM:*** *basal ration + 2% of probiotics cultured in cow milk (50%), soybean emulsion (25%), and mungbean emulsion (25%) media; ** P<0.01; *** P<0.0001.* ***T:*** *fixed effect of treatment;* ***W:*** *fixed effect of week (time);* ^{a,b,c} *Means with different superscripts within the same row are different in accordance with respective significance levels*

Table 6. *Effects of different lactic acid bacteria culture media on natural antibodies binding Keyhole Limpet Hemocyanin (KLH)*

Item	Treatment (T)				SEM	Week			SEM	P-values	
	CT	CM	SM	MM		0	2	4		T	W
IgY titer	5.34^{a}	3.83^{b}	3.33^{c}	3.13^{a}	0.34	4.41	1.94	5.37	0.28	<.001	0.1072
IgM titer	5.40^{a}	4.39^{b}	4.57^{b}	3.46^{c}	0.36	4.80	3.72	4.85	0.29	0.0025	0.325
Bursa of Fabricius weight (g)	1.34	1.40	1.12	0.96	0.09				0.09	0.22	

CT: *control (no probiotics supplementation);* ***CM:*** *basal ration + 2% of probiotics cultured in cow milk (100%) medium;* ***SM:*** *basal ration + 2% of probiotics cultured in cow milk (50%) and soybean emulsion (50%) media;* ***MM:*** *basal ration + 2% of probiotics cultured in cow milk (50%), soybean emulsion (25%), and mungbean emulsion (25%) media; * P<0.05; ** P<0.01; *** P<0.0001;* ***T:*** *fixed effect of treatment;* ***W:*** *fixed effect of week (time);* ^{a,b,c} *Means with different superscripts within the same row are different in accordance with respective significance levels*

Table 7. *Effects of different lactic acid bacteria culture media on lipid metabolism*

Item	Treatment				SEM	Week			SEM	P-values		
	CT	CM	SM	MM		0	2	4		T	W	T×W
Plasma cholesterol (mg/dL)	186.23	192.12	184.26	186.9	4.70	280.08^{a}	139.55^{c}	142.5^{b}	3.96	0.68	<.001	0.001
Meat cholesterol	36.19^{a}	31.51^{b}	27.97^{b}	29.07^{b}	1.05	n.a	n.a			<.001	n.a	n.a
Carcass percentage (%)	67.32^{b}	67.56^{b}	68.43^{a}	63.82^{c}	0.76					<.001		
Abdominal fat (g)	1.42^{ab}	1.59^{a}	1.11^{b}	1.10^{b}	0.12					0.01		

CT: *control (no probiotics supplementation);* ***CM:*** *basal ration + 2% of probiotics cultured in cow milk (100%) medium;* ***SM:*** *basal ration + 2% of probiotics cultured in cow milk (50%) and soybean emulsion (50%) media;* ***MM:*** *basal ration + 2% of probiotics cultured in cow milk (50%), soybean emulsion (25%), and mungbean emulsion (25%) media; * P<0.05; ** P<0.01; *** P<0.0001;* ***T:*** *fixed effect of treatment;* ***W:*** *fixed effect of week (time). n.a: not applicable;* ^{a,b,c} *Means with different superscripts within the same row are different in accordance with respective significance levels*

mobilization. Levels of total protein were higher in the CT-fed group compared to other treatment groups (P<0.01), suggesting a high protein transport. However, plasma albumin levels were lower in the CT-fed group, but insignificant. This suggestion is supported by the fact that levels of IgY-binding KLH in the CT-fed group were significantly higher compared to other treatment groups. (P<0.001). Titers of natural antibodies IgY and IgM binding KLH in SM-fed birds were higher than CM and MM-fed birds (P<0.05).

Lipid Metabolism

In week 4, plasma triglyceride and cholesterol levels in the SM and MM-fed groups were significantly lower compared with CT and CM groups (P<0.01), suggesting a low level of fat mobilization. Birds fed SM had lower cholesterol levels in meat *(Table 7)*. In terms of abdominal fat, a lower percentage was found in SM and MM fed groups, compared to the CT and CM fed group (P<0.01). The SM fed group had a higher carcass percentage compared to other treatment groups (P<0.01), suggesting a higher inedible parts percentage in MM fed birds.

Discussion

The week 0 data depict the actual situation of commercial poultry practices in Indonesia, showing extended levels of variation in day-old chick conditions.

While pH, lactic acid levels, and total bacterial counts were similar across treatments, the MM culture medium supported significantly higher LAB growth. This may be attributed to the specific nutrient composition of the MM formulation, which combines cow milk, soybean emulsion, and mung bean emulsion [33,34]. These components may create a synergistic effect, enhancing microbial proliferation. Although the pH values in our study (3.78-4.03) were below the optimal bacteriocin production range of 6.2-8.5 [35], the MM medium's relatively higher pH (4.03) may still offer a more hospitable environment for LAB survival. Prior findings have suggested that cow milk provides readily available lactose for LAB, while soybean and mung bean components contribute prebiotic fibers and bioactive compounds that may further promote LAB activity [34].

The differing carbohydrate profiles of cow milk, soybean, and mung bean may explain the variations in LAB viability. Cow milk offers simple sugars like lactose, which LAB strains such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* readily utilize [36]. In contrast, soybean and mung bean provide oligosaccharides such as raffinose and stachyose, substrates more selectively metabolized by *Bifidobacterium bifidum* [37].

Current study showed a significant difference on growth performance, carcass yield, abdominal fat including other lipid metabolism among treatments especially at week 4. Week 4 in the present study can be regarded as a final time point that reflects the condition of birds to be harvested in commercial poultry circumstances. The final body weight of birds in SM fed group was significantly higher compared to other groups, especially compared to the CM fed group. This is in line with the feed intake in the SM fed group which is significantly higher compared to other treatment groups. The lower feed FCR in the SM fed group also reflected this. The carcass percentage was also higher in the SM fed group compared to other treatment group, but not significant. In terms of lipid metabolism including plasma triglyceride, levels of cholesterol in plasma and in meat and abdominal fat, a lower percentage was found in SM fed groups compared to the CT and CM fed group (P<0.01).

The SM fed birds not only had higher final body weight, but also higher body weight gain and feed intake compared with the other treatments. In addition, SM fed birds had better FCR compared with the control group. The MM fed group in week 2 had a lower carcass percentage compared to other treatment groups (P<0.01), suggesting a higher inedible parts percentage in MM fed birds.

With respect to blood parameters, significantly higher plasma triglycerides levels in the CT fed were found compared to other treatment groups (P<0.0001), suggesting a higher adipose fat transport and glucose transfer in the CT fed group at week 2 compared to the other treatment groups. Previous studies have shown that probiotic supplementation in laying hen feed has a significant effect in reducing blood triglyceride levels [38]. Meanwhile, other studies have shown that probiotic supplementation in layer feed tends to reduce blood triglyceride levels although not significantly different [33]. In this study, we observed that supplementation of probiotic with different culture media in the diet of broiler also reduce plasma triglyceride levels. The decrease in plasma triglyceride levels could be due to the supplementation of probiotic, which affects the fatty acid synthesis process in the body of the hens. According to previous studies, microbiota in probiotics can effectively reduce the activity of acetyl-CoA carboxylase (ACC), which is an enzyme involved in the rate of fatty acid synthesis. Less secretion of ACC results in less formation of fatty acids and decreased fatty acid formation lowers blood triglyceride levels [38]. Thus, decreased blood triglyceride levels lead to decreased triglyceride levels synthesized in the liver. In addition, according to Mayasari and Adriani [38], probiotics can also assimilate cholesterol, leading to impaired micelle formation. Lower micelle formation decreases the uptake of lipids in the intestinal lumen, ultimately reducing the number of circulating triglycerides in the blood. This

will lead to a decrease in the uptake of lipid profile in the blood. The change in lipid profile distribution especially cholesterol may be due to increased biosynthesis and accumulation in the liver. A previous study showed that probiotic supplementation decreased plasma cholesterol levels in broilers [39]. Supplementation of lactic acid bacteria in diet or water was related to low cholesterol levels in the plasma which may be due to LAB causing a decrease in gallbladder acid secretion. Low secretion of gallbladder acid decreased the ability of fat digestion and therefore decreased lipid levels in the blood [40]. In addition, bioactive compounds both in soybean (glycinin, lecithin) and mung bean (phytosterol) were shown to decrease cholesterol in plasma [41]. Moreover, soybean and mung bean contain resistant starch which increased the production of cecal short-chain fatty acid (SCFA, eg. Butyrate), and elevated fecal neutral sterol excretion, thereby reducing the serum total cholesterol level [40]. Regarding the blood parameters, the levels of total protein were significantly higher in the MM-fed group compared to other treatment groups ($P<0.01$), suggesting a high protein transport. However, plasma albumin levels were lower in the CT-fed group, but insignificant. A critical factor in the protein depositing into meat is the levels of protein and albumin. The high level of total protein and low level of albumin might suggest that the proportion of the major protein type transported is not albumin, but globulin. This lower proportion of albumin compared to globulin might suggest a high mobilization in immune system build-up, especially for IgY formation. This suggestion is supported by the fact that levels of IgY-binding KLH in the CT-fed group were significantly higher compared to other treatment groups. ($P<0.001$). Titers of natural antibodies IgY and IgM binding KLH in SM-fed birds were higher than CM and MM-fed birds ($P<0.05$).

This lower proportion of albumin compared to globulin might suggest a high mobilization in immune system build-up, especially for IgY formation. The suggestion is supported by the fact that levels of IgY-binding KLH in the CT-fed group were significantly higher compared to other treatment groups. ($P<0.001$). Titers of natural antibodies IgY and IgM binding KLH in SM-fed birds were higher than CM and MM-fed birds ($P<0.05$). This aligns with a previous study that dietary supplementation of fermented soybean meal could increase immune responses in broilers [42]. Probiotics effectively remove anti-nutritional elements and increase the nutritional value by fermenting soybean meal, which results in the production of numerous enzymes [43], therefore the performance is enhanced as well as the immune response.

With respect to levels of plasma Nabs, the IgM anti-KLH titer tended to be higher in the SM fed group compared to the CM fed group. The plasma IgM titer decreased in the CT, CM, and SM fed birds from week 0 to 2, while, in contrast, the plasma IgM titer in MM fed birds increased in the same period. The observed elevation of IgM might be related to activation of the immune system [41]. In terms of IgY, a decreased level was observed in all treatments from week 0 to week 2. A previous study suggested that chicks' IgY was maternally derived until at least 8 days of life [44], implying that the decreased level of IgY in week 2 could reflect the remnants of this maternal derivation. Regarding treatment effects, MM-fed birds exhibited a significantly lower IgY level in week 2 compared to CT-fed birds ($P<0.05$), indicating an accelerated breakdown or usage of the maternal antibody remnants. Nabs are immunoglobulins that are present in individuals who have had no known exposure to antigens in the past [45]. Recent studies have suggested that the levels of Nabs in an individual animal may serve as a reliable indicator of their immune competence [26], with higher Nabs levels in poultry indicating a stronger and more efficient immune response [46]. The availability of nutrients in broilers may have been increased by microbial fermentation since it was shown that fermentation reduced antinutritional factors in soybean and boost small-size peptides [47].

Although the diets were not strictly isoenergetic, the linear trends observed across treatments for several parameters (e.g., growth performance and lipid profiles) suggest that the positive effects were not solely due to variations in nutrient content. Rather, the results likely reflect a combined influence of probiotic activity and nutritional composition, with the probiotic effect becoming more evident as the complexity of the culture media increased.

In conclusion, different culture media of lactic acid bacteria (cow's milk, soybean milk and mung bean milk) affected performance, plasma metabolites, and immune status. We tried to determine culture media that support growth and activity of LAB as alternative for cow milk. The best result was obtained with probiotic in a combination of cow's milk and soybean milk. Based on the results of this research, birds fed probiotics cultured in cow's milk and soybean milk showed better growth performance, an increased carcass percentage, lower abdominal fat, lower levels of plasma cholesterol, and higher titers of IgY binding KLH representing natural antibodies. Thus, supplementation of probiotics with combination of specific culture media can be effectively used to ensure favorable results, not only for growth performance and immune response but also to replace antibiotics.

However, this study was limited by its relatively short trial duration and the absence of molecular identification of microbial changes in the gut. Additionally, the strain-specific effects of probiotics were not distinguished. Future studies are encouraged to investigate the underlying microbiota shifts using metagenomic tools,

explore longer-term impacts through the full production cycle, and assess cost-effectiveness at scale for commercial application.

Declarations

Availability of Data and Materials: The authors declare that data supporting the study findings are also available from the corresponding author (N. Mayasari) on reasonable request.

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Research Article

Epidemiology of Tick Infestation in Dogs: Prevalence, Risk Factors and Seasonal Trends in Pakistan

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Abstract

Dog ticks are the sever threat to both human and animal life due to their medical importance in transmission of tick borne diseases. The current study aimed to investigate tick infestation, associated risk factors, species diversity and seasonal abundance of ticks in selected regions of Pakistan. A total of 940 dogs (both free roaming and owned) were examined during 2023-24 and 712 tick samples were collected. These ticks were morphologically identified into three species comprising of *Rhipicephalus sanguineus* **(81.5%)**, *Rhipicephalus haemaphysaloides* (10.2%) and *Rhipicephalus turanicus* **(8.3%)**. All demographic and epidemiological information were recorded and chi-square test and logistic regression was performed. Overall prevalence of tick infestation in dogs was 27% (254/940). Infestation rates varied by age groups with highest prevalence observed in puppies <1 year (30.50%). Female dogs showed higher infestation rate (30.60%) compared to male dogs (23.72%). Dogs with short hair had significantly higher infestation rates (31.94%) than long-haired breeds (6.15%) and summer had the highest tick infestation rate (32.42%). The tick prevalence was highest in Gujar Khan while least prevalent in Taxila. Simpson's Diversity Index (D) and Shannon-Wiener Diversity Index (H') indicated a relatively low and moderate level tick diversity, which highlighted the need for dedicated tick control measures, particularly in regions of high infestation of tick in the dog population.

Keywords: Tick infestation, Prevalence, Risk factors, Seasonal trends, *Rhipicephalus sanguineus*, Dogs, Pakistan

Introduction

Ticks are ectoparasites that rely solely on blood of hosts including canines, bovines, and sometimes humans in all climatic zones of the world [1]. Ticks pose significant threats to animal and human health, being considered the second most important vector of human diseases after mosquitoes [2]. Dogs are among the first animals to be domesticated and kept as pet animals throughout the world [3]. The precise figures are lacking however global population of dog is estimated about 700 million exist, in which 75% are free roaming [4]. A developing country like Pakistan is experiencing rapid urbanization and socio-economic challenges [5]. The unregulated increase in dog populations has raised significant concerns regarding environmental sanitation, animal welfare and transmission of zoonotic diseases, particularly in densely populated urban and peri-urban areas [6]. Although official statistics on dogs population in Pakistan are lacking, however it is estimated that approximately 3 million dogs reside in the country [7,8]. Large-scale studies on tick from companion animals have recently gained attention due to the close relationship between humans and pets, as well as the shared disease risk posed by ticks [9]. Tick infesting dogs can infest humans and spread zoonotic diseases [10]. Dogs inhabiting urban and peri-urban environments are commonly infested by various hard tick species including *Rhipicephalus microplus, Rhipicephalus sanguineus, Rhipicephalus haemaphysaloides, Haemaphysalis erinacei,* and *Haemaphysalis parva* [11,12]. Accurate identification of these ectoparasites is essential for effective surveillance and control strategies, as these vectors are the confirmed

carriers of several tick-borne pathogens with significant zoonotic potential [13]. Tick infestation and seasonal fluctuation are influenced by biotic and abiotic factors. Host availability, roaming behavior and habitat type are key biotic factors affecting the occurrence. Abiotic factors such as temperature, humidity, rainfall and other climatic factors impact ticks' survival and activity [1].

Several pathogens such as *Rickettsia, Borrelia burgdorferi* (Lyme disease), *Anaplasma, Ehrlichia,* and *Babesia* can infect both humans and animals, leading to severe health complications. These infections not only cause health risks but also result in financial losses and threaten the well-being of people, animals, and the environment. *Rhipicephalus sanguineus* is the most common species of dogs, transmits *Anaplasma capra, Anaplasma platys, Ehrlichia canis, Ehrlichia minasensis, Babesia canis, Rickettsia massiliae, Rickettsia aeschlimannii,* and *Rickettsia rickettsii*. More temperate tick species such as *Ixodes scapularis* and *Ixodes ricinus* are vectors of: *Borrelia burgdorferi* (causative agent of Lyme disease), *Anaplasma phagocytophilum* and *Babesia* spp. In addition to affecting canine health, these ticks can also transmit pathogens to humans [13-16]. The presence of tick in livestock or companion animals has been associated with several risk factors. Tick dispersion varies globally depending on host demographics (e.g., age, gender, and breed) and management practices (e.g., acaricidal usage, dogs roaming) [14]. Understanding the distribution of dog population and their associated tick diversity is critical to mitigating public health risks associated with the transmission of vector borne diseases [15]. In Pakistan, studies on risk factors for tick infection in companion animals have been conducted in only a few regions [7,16-20]. However, precise tick species identification is essential for developing effective tick management strategies, and this primarily relies on morphological keys [17,21]. The current study has been designed to investigate the diversity, prevalence and associated risk factors of hard tick species infesting dogs in the districts of Rawalpindi and Islamabad, Pakistan. The findings of the current research will contribute as a baseline in enhancing knowledge regarding future ticks' surveillance and control strategies against ticks and tick-borne diseases in both animal and humans.

Material and Methods

Ethical Approval

The study was approved by the Institutional Review Board (IRB) (Approval No: CUI/Bio/ERB/2024/43) of the Department of Biosciences, COMSATS University Islamabad Pakistan.

Study Area

Pakistan is primarily an agricultural country that is divided into five agroecological zones based on the examination of climatic/aridity data using the remote sensing climate compound index [22]. The northern cities of Pakistan including Islamabad and Rawalpindi were chosen as the study locations for this investigation on dog tick infestation. Its twin city, Rawalpindi, is a metropolitan center with a higher population density and more diverse climatic conditions, Islamabad, the capital, is renowned for its contemporary infrastructure, open spaces, and reasonably managed urban environment. According to the Köppen-Geiger classification [23], Islamabad has hot and humid summer with mild winter weather. The city has a temperate climate because the annual temperature averages is 21.3°C and annual precipitation is approximately 1201 mm. The weather in Islamabad shows a clear pattern between seasons as spring runs from March to May and summer arrives in June continuing through August. The following shift is autumn from September to November and the cold winter lasts all through December up until February. Sandstorms and heat create dry spring weather known as pre-monsoon that usually experiences drought conditions. The summer monsoon provides enough rainfall to counteract hot weather but has high moisture content in the air *(Fig. 1)*.

Tick Sampling and Morphological Identification

In the present study, we investigated tick infestation in dogs from May (2023) to April (2024). Dogs belonging to willing owners including caged pet dogs, were observed using random sampling method. A questionnaire was prepared to get data of dogs that were part of the study. The data regarding breed, age, gender, roaming behavior, acaricide usage, and bathing from the owners of dogs was collected. We also asked about tick infestations and whether they had used acaricides on their dogs. The dog' age was divided into three groups comprising puppy (<1

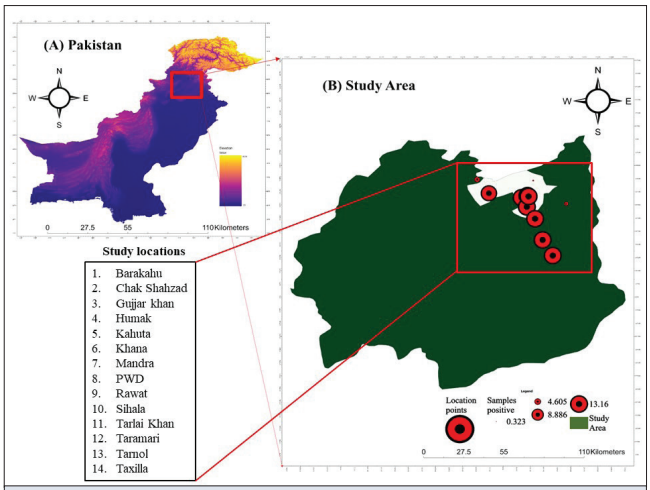

Fig 1. Map of the study area (red circles indicating sampling sites of dog ticks collection)

year), juvenile (1-3 years), and adult (>3 years). In the study, Specific breed data was lacking, so we classified dogs based on the length of their tail (long-haired or short-haired dogs) [24]. The term infestation rate describes the number of infected ticks as a proportion of the total number of ticks collected for each class.

Dogs were categorized as infested (at least one tick detected) or non-infested (no ticks found). This binary classification enabled us to determine the prevalence of tick infestation and to study potential risk factors associated with infestation status. In this study, 14 localities including towns and localities of the areas (Barakahu, Chak Shahzad, Gujjar khan, Humak, Kahuta, Khana, Mandra, PWD, Rawat, Sihala, Tarlai Khan, Taramari, Tarnol, and Taxilla) and dogs (n=940) were examined of tick incidence. Standard techniques were used such as by wearing gloves and using forceps to gather tick samples on the dogs; no physical strain or harm was put on the dogs when tick samples were gathered. To ensure a thorough examination, dogs were restrained using a mouth gag or muzzle by the owner or handler and Tick were carefully captured. The collected ticks were preserved, and stored in the Department of Biosciences, COMSATS University Islamabad, Pakistan for further research. Morphological identification to the species level was only for previously unfed ticks, using conventional identification keys [25,26] and a stereo zoom microscope (SZ61, Olympus®, Tokyo, Japan).

Statistical Analysis

Basic frequencies were determined, and Microsoft Excel was used for data recording. The data was then analyzed by using descriptive statistics, and the variables were first identified as independent and dependent variables. Independent factors included age, gender, breed, use of acaricides, bathing, dog roaming range, tick species, month, and season, whereas tick infestation (positive or negative) was the dependent variable. A chi square test and logistic regression were performed in SPSS to assess the association and peculiarity between independent and dependent variables. Results with P<0.05 were significant for hypothesis testing. Tick species diversity was computed using Shannon-Wiener diversity Index (H′) and Simpson's Diversity Index (D) in Microsoft Excel. Graphs were generated in GraphPad Prism software (10.0.1), and the study site mapping was conducted using ArcGIS Pro.

Results

In the present study, overall tick prevalence among dogs was 27% (254/940). With total collection of 712 ticks, three tick species including *Rhipicephalus sanguineus, R. turanicus,* and *Rhipicephalus haemaphysaloides* were identified by using taxonomic keys *(Fig. 2)*.

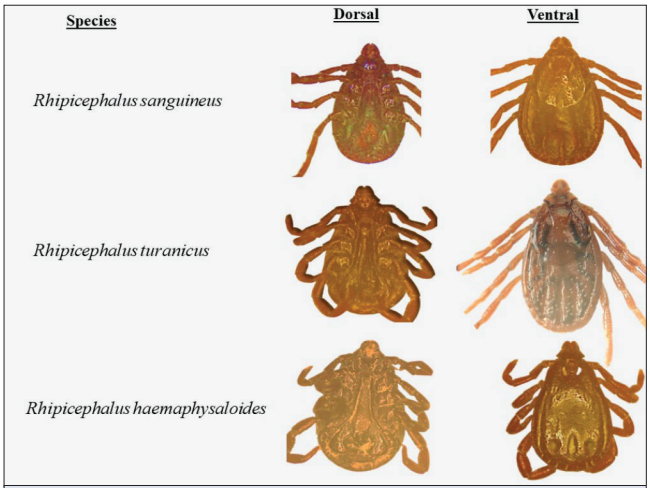

Fig 2. Representative images of three hard tick species including *Rhipicephalus sanguineus*, *Rhipicephalus turanicus* and *Rhipicephalus haemaphysaloides*, collected from dogs

Prevalence and Species Composition of Tick Infestation in Dogs

Rhipicephalus sanguineus had the highest (81.5%) prevalence in which female ticks (54.8%) were higher than males (26.7%) and *R. haemaphysaloides* (10.2%) had infestation in which female ticks (6%) showed higher prevalence as compared to male (4.2%) while *R. turanicus* had lower (8.3%) prevalence of tick infestation, female tick prevalence was little higher (4.9%) than male (3.4%). These results emphasized the abundance and the gender specific distribution of different tick species infesting dogs within the studied population *(Table 1)*.

Table 1. *Prevalence and species composition of tick infestation in dogs*

Species	Gender (n)		Prevalence (%)
R. turanicus	Male	24	3.4
	Female	35	4.9
	Total	59	8.3
R. sanguineus	Male	190	26.7
	Female	390	54.8
	Total	580	81.5
R. haemaphysaloides	Male	30	4.2
	Female	43	6
	Total	73	10.2

Risk Factors Studied in Dog Population

The infestation rate was higher in the puppies (30.50%), followed by juveniles (26.78%) and adults (24.65%), although the association was not statistically significant (P=0.525). Female dogs had the highest (30.60%) tick infestation than males (23.72%, Odd Ratios (OR)=0.73)

Table 2. *Potential risk factors facilitating tick infestation of the dog population*

Categories		Tick Infestation		Total	Infestation Rate (%)	Coefficient (β)	Odds Ratio (OR)	95% CI for OR	Chi-Square	df	P-value
		Non-infested	Infested								
Age	Puppy (< 1)	98	43	141	30.50	0.24	1.27	0.79–2.04	1.287[a]	2	.525
	Juvenile (1-3)	481	176	657	26.78	0.11	1.12	0.73–1.71			
	Adult (>3)	107	35	142	24.65	0	1.00	-			
Gender	Female	313	138	451	30.60	0	1.00	-	5.626[a]	1	.018
	Male	373	116	489	23.72	-0.32	0.73	0.56–0.95			
Breed	Long-haired	168	11	179	6.15	0	1.00	-	48.864[a]	1	< 0.001
	Short-haired	518	243	761	31.94	1.77	5.87	3.16–10.91			
Acaricides usage	Irregular	41	30	71	42.25	1.47	4.35	2.38–7.94	33.771[a]	2	< 0.001
	No use	489	205	694	29.53	1.06	2.89	1.75–4.76			
	Regular	156	19	175	10.86	0	1.00	-			
Dog-bathing	No	564	244	808	30.20	1.49	4.44	2.31–8.55	29.446[a]	1	< 0.001
	Yes	122	10	132	7.58	0	1.00	-			
Dog roaming range	Free-roaming	321	213	534	39.89	0	1.00	-	102.284	1	< 0.001
	Non-roaming	365	41	406	10.10	1.76	5.81	4.03–8.38			

Table 3. *Spatial distribution of ixodid tick species across the study area*

Study Sites	Latitude N	Longitude E	Non-infested	Infested	Total	Infestation Rate (%)	Coefficient	Odds Ratio	95% CI for OR	Chi Square X^2	df	P-value
Barakahu	33.73806085	73.18512218	52	1	53	1.89	0	1.00	-	178.9	13	< 0.001
Chak Shahzad	33.66160139	73.13995099	44	5	49	10.20	1.78	5.93	0.67-52.34			
Gujjar khan	33.26246316	73.30543805	49	68	117	58.12	5.12	167.43	22.63-1238.72			
Humak	33.53900802	73.14828967	27	0	27	0.00	-19.61	0.00	-			
Kahuta	33.59050237	73.38845047	43	3	46	6.52	1.28	3.60	0.36-35.67			
Khana	33.62912374	73.11354477	53	17	70	24.29	2.83	16.96	2.16-133.23			
Mandra	33.36149277	73.24286275	46	38	84	45.24	4.32	75.19	9.96-567.45			
PWD	33.5708391	73.14545694	68	15	83	18.07	2.34	10.38	1.34-80.52			
Rawat	33.49556111	73.19638253	56	32	88	36.36	3.82	45.60	5.98-347.81			
Sihala	33.54580694	73.1953947	36	0	36	0.00	-19.61	0.00	-			
Tarlai Khan	33.64238067	73.14955488	36	24	60	40.00	3.99	53.99	7.02-415.29			
Taramari	33.63715527	73.15620495	39	35	74	47.30	4.56	95.62	12.47-733.24			
Tarnol	33.65728874	72.91174182	78	12	90	13.33	2.03	7.62	0.96-60.68			
Taxilla	33.74487082	72.83878527	59	4	63	6.35	1.30	3.67	0.40-33.90			

(95% Confidence Interval (CI): 0.56-0.95) (P=0.018). The infestation rates varied significantly in breeds, with short-haired dogs having a higher infestation rate (31.94%, OR=5.87) (95% CI: 3.16-10.91) compared to long-haired breeds (6.15%) (P<.001). Preventive measures such as acaricide usage and regular bathing were also considered in this study. The highest infestation rate (42.25%) was observed in dogs that received the irregular acaricide application, while infestation was 1.5 times higher (29.53%) in dogs without acaricide usage. Irregular and no-use groups had significantly higher odds of infestation (OR: 4.35) compared to regularly treated dogs (OR: 2.89). Bathing was also a significant factor, infestation rate for non-bathed dogs was higher (30.20%) and the odds

ratios were 4.44 times higher in unbathed dogs (95% CI: 2.31-8.55) than bathed dogs (7.58%) (P<0.001). Roaming behavior also played a significant role, with roaming dogs (39.89%) experiencing a much higher infestation compared to non-roaming dogs (10.10%) (P<0.001) *(Table 2)*.

Spatial Distribution of Dog Ticks

Infestation rates varied across different locations in current study. The highest tick infestation was observed in Gujjar Khan (58.12%, OR=167.43) (95% CI: 22.63-1238.72), followed by Taramari (47.30%, OR=95.62), Mandra (45.24%, OR=75.19) and Tarlai Khan (40%, OR=53.99) all showed a strong statistical association. Moderate infestation rates were noted in Rawat (36.36%), Khana (24.29%), and PWD (18.07%) (OR=10 to 45 times higher. The lowest infestation rates were found for Humak (0%), Sihala (0%), and Barakahu (1.89%). The association between location and infestation rates was statistically significant *(Table 3)*.

Temporal Dynamics of Tick Infestation in Dogs

The temporal dynamics varied significantly in the present study. Total 940 dogs were inspected, and 254 were extracted. The results showed the monthly variation in infestation rates, with the highest prevalence recorded in August (51.05%, OR=5.47) (95% CI: 2.89-10.36) followed by June (45.90%, OR=4.95) (95% CI: 2.48-9.87), May (43.90%, OR=4.48) (95% CI: 2.01-9.98). The lowest tick infestation rates were observed in December (OR=0.38, 95% CI: 0.19-0.75), November, and October.

Seasonal analysis revealed that ticks had the highest infestation rate during summer (32.42%), followed by spring (25.81%), winter (7.53%), and fall (7.32%). Chi-square analysis and odds ratios showed a statistically significant relationship between tick infestation and monthly and seasonal variations, indicating higher tick infestation risk during warmer periods *(Table 4)* *(Fig. 3)*.

Assessment of Diversity of Tick Species Infesting Dogs

The current study determined the diversity of tick species infesting dogs through Shannon-Wiener and Simpson's Diversity Indices. *Rhipicephalus sanguineus* ticks dominated the others but *R. haemaphysaloides* ticks

Table 4. *Temporal dynamics of tick infestation in dogs*

Category		Tick' Infestation		Total	Infestation Rate (%)	Coefficient (β)	Odds Ratio	95% CI for OR	Chi-Square	df	P-value
		Non-infested	Infested								
Months	January	18	2	20	10.00	0	1.00	-	119.8[a]	11	0.0001
	February	10	4	14	28.57	1.08	2.94	0.92-9.39			
	March	26	9	35	25.71	0.96	2.61	1.16-5.85			
	April	20	7	27	25.93	0.98	2.66	1.10-6.45			
	May	23	18	41	43.90	1.50	4.48	2.01-9.98			
	June	66	56	122	45.90	1.60	4.95	2.48-9.87			
	July	308	77	385	20.00	0.69	2.00	1.26-3.18			
	August	70	73	143	51.05	1.70	5.47	2.89-10.36			
	September	5	1	6	16.67	0.51	1.67	0.29-9.62			
	October	33	2	35	5.71	-0.63	0.53	0.26-1.08			
	November	57	3	60	5.00	-0.69	0.50	0.29-0.86			
	December	50	2	52	3.85	-0.96	0.38	0.19-0.75			
Seasons	Winter (November - February)	135	11	146	7.53	0	1.00	-	46.43	3	0.0001
	Spring (March - April)	46	16	62	25.81	1.32	3.74	1.83-7.65			
	Summer (May - August)	467	224	691	32.42	1.80	6.05	3.22-11.37			
	Fall (September - October)	38	3	41	7.32	-0.03	0.97	0.28-3.36			

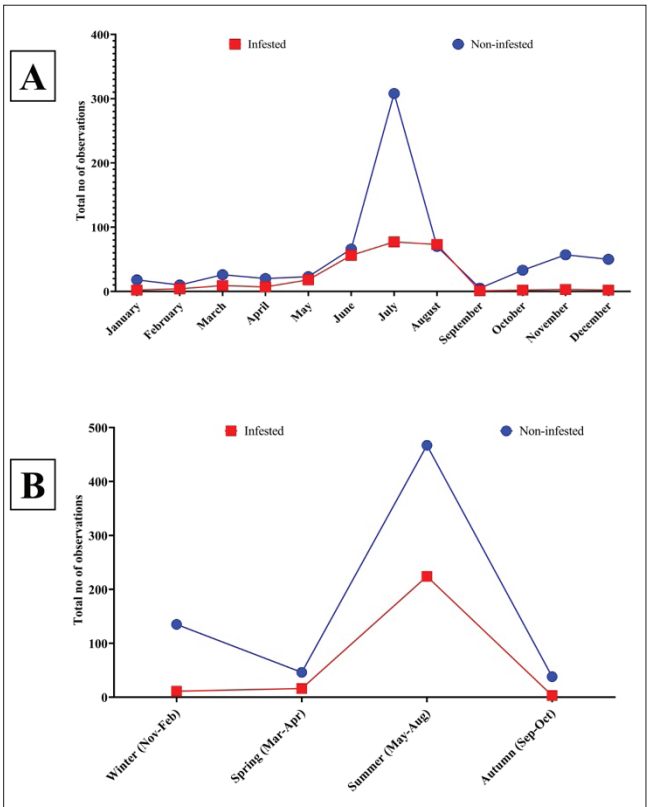

Fig 3. Temporal distribution of host wise prevalence of ticks in dogs (A: month wise data, B: seasonal pattern)

stimulated by the environmental conditions in Pakistan. However, previous reports have confirmed several tick species on different hosts across various regions of Pakistan, highlighting the need for further research focused specifically on canine tick infestations in urban settings [7,18-20,27-32].

The seasonal tick infestation pattern observed in this study were influenced by both biotic and abiotic factors. Warm temperatures and high humidity during summer and spring favored tick activity and survival. Dense vegetation and suitable habitats also supported tick growth in some areas. Dog related factors like roaming poor grooming and high population density raised infestation risks [9-11]. The overall infestation rate of the current study was 27%. The overall tick infestation rate in our study on dogs was relatively lower than reported studies conducted in Pakistan [11,20]. This difference could be attributed to Islamabad's relatively more urbanized and developed environment, lower livestock density, and better waste management [33,34], all of which likely contribute to reduced tick proliferation. Notably, our study found a high prevalence of *R. sanguineus*, which were consistent with previous studies [7,11,18,35-38] followed by *R. haemaphysaloides,* and *R. turanicus* [7,18,39]. These species have been associated with dogs in both rural

Table 5. *Assessment of the diversity of tick species infesting dogs*

Category	**Shannon-Wiener Diversity Index (H')**				**Simpson's Diversity Index (D)**			
Species	Total (n_i)	Proportion (p_i)	pi·ln(pi)	(H')	ni (ni−1)	Sum the values of ni(ni−1)	*N* (*N*−1)	(D)
R. turanicus	59	0.0829	−0.2065	0.6070	3422	344.498	506.232	0.3195
R. sanguineus	580	0.8146	−0.1670		335.820			
R. haemaphysaloides	73	0.1025	−0.2335		5.256			

[(H') is the Shannon-Wiener Diversity Index, where: pi is the proportion of each species in the total sample and ln pi is the natural logarithm of pi. While (D) is the Simpson's Diversity Index, ni = Number of individuals of each species and N = Total number of individuals of all species]

and *Rhipicephalus turanicus* were reported in lower ratios. The Shannon-Wiener Diversity Index (H') indicated a moderate level of species diversity because other species also inhabited in study area. Similarly, the Simpson's Diversity Index (D), which represented the probability that two randomly selected individuals belong to different species, showed a level of population diversity. This finding indicated that the majority of dogs were infested with *R. sanguineus* tick. Despite the presence of three tick species, one particular species exhibited dominance over others in terms of abundance *(Table 5)*.

Discussion

The current study investigated the diversity and prevalence of tick species infesting dogs in the cities of Rawalpindi and Islamabad. Tick reproduction and development are

and urban areas, as well as confirmed with detection of several rickettsial, protozoan pathogens, threatening both animal and public health in the region [13,15,38]. Differential accuracy often requires the use of molecular tools for tick species identification [40-43], which were outside the frame of our present study. The presence of *Rhipicephalus* species on canine hosts demonstrates the need for additional taxonomic studies, including at least genetic markers that will be helpful to determine species distribution patterns and host associations in this area.

There is significant variation in the risk factors associated with tick infestation across different research studies. Younger dogs had slightly higher prevalence than adults [17,44]. The active and exploratory behavior of younger dogs may increase their risk of encountering tick-infested environments such as fields and wooded areas as well

as kennels and younger dogs fail to maintain proper self-grooming, allowing tick to remain attached longer periods [45,46].

Female dogs were statistically more infested than males. Our findings were consistent with reported studies [7,18-20,47]. The rest periods of pregnant or lactating female dogs are longer and more frequent [48] when they choose shaded areas or dense vegetative areas, increasing their risk of tick encounters. Coat length was the most significant factor influencing infestation rates; short haired breeds had significantly higher infestations than the long-haired breeds [21,49]. This is because short-haired dogs have more exposed skin, providing ticks with easier access to attach and feed, whereas long-haired breeds may offer a protective barrier that hinders tick attachment [20, 45]. Acaricide treatment and regular bathing were also important in keeping infestation rates at low levels. Dogs with irregular acaricide treatment showed the highest tick infestation. Dogs that were never received acaricide treatment had 1.5 times higher tick infestation than those treated regularly. Similarly, non-bathed dogs had a higher incidence of tick infestation than bathed dogs, which was consistent with previous studies [20,35]. Free-roaming dogs exhibited significantly higher infestation rates than confined dogs [50,51], which reported that free roaming behavior and exposure to the tick population of natural habitat had a direct association with tick infestation. Risk factors in present study such as, age, short haired breeds, use of acaricide, bathing, and roaming behavior [20,52].

Further, spatially distributed variations of tick infestations showed that the highest infestation rates were in Gujjar Khan, while Humak, Sihala, and Barakahu had significantly lower infestation rates. These differences could be due to because most of the dogs studied in Gujjar Khan were semi-stray or free roaming and in most instances were confined in peri-urban or rural environments where they had direct contact with vegetation, animal pens, and other possible tick foci. These dogs were not likely to get regular veterinary treatment or acaricidal treatment, hence exposing them to more ticks. On the other hand, dogs in Humak, Sihala, and Barakahu were companions of a more urbanized nature, with better hygiene, limited exposure to the external wild, and wider access to tick repellent strategies, which could all affect the lower instances of infestation. Summer and spring had the highest tick infestation than autumn and winter, which were consistent with previous literature [19,20,30,39,53] reported that tick activity peaks during warm and humid seasons when survival and host-seeking behavior are most favorable. Increased moisture and high temperature are responsible for the high tick infestation rate observed in the summer [54].

Simpson's Diversity Index (D) indicated a relatively low level of population diversity, demonstrating that many dogs were infested with *R. sanguineus*. In contrast, the Shannon-Wiener Diversity Index (H') suggested a moderate level of species diversity. Ecological studies on similar habitats often report the predominance of a single tick species due to habitat and host specificity; such a predominance may signal such specificity [55-57], but a study conducted in Lakki Marwat district, Khyber Pakhtunkhwa, Pakistan reported low diversity in dogs [58]. The current study highlights the necessity of specific tick management strategies, particularly in high-risk areas and during times when the dog population is at high risk of infestation. Future studies that incorporate molecular tools are highly recommended for dog-related tick research, and long-term surveillance of tick species should be done to assess the emerging species and tick distribution in Islamabad and Rawalpindi, Pakistan.

The present study provides comprehensive insight into the prevalence, diversity and risk factor factors of infestation in dogs from Rawalpindi and Islamabad, Pakistan. Our findings revealed *R. sanguineus* as the dominant species, with seasonal patterns showing peak infestation during summer. Several risk factors including young age, female gender, short hair, irregular acaricides use, lake of bathing, and free roaming, were significantly associated with higher infestation rates of ticks. Spatial variations highlighted certain areas hotspot for tick occurrence. The moderate species diversity and high prevalence emphasizes the need for targeted control measures in dogs. Regular acaricide treatments, proper hygiene, and restricted roaming could significantly reduce infestation risks. This current study also underscores the need for continued surveillance and future research employing molecular identification methods to better understand it ecology and control strategies in the region.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author (H. Ahmed) on reasonable request.

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RESEARCH ARTICLE

Comparative Analysis of Diet Composition and Gut Parasite Diversity in Bar-Headed Geese and Ruddy Shelducks Using Environmental DNA Metabarcoding

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Abstract

We comprehensively analyzed the dietary preferences and intestinal parasite diversity of two waterbirds, the Ruddy shelduck *(Tadorna ferruginea)* and the Bar-headed goose (*Anser indicus*), using environmental DNA metabarcoding with the 18S rDNA V9 (for detecting plankton and parasites) and trnLg - trnLh (for detecting terrestrial plants) amplicon primer pairs. Our results showed that both species fed on multiple types of phytoplankton and terrestrial plants, but with different abundances. The ruddy shelduck mainly consumed *Chlorophyta*, *Bacillariophyta*, and *Poa*, while the bar-headed goose preferred *Chlorophyta*, *Xanthophyta*, *Pyrrophyta*, and *Potentilla*. Alpha - and beta-diversity analyses revealed significant niche differences in their food choices, enabling coexistence through different food selection or different consumption levels of the same food. Moreover, we detected the main intestinal parasites in both species: *Eimeria sp.* and *Tetratrichomonas sp*. in the bar-headed goose, and *Eimeria sp.* and some endogenous protozoan parasites in the ruddy shelduck. The dominance, species, and genetic variation range of this host-parasite system require further study and attention in future work. Our findings enhance the understanding of the ecological roles and dietary preferences of these two waterbirds in the Tibetan Plateau wetland ecosystem of China, and are significant for wetland environmental protection and species conservation.

Keywords: Bar-headed goose, Dietary analysis, Environmental DNA, Metabarcoding, Parasites, Ruddy shelduck

INTRODUCTION

Dietary analysis plays a pivotal role in animal ecology, constituting an essential component of nutritional ecology. It is used to analyze the survival conditions, habitat preferences, and ecosystem functions of individuals or animal populations. Additionally, it reflects the interrelationships between species (including predator-prey relationships and food web interactions) and elucidates the ecological roles of various organisms within communities [1,2]. Furthermore, analyzing animal diets serves as a critical tool for tracing the origins and transmission pathways of zoonotic diseases [3], Consequently, studies on animal diets hold immense research value for wildlife conservation from a dietary perspective and ensuring human health. Birds, with their large populations, wide distribution, and sensitivity to environmental changes and human disturbances, act as sensitive indicators of ecological conditions [4]. Thus, research on avian feeding habits has long been a central focus in ornithology [5]. However, traditional methods have limitations in accurately and comprehensively analyzing avian diets [6]. Traditional methods for studying bird diets, including photographing [7], feces collection, stomach content analysis [8], and chick neck-tie sampling [9], have several limitations. These methods struggle with precise identification and quantification of food components, especially when dealing with small, quickly digested, or fragmented food items [10-13]. Additionally, these techniques can impact species by altering behaviors and even causing death [14]. Environmental DNA (eDNA) metabarcoding has revolutionized avian diet research by detecting prey

DNA in feces, vomit, or other environmental samples. This technique enables identification of consumed prey, quantification of dietary proportions, and detection of parasites [15]. Compared with traditional research methods, the advantage of environmental DNA sequencing and analysis lies in its capability to provide large-scale data. This approach facilitates a more comprehensive analysis of the spatial distribution and dynamic changes within animal populations [16].

Both the Bar-headed Goose and the Ruddy Shelduck are waterfowl belonging to the *Anatidae* family of the *Anseriformes* order. They have wide distribution ranges and possess edible value, contributing to economic benefits [17]. Unfortunately, habitat destruction on a global scale and hunting pressures have led to a sharp decline in their population numbers [18]. As two representative species widely distributed and abundant within the wetland wildlife resources of the Tibetan Plateau, both the Bar-headed Goose and the Ruddy Shelduck hold considerable economic and ecological value. Therefore, dietary and parasitological studies of these two bird species could provide valuable insights into their habitat preferences, resource consumption behaviors, and physiological characteristics such as dietary niche differentiation, contributing to the conservation of wetland bird diversity and effective management of wetland ecosystems. Despite recent research focusing on aspects such as avian migration [19] and gut microbiota [20]. for both the Ruddy Shelduck and the Bar-headed Goose, issues related to their feeding habits and parasites have been less explored. This study aims to investigate the dietary preferences and differences between the Bar-headed Goose and the Ruddy Shelduck using environmental DNA metabarcoding with two primer pairs (18s-V9F and trnLg-trnLh) to determine whether there is dietary niche differentiation between the two species and to analyze their internal parasites.

Material and Methods

Ethical Approval

This study conformed to the guidelines for the care and use of experimental animals established by the Ministry of Science and Technology of the People's Republic of China (Approval number: 2006-398). The research protocol was reviewed and approved by the Ethical Committee of Qinghai University. This study did not involve capture or any direct manipulation or disturbance of Bar-headed goose and the Ruddy shelduck.

Sample Collection and Processing

In this study, environmental DNA samples were collected from feces of five Ruddy shelducks *(Tadorna ferruginea*, abbreviated as RSD group) and five Bar-headed geese *(Anser indicus*, abbreviated as BHG group), resulting in a total of ten fecal samples (five from each species). The sampling location was within the province of Qinghai, China *(Fig. 1)*. Fresh fecal samples were collected and stored in sterile tubes. All freshly collected fecal samples were transported to the laboratory using liquid nitrogen and subsequently stored in a -80°C freezer until further processing.

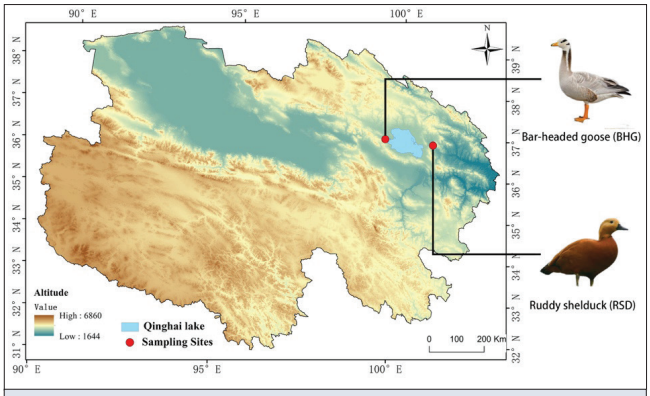

Fig 1. Map of the Qinghai-Tibetan Plateau with sampling sites

DNA Extraction from Feces and PCR Amplification

DNA was extracted from fecal samples using the Qiagen QIAamp DNA Stool Mini Kit (Qiagen, Germany) following the manufacturer's instructions. Genomic DNA integrity was assessed via 1% agarose gel electrophoresis. Metabarcoding analysis was performed by amplifying food DNA present in fecal samples using two sets of universal primers targeting different regions. The first primer set targeted the V9 region of the eukaryotic 18S rRNA gene using the primer pair 18Sv9F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The second primer set targeted the P6-trnLh region of the terrestrial plant trnL gene using the primer pair g: 5'-GGGCAATCCTGAGCCAA-3' and h: 5'-CCATT GAGTCTCTGCACCTATC-3'. PCR amplification was conducted using an ABI GeneAmp® 9700 PCR System. Custom barcoded primers were synthesized for the designated sequencing regions. Each 20 µL reaction mixture contained 4 µL of 5x FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each forward and reverse primer (5 µM), 0.4 µL of FastPfu Polymerase, 10 ng of template DNA, and ddH_2O to make up the total volume. The PCR program started with an initial denaturation at 95°C for 5 min, followed by 30 cycles of amplification (95°C for 30 sec, 58°C for 30 sec, and 72°C for 45 sec), and ended with a final extension at 72°C for 10 min, cooling down to 4°C. Based on preliminary quantification results obtained by agarose gel electrophoresis, the PCR products were accurately quantified using the QuantiFluor™ -ST Fluorometer (Promega) system. According to the DNA

concentration of each sample and the sequencing requirements, the PCR products were pooled in appropriate ratios.

Construction of High-Throughput Sequencing (NGS) Libraries

Library preparation for NGS was outsourced to Shanghai BioZeron Biotechnology Co., Ltd. The libraries were constructed for Illumina PE250 sequencing. Initially, 'Y'-shaped adaptors were ligated to both ends of the DNA fragments. Subsequently, non-specific fragments, including adaptor dimers, were removed using magnetic bead selection technology. PCR amplification was then performed to increase the library template quantity, effectively enriching the library. Upon completion of library construction, sodium hydroxide treatment was applied to denature the double-stranded DNA within the library into single-stranded DNA fragments, preparing them for subsequent bridge PCR and Illumina PE sequencing.

Data Accessibility

Based on the effective sequences from all samples, the Trimmomatic software was utilized to perform quality control filtering of the reads. Reads with quality scores below 20 at their tails were trimmed using a sliding window of 10 base pairs; if the average quality within the window fell below 20, the end of the read was removed starting from that point. After quality control, reads shorter than 50 base pairs were discarded. Paired-end (PE) reads were merged into a single sequence based on their overlap, with a minimum overlap length of 10 base pairs and an allowable maximum mismatch rate not exceeding 20%; sequences that did not meet these criteria were excluded. The orientation of sequences was corrected according to the forward and reverse barcodes and primers, and chimeric sequences were removed. The high-quality sequences were then separated by barcode and primer sequence to obtain the high-quality sequences for each sample. Subsequently, duplicate sequences were removed, and the remaining reads were processed through the DADA2 algorithm in QIIME 2 (version 2020.11) for quality filtering, denoising, merging, and chimera removal. Sequences with 100% similarity were grouped into amplicon sequence variants (ASVs), and representative sequences were generated. Taxonomic annotation of the representative sequences was performed using the uclust algorithm (with a confidence threshold of 0.8) to assign classification information at various taxonomic levels including domain, kingdom, phylum, class, order, family, genus, and species. Comparative analysis was conducted using databases such as Silva for plankton, PR2 for protists, and NT for general nucleotide sequences. The community composition and phylogenetic structure were further analyzed and visualized using the vegan package in R software (version 3.6.3). Bar charts were created using the ggplot2 package. And significant differences between sample groups were identified using the Analysis of Similarities (ANOSIM). Statistical comparisons were carried out using the non-parametric Wilcoxon test, and multiple testing corrections were made using the Bonferroni method. For all statistical tests, p-values less than 0.05 were considered statistically significant. Additionally, rarefaction curves were constructed using Mothur (version 1.21.1) to evaluate diversity indices such as Chao1, Pielou J, and Shannon. Beta diversity analysis was performed using principal component analysis (PCoA) plots were generated using the Vegan 2.0 package available on R-Forge.

Results

Sequencing Outcome Statistics

Following quality control, filtering, and merging procedures, 923.360 valid sequences were obtained from amplification using the 18S-V9 region primers. Of these, 923.356 sequences had lengths between 101-200 bp, with an average length of 138 bp. Detailed statistics regarding the optimized sequence counts, base pairs, and average sequence lengths for each sample were presented in *Table 1*. Additionally, 943,910 valid sequences were acquired through amplification using the trnLg-trnLh region primers. Among these sequences, 943.863 were found to have lengths within the 1-100 bp range, averaging 64 bp. Comprehensive sequence information for each sample was tabulated in *Table 2*.

Phytoplankton Plant Species Composition and Abundance Statistics

In the field of phytoplankton, a total of 213 ASVs were detected. At the phylum and family levels, all ASVs were

Table 1. *Quality control results of sequencing data (18SV9F).*

Primer	Samples	Sequences	Bases (bp)	Average Length (bp)
18SV9F-18SV9FR	BHG1	63.178	8.429.955	133.43
18SV9F-18SV9FR	BHG2	62.028	8.600.322	138.65
18SV9F-18SV9FR	BHG3	56.152	7.756.751	138.14
18SV9F-18SV9FR	BHG4	67.496	9.422.336	139.6
18SV9F-18SV9FR	BHG5	65.172	8.976.968	137.74
18SV9F-18SV9FR	RSD1	129.737	18.017.519	138.88
18SV9F-18SV9FR	RSD2	121.234	16.859.068	139.06
18SV9F-18SV9FR	RSD3	126.258	17.554.538	139.04
18SV9F-18SV9FR	RSD4	112.763	15.675.603	139.01
18SV9F-18SV9FR	RSD5	119.342	16.575.467	138.89

Table 2. *Quality control results of sequencing data (trnLg).*

Primer	Samples	Sequences	Bases (bp)	Average length (bp)
trnLg-trnLh	BHG1	62,468	3,988,623	63.85
trnLg-trnLh	BHG2	62,343	4,120,298	66.09
trnLg-trnLh	BHG3	56,125	3,630,090	64.68
trnLg-trnLh	BHG4	57,426	3,852,546	67.09
trnLg-trnLh	BHG5	61,904	4,140,291	66.88
trnLg-trnLh	RSD1	125,894	8,042,020	63.88
trnLg-trnLh	RSD2	118,573	7,509,705	63.33
trnLg-trnLh	RSD3	137,940	8,819,061	63.93
trnLg-trnLh	RSD4	126,661	8,132,803	64.21
trnLg-trnLh	RSD5	134,576	8,633,477	64.15

Table 3: *Statistical information on the annotation of each sample to the taxonomic levels of phytoplankton (phylum, family, genus, species)*

Taxonomy	BHG1	BHG2	BHG3	BHG4	BHG5	RSD1	RSD2	RSD3	RSD4	RSD5	Total
Phylum	4	3	1	6	5	7	4	3	7	6	8
Family	6	4	1	6	13	21	9	6	14	13	32
Genus	6	3	1	5	18	25	12	7	15	15	52
Species	3	0	0	2	2	2	1	0	0	1	7

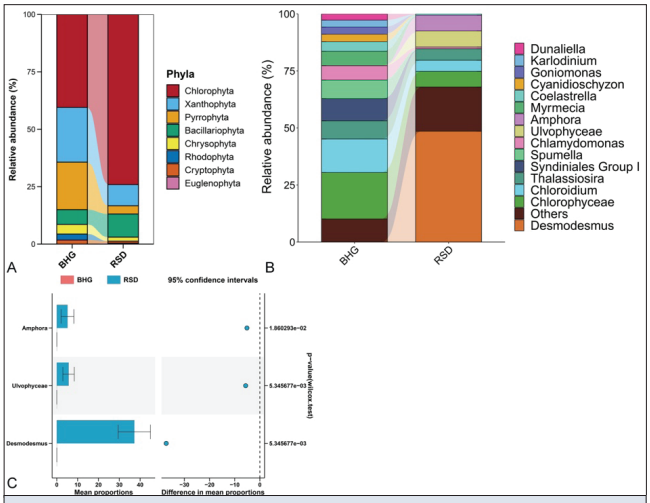

Fig 2. Phytoplankton composition in bar-headed goose (BHG) group and ruddy shelduck (RSD) group. **A**- Relative abundance of the dominant phytoplankton phyla in each group, **B**- Relative abundance of the dominant phytoplankton genera in each group, **C**- Phytoplankton composition at the genus level showing inter-group differences

annotated. At the genus level, 62.91% of ASVs were successfully annotated, while at the species level, only a modest 3.76% of ASVs received annotations. The results indicated that the phytoplankton consumed by the BHG and the RSD covered 8 phyla, 32 families, 52 genera, and 7 species. The number of distinct taxonomic levels annotated for each sample was detailed in *Table 3*. At the phylum level *(Fig. 2-A)*, the dominant phytoplankton groups for BHG were *Chlorophyta* (40.48%), *Xanthophyta* (23.86%), and *Pyrrophyta* (20.67%). For RSD, the major phytoplankton groups were *Chlorophyta* (74.1%), *Bacillariophyta* (10.08%), and *Xanthophyta* (9.23%). Furthermore, the comparative analysis at the phylum level revealed that there were no significant differences in the relative abundances of these phyla between the two groups. At the genus level *(Fig. 2-B)*, the dominant phytoplankton groups for BHG were *Chlorophyceae* (20.38%), *Chloroidium* (14.66%), and *Syndiniales Group*

I (9.70%). For RSD, the major phytoplankton groups were *Desmodesmus* (48.58%), *Ulvophyceae* (7.00%), and *Amphora* (6.93%). Furthermore, the comparative analysis at the genus level revealed that a total of three genera showed significant differences between the two groups *(Fig. 2-C)*.

Terrestrial Plant Species Composition and Abundance Statistics

Analysis of the sequencing data for terrestrial plants revealed that the terrestrial food groups consumed by the BHG and the RSD encompassed 1 domain, 1 phylum, 1 class, 19 orders, 34 families, 172 genera, and 261 species *(Table 4)*. The terrestrial food groups for BHG consisted of 17 orders, 26 families, 150 genera, and 223 species, while those for RSD consisted of 15 orders, 21 families, 62 genera, and 91 species. From the statistical results at the taxonomic levels, BHG had higher totals of species at the order, family, genus, and species levels compared to RSD. The analysis at the phylum level revealed that the terrestrial plant composition of both BHG and RSD groups was dominated by *Streptophyta*, with no significant difference in relative abundance observed between the two groups *(Fig. 3-A)*. In the analysis of the BHG group, it was found that the genus exhibiting the highest abundance was *Potentilla* (46.99%), followed by *Elymus* (8.86%) and *Avena* (6.02%), amongst others *(Fig. 3-B)*. In the analysis of the RSD group, it was identified that the genus exhibiting the highest abundance was *Poa* (60.92%), followed by *Potentilla* (18.03%) and *Puccinellia* (13.81%), among others *(Fig. 3-B)*. A total of 31 genera exhibited significant differences in abundance between the two groups. Furthermore, Linear discriminant analysis effect size (LEfSe) analysis was employed to identify taxa exhibiting significantly different abundances between groups. The LDA score plot illustrated that several taxa showed marked distinctions across the compared groups *(Fig. 3-C)*. Specifically, in the BHG group, 18 taxa, including *Symphyllocarpus*, *Barnadesia*, and *Hordeum*, demonstrated statistically significant differences in abundance ($P<0.05$). Similarly, for the RSD group, 18 taxa such as *Poa*, *Poales*, and *Puccinellia* also exhibited statistically significant differences in their abundance levels ($P<0.05$).

Analysis of Alpha Diversity

The dilution curves and species accumulation curves derived from the sequencing of phytoplankton and terrestrial plant communities indicated that the current sequencing depth is sufficient for the dietary diversity

Table 4: *Statistical information on the annotation of each sample to the taxonomic levels of terrestrial plants (phylum, class, order, family, genus, species)*

Taxonomy	BHG1	BHG2	BHG3	BHG4	BHG5	RSD1	RSD2	RSD3	RSD4	RSD5	Total
Phylum	1	1	1	1	1	1	1	1	1	1	1
Class	1	1	1	1	1	1	1	1	1	1	1
Order	10	13	13	9	11	11	12	8	10	13	19
Family	13	17	17	11	19	14	14	10	12	14	34
Genus	59	73	93	32	81	46	41	32	30	35	172
Species	88	97	127	51	105	63	56	41	42	49	261

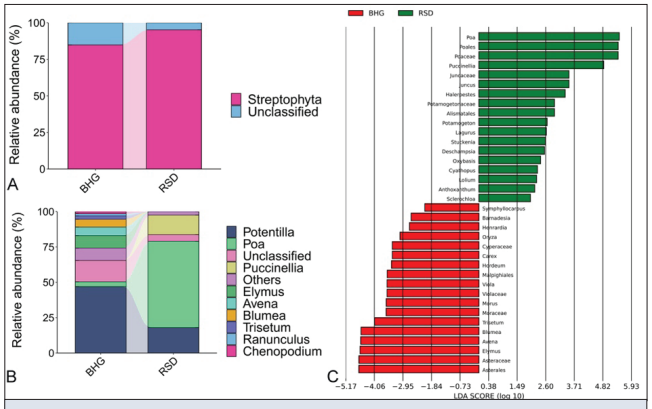

Fig 3. Terrestrial plants composition in bar-headed goose (BHG) group and ruddy shelduck (RSD) group. **A**- Relative abundance of the dominant terrestrial plants phyla in each group, **B**- Relative abundance of the dominant terrestrial plants genera in each group, **C**- A plot displaying the LDA scores obtained through LDA analysis (linear discriminant analysis) for taxa that have a significant role in the two groups

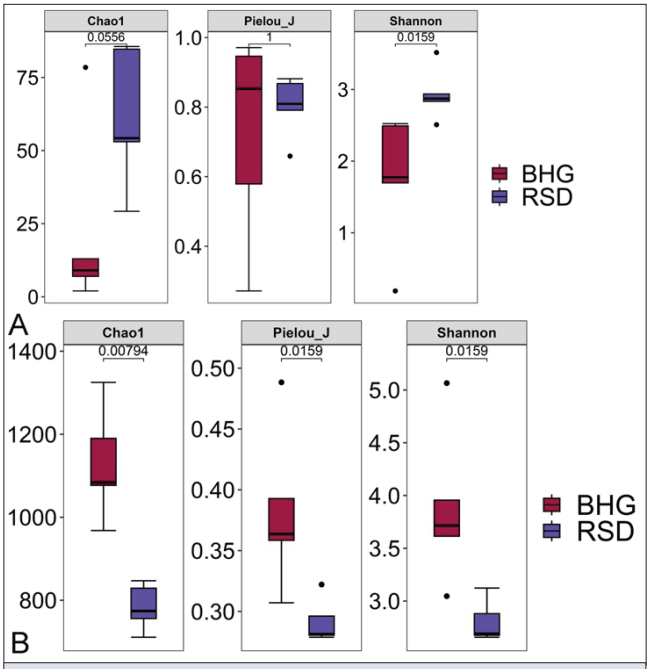

Fig 4. Comparison of alpha diversity indices. **A**- A boxplot representing the alpha diversity indices for phytoplankton, **B**- A boxplot representing the alpha diversity indices for terrestrial Plant(s)

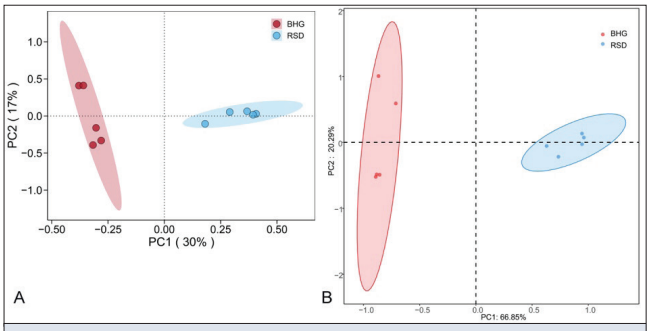

Fig 5. A- the Principal Component Analysis (PCoA) plots for phytoplankton, **B**- the Principal Component Analysis (PCoA) plots for terrestrial plants

analysis of the BHG and the RSD. The sample size is adequate for estimating species richness. Box plots of various alpha diversity indices, including the Chao1, Pielou_J, and Shannon indices, were compared between the phytoplankton communities of two groups *(Fig. 4-A)*. These analyses indicated that the phytoplankton communities consumed by RSD had higher community diversity compared to those of BHG. Similarly, alpha diversity indices of terrestrial plant communities were compared between the two groups *(Fig. 4-B)*. These results indicated that the terrestrial plant communities consumed by BHG had a higher number of species, greater community diversity, and more even species distribution, without any single species dominating the community.

Beta Diversity Analysis

PCoA was performed on the phytoplankton and terrestrial plant communities of BHG and RSD. In the

Table 5: *Statistical information on the annotation of each sample to the taxonomic levels of zooplankton (phylum, family, genus, species).*

Taxonomy	BHG1	BHG2	BHG3	BHG4	BHG5	RSD1	RSD2	RSD3	RSD4	RSD5	Total
Phylum	3	5	4	5	5	7	4	3	4	6	9
Family	6	11	7	8	15	16	7	7	8	14	31
Genus	7	8	6	5	14	15	6	7	8	10	35
Species	2	1	1	1	1	3	3	2	2	2	8

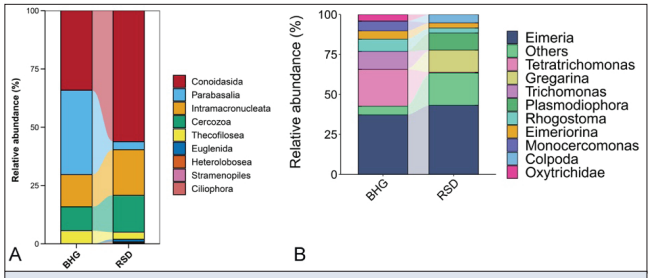

Fig 6. A- Relative abundance of the dominant zooplankton phyla, B- relative abundance of the dominant genera in each group

PCoA analysis of phytoplankton communities *(Fig. 5-A)*, the first principal component explained 30% of the variation, and the second principal component explained 17%. The samples from RSD and BHG were distantly located, indicating low similarity between the community compositions. Further ANOSIM analysis indicated that the differences between RSD and BHG were greater than the differences within each group for phytoplankton communities (R=0.556, P=0.015) and that these differences were statistically significant.In the PCA analysis of terrestrial plant communities, the first principal component explained 66.85% of the variation, and the second principal component explained 20.29% *(Fig. 5-B)*. The samples from RSD and BHG were again distantly located, indicating low similarity between the community compositions. For terrestrial plant communities, ANOSIM analysis also showed that the differences between RSD and BHG were greater than the differences within each group (R=0.596, P=0.007) and were statistically significant.

Parasite and Protozoan Composition Analysis

A total of 401 ASVs were annotated to zooplankton. The sequencing results indicated that the protozoans and parasites in BHG and RSD covered 9 phyla, 31 families, 35 genera, and 8 species *(Table 5)*. At the phylum level *(Fig. 6-A)*, the dominant phyla for BHG were *Parabasalia* (36.2%), *Conoidasida* (34.12%), and *Intramacronucleata* (13.81%). For RSD, the dominant phyla were *Conoidasida* (56.19%), *Intramacronucleata* (19.58%), and *Cercozoa* (15.77%). The top 10 genera *(Fig. 6-B)* revealed that the parasites in BHG were predominantly by *Eimeria sp.* and *Tetratrichomonas sp.*, which are common endoparasites in vertebrates. In RSD, the parasites were mainly *Eimeria* sp., along with some endoparasitic protozoa genera that infect animals *(Gregarina* sp.) and plants *(Plasmodiophora* sp.).

Discussion

This study used an environmental DNA (eDNA) metabarcoding method for the first time. With two primer pairs, it determined the phytoplankton and terrestrial plant diet compositions of BHG and RSD on the Qinghai-Tibet Plateau in China. The results revealed that the BHG primarily fed on *Chlorophyta*, *Xanthophyta*, and *Pyrrophyta* in phytoplankton, while it showed a preference for *Potentilla* within *Streptophyta* in its terrestrial diet. In contrast, the Ruddy Shelduck predominantly consumed *Chlorophyta* and *Bacillariophyta* in phytoplankton, and mainly fed on *Poa* within *Streptophyta* in its terrestrial diet. Although the experimental results revealed the presence of zooplankton group sequences, the annotations were primarily dominated by protists. These included several single-celled eukaryotes *(Heterolobosea, Cercozoa, Ciliophora, Euglenida)* and parasitic organisms *(Parabasalia, Conoidasida)*. However, this did not necessarily indicate that these organisms were directly consumed by the two waterbird species. Both the BHG, an omnivorous waterbird that feeds primarily on seeds of herbaceous plants -including grasses, leaves, roots, tubers, grains, and nuts- but also consumes small fish and aquatic macro-invertebrates when seed availability is limited [21], and the RSD, another omnivorous waterbird that adjusts its diet according to seasonal and habitat-specific food resources [18], are unlikely to directly prey on protists. Instead, they are more likely to indirectly ingest these microorganisms through intermediate hosts such as small fish, aquatic invertebrates, mollusks, or other planktonic organisms While these protozoa cannot serve as direct food sources for these waterfowl, they play a critical role in aquatic ecosystems as primary producers, supporting the base of the food chain [22,23]. Based on the current findings, future studies should employ stable isotope analysis and behavioral observations [24] to directly verify the types of zooplankton consumed by these two waterbird species and their utilization of resources at different trophic levels. In addition to the dominant eukaryotic protist group *Cercozoa* found in free-living aquatic environments [25], the identification of these zooplankton groups provided additional information about parasitic taxa. *Parabasalia* and *Conoidasida* were most abundant in BHG, while *Conoidasida* and *Intramacronucleata* were most abundant in RSD. Among these, *Eimeria sp.*, a common endoparasite in vertebrates [26], had relatively high abundance. Additionally, the composition of parasites was found to be highly correlated with diet composition [27]. Therefore, further research was suggested to investigate

the relationship between the diet composition and parasite load in the BHG and RSD, which could provide insights into their health status.

The selection of fecal samples from BHGs and RSDs for dietary analysis and interspecific comparison was methodologically justified, as both species are omnivorous waterbirds exhibiting highly congruent habitat preferences [20]. However, two species with identical niches cannot stably coexist in the same environment for a long time [28]. To achieve stable coexistence, species had to differentiate their niches in aspects like time, space, or food. They could reduce resource competition and realize trophic niche segregation by selecting different food, staggering activity times, or occupying different locations [29]. At the taxonomic level, zooplankton exhibited significantly more ASVs than phytoplankton (401 vs. 213), suggesting that both waterfowl species preferred zooplankton when feeding on plankton. This preference likely resulted from the ingestion of zooplankton while drinking water [30], or due to trophic interactions within the food chain. Alpha-diversity analysis revealed that RSD had a higher number of species and a more even distribution in their phytoplankton food sources, indicating efficient utilization of diverse phytoplankton resources. In contrast, BHG exhibited richer species numbers and higher community diversity in terrestrial plant food sources. These findings suggest that BHG and RSD reduce resource competition and achieve trophic niche segregation by selecting different food sources or varying the intake amounts of the same food source. This allows them to coexist under limited resources and maintain ecosystem diversity and balance.

Birds' food choices mirror their habitat use. Food resources greatly impact birds' reproduction and habitat selection, and are closely linked to birds' energy needs and habitat resource abundance [31]. According to the optimal foraging theory, foragers seek food options that offer the highest energy returns [32]. RSD's preference for zooplankton may stem from these foods' high protein and energy content, meeting the species' needs during rapid growth or reproduction. In contrast, BHG's preference for terrestrial plants could be due to the stable supply of these foods, supporting their nutritional requirements across seasons and reproductive cycles. These dietary differences reflect divergent habitat resource use and niche partitioning, thereby reducing interspecific competition and promoting coexistence. The specific reasons for these differences remained unclear. However, PCoA analyses indicated significant dietary differences between the two waterfowl species, showing that they occupied distinct ecological niches. The niche differentiation hypothesis posited that coexisting species in the same geographic area could reduce interspecific competition and promote coexistence and ecosystem stability through niche differentiation, despite similarities in morphology, behavior, or resource use [33].

Limitations within this study warrant acknowledgment. Firstly, the restricted sample size may compromise the precision of certain conclusions. Secondly, no primer set can perfectly amplify DNA from every species, despite some primer combinations showing high efficacy under specific conditions. Even with the design of two primer sets for amplification in this study, the ideal species results for zooplankton consumed by the two waterbird species were not obtained. Furthermore, since these waterbirds are not obligate specialists, they incidentally ingest non-target items such as plants or zooplankton during feeding, which might be taken up with water. The presence of these species in fecal samples and their detection could influence the interpretation of the final results.

In summary, this study employed environmental DNA (eDNA) metabarcoding using two primer pairs to comprehensively investigate the dietary composition and feeding preferences of the BHG and the RSD, as well as to analyze the parasite species present in both species. Through comparative analysis of their diets, we have uncovered differences in their utilization of trophic niches. Although the precise causes of these distinctions remain to be fully elucidated, these findings significantly enhance understanding of avian dietary adaptability and ecological strategies. They also showcase a novel perspective and methodology for studying wildlife diets using modern molecular biology techniques. The application of environmental DNA metabarcoding has not only improved the accuracy and efficiency of dietary analysis but also provided a powerful tool for monitoring biodiversity, assessing ecosystem health, and informing conservation management strategies. Moving forward, interdisciplinary research combining behavioral ecology, physiological ecology, genetics, and other fields will further unravel the ecological and evolutionary mechanisms underlying avian feeding choices.

Declarations

Availability of Data and Materials: The data given in this study may be obtained from the corresponding auther (W W) on reasonable request.

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Ethical Approval: This study conformed to the guidelines for the care and use of experimental animals established by the Ministry of Science and Technology of the People's Republic of China (Approval number: 2006-398).

Competing Interests: The authors declared that there is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The authors declare that the article, tables and figures were not written/created by AI and AI-assisted Technologies.

Author Contributions: WW conceived and designed the study. WW and YGD drafted the manuscript and provided critical revisions. YGD, YL H, FY, XLW, and ZMLC performed the experiments and analyzed the data. All authors read and approved the final manuscript.

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Research Article

Mechanistic Insights into the Mitigating Role of Beta-Caryophyllene on Cadmium-Induced Liver Injury

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Abstract

Cadmium (Cd) is a toxic heavy metal that can cause severe hepatotoxicity in the body. This study investigates the hepatoprotective effects of Beta-Caryophyllene (BCP), a natural sesquiterpene, against Cd-induced liver injury. In the study, the protective effects of BCP on biological processes such as oxidative stress, inflammation and apoptosis were investigated through TLR4/NF-κB, SIRT1/KEAP1/Nrf2/HO-1 and Bax/Bcl-2/Caspase3 signaling pathways. For this purpose, 50 male rats were divided into 5 groups: Control, Cd, BCP100+Cd, BCP200+Cd and BCP200, 10 in each group. At the end of the study, it was determined that Cd exposure caused damage to cells by increasing lipid peroxidation, oxidative stress and inflammation in liver tissue. However, BCP treatment was found to reduce oxidative damage by increasing antioxidant enzyme activities (SOD, GSH, CAT) and reducing lipid peroxidation (MDA). Furthermore, it was determined that BCP lowered proinflammatory cytokine levels (TNF-α, IL-1β) by inhibiting TLR4/NF-κB signaling activity, while also increasing anti-inflammatory IL-10 levels. It was also observed that BCP inhibits the suppression of Nrf2 through KEAP1 by activating the Nrf2 signaling pathway, resulting in elevated levels of SIRT and HO-1. In the analyses of apoptosis, it was determined that BCP inhibited Caspase3 activity and reduced apoptosis in liver cells by balancing the Bax/Bcl-2 ratio. These findings suggest that BCP provides potent protection against Cd-induced liver toxicity by regulating various signaling pathways and could potentially be used as a hepatoprotective agent.

Keywords: Apoptosis, Beta Caryophyllene, Cadmium, Hepatotoxicity, Inflammation, Oxidative stress

Introduction

Cadmium (Cd) is one of the heavy metals that has no physiological function, such as arsenic, lead, mercury and chromium, and is considered toxic to human health [1-4]. Smokers are 3-4 times more exposed to Cd than non-smokers. The amount of Cd ingested through food usually varies between 10-25 µg, but this value can be much higher in contaminated areas [5-7]. The accumulation of this metal in the body, the production of free radicals, leads to peroxidation in cell membranes, disruption of DNA repair mechanisms and cellular changes, causing serious damage to the body [8,9]. Cd triggers harmful biological processes in liver cells, such as oxidative stress, inflammation, and apoptosis. Furthermore, Cd has been shown to suppress antioxidant defense mechanisms by increasing the production of reactive oxygen species (ROS), trigger inflammation by activating the TLR4/NF-κB signaling pathway, and weaken cellular protection mechanisms by inhibiting the Nrf2/HO-1/SIRT1 signaling pathway via KEAP1 [10,11].

Beta-caryophyllene (BCP) is an herbal sesquiterpene noted for its powerful antioxidant, anti-inflammatory, and antiapoptotic properties [12,13]. BCP, a variety of herbal it is abundant in springs, especially in herbs such as black pepper, cloves, lavender, thyme and rosemary. In particular, CB2 is known to exert anti-inflammatory and antioxidant effects by interacting with cannabinoid receptors [14]. BCP has been shown to have the potential to protect cells by reducing oxidative stress and suppressing inflammatory signaling pathways [13].

It is thought that BCP may have a protective effect against oxidative stress, inflammation and apoptosis that occur in Cd-induced liver damage via TLR4/NF-κB, KEAP1/Nrf2/HO-1/SIRT1 and Bax/Bcl-2/Cas3 pathways.

Materials and Methods

Ethical Approval

Ethics committee approval for this study was obtained from Atatürk University Animal Experiments Local Ethics Committee (Ethical No: 2025/57).

Chemicals

CD (≥99%) (CAS No: 7440-43-9) and BCP (≥80%) (CAS No: 87-44-5) were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA). ELISA kits were obtained from SunRed.

Animals

The Sprague Dawley rats used in this study were provided by Atatürk University Experimental Research and Application Center (ATADEM). The average weight of each of the 50 male rats selected for the study was between 200-250 g. A power analysis program was used to determine the number of animals per group (G-Power 3.1.9.7). A minimum of 10 rats per group and a total of 50 rats were calculated to require 99% power (Type II error, β) with an error of 0.05 (Type I, α). Data from a previous study were used for this analysis *(Fig. 1)* [15].

Experimental Design

Prior to the start of the experiment, all rats were weighed and randomly assigned to one of five groups: Group I (Control): Intragastric (IG) administration of 1 mL ddH_2O for 7 days. Group II (CD): Intraperitoneal (IP) administration of Cd at a dose of 6.5 mg/kg [15] for 7 days. Group III (BCP100+Cd): Administration of BCP at a dose of 100 mg/kg [16,17] IG, followed by administration of Cd at the 6.5 mg/kg dose for 7 days. Group IV (BCP200+Cd): Administration of BCP at a dose of 200 mg/kg [16,17] IG, followed by administration of Cd at the 6.5 mg/kg dose for 7 days. Group V (BCP200): IG administration of BCP at a dose of 200 mg/kg/day for 7 days. For Groups III and IV, BCP was administered 30 min before Cd each day. On the 8th day, the rats were weighed again and then euthanized under sevoflurane anesthesia. Liver tissues were harvested, weighed, rinsed with physiological saline, and stored at -80°C for subsequent analyses

Oxidative Parameters and Antioxidant Enzymes Analysis

Oxidative parameters and antioxidant enzyme activities were analyzed with an ELISA plate reader (Bio-Tek, Winooski, VT, USA) capable of measuring absorbance at a wavelength of 450 nm. Using previously obtained supernatants, malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) levels were determined in accordance with the guidelines of the relevant ELISA kits [18].

Inflammation Markers Analysis

Interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), interleukin-10 (IL-10), nuclear factor kappa-B (NF-κB) and Toll-like receptor 4 (TLR4) levels in supernatants were evaluated in accordance with the protocols of the relevant ELISA kits.

Histopathological Examination

When the study was completed, the liver tissues were fixed in 10% buffered formalin solution. The tissues were then followed with routine tissue and embedded in paraffin. Five μm thick sections were taken from the paraffin blocks. The sections were stained with Hematoxylin Eosin (H&E) and Masson's Trichrome (MT). The stained sections under a light microscope were examined and visualized (Leica, Flexacam i5, Germany). The levels of degeneration and necrosis in hepatocytes were examined in H&E staining, while the level of fibrosis in the tissue was determined in MT staining. The findings were evaluated as absent (No cells were affected) (0), mild (1-5 cells affected) (+1), moderate (6-15 cells affected) (+2) and severe (15< cells affected) (+3) [19].

Immunohistochemical Examination

After routine rehydration and deparaffinization of the sections taken on adhesive slides for immunohistochemical examinations, endogenous peroxidase was inactivated by

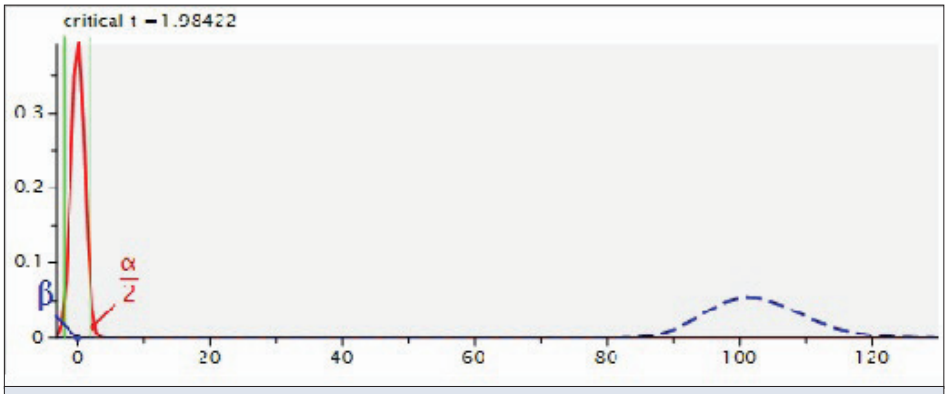

Fig 1. G-Power analysis findings to determine the number of animals in groups

keeping the tissues in 3% H_2O_2 for 10 min. The tissues were then boiled in 1% antigen retrieval solution (TRIS EDTA buffer (pH+6.1) 100X). Sections were incubated with protein block for 5 min to prevent nonspecific background staining. The tissues were then incubated with primary antibody (SIRT1 Cat No: BT-AP07980, Reconstitution Ratio: 1/100; Keap1 Cat No: E-AB-60460, Reconstitution: 1/200, US; Bcl2 Cat No: sc-7382, Reconstitution Ratio: 1/100, US) were added and incubated according to the instructions for use. 3-Amino-9-Ethylcarbazole (AEC) chromogen was used as chromogen in the tissues. Stained sections were examined by light microscopy (Leica, Flexacam i5, Germany). ImageJ analysis software was used to determine the intensity of immunopositivity in immunohistochemical analysis [20].

Immunofluoresence Examination

For immunofluorescence examination, tissue sections taken on adhesive slides were deparaffinized and rehydrated. The tissues were then boiled in 1% antigen retrieval solution (TRIS EDTA buffer (pH+6.1) 100X). Sections were incubated with protein block for 5 min to prevent nonspecific background staining. The tissues were then treated with primary antibody (Nrf2 Cat. No: E-AB-68254, Reconstitution Ratio: 1/200, US; HO-1 Cat No: E-AB-66079, Reconstitution: 1/200, US; BAX Cat No: sc-7480, Reconstitution Ratio: 1/100, US; Caspase 3 Cat No: sc-56053, Reconstitution Ratio: 1/100, US) were added and incubated according to the instructions for use. Immunofluorescence secondary antibody was used as secondary marker (FITC Cat No: ab6785 Diluent Ratio: 1/1000) and kept in the dark for 45 minutes. Then, DAPI with mounting medium (Cat no: D1306, Diluent Ratio: 1/200 UK) was added to the sections and kept in the dark for 5 min and the sections were covered with coverslips. The stained tissues were examined under a fluorescence attachment microscope (Zeiss AXIO, Germany). ImageJ analysis software was used to determine the intensity of immunopositivity in immunoforesence analysis [21].

Statistical Analysis

Statistical analyses of the study data were calculated in GraphPad Prism 8.0.2 software and $P<0.05$ was considered significant. In histopathologic analyses, nonparametric Kruskal-Wallis test was used to determine group interaction and Mann Whitney U test was used to determine the differences between groups

Results

Effect of Cd and BCP Applications on Starting, Ending and Liver Weights of Rats

At the outset of the study, there were no discernible differences in initial body weights among the experimental groups. Upon completion of the experiment, a reduction in body weight was observed across all groups subjected to Cd intoxication and subsequent treatment. The animals in the control group specifically exhibited significantly greater body weights compared to those in the Cd-treated groups. In terms of liver weight, the Cd group exhibited the highest liver weight, followed by the BCP100+Cd group, which also showed a relatively higher liver weight compared to the other three groups. The liver weights in the remaining groups were comparatively lower, though this disparity did not achieve statistical significance *(Table 1)*.

Table 1. *Effects of Cd and BCP on the starting, ending, and liver weights of rats. Weight of organ in g/kg body weight in the experimental groups (P<0.05, n=10) (the results are expressed as mean±SD)*

Groups	Weight of Liver (g/kg body weight)
Control	0.47±0.02^{b}
CD	0.58±0.01^{a}
BCP100+CD	0.53±0.01^{c}
BCP200+CD	0.49±0.03^{bc}
BCP200	0.44±0.02^{b}

a, b, c: *Values different from a indicate "a" statistically significant difference compared to the CD group*

Oxidative Stress Parameters in Liver Tissue

MDA levels were significantly increased in the Cd group compared to the control group. However, it was found that this increase was significantly suppressed in the BCP200+Cd group and MDA levels were found to be close to the control group. SOD, GSH and CAT activity was significantly decreased in the Cd group compared to the control group, but a statistically significant increase was observed in the BCP200+Cd group *(Table 2)*.

Parameters Related to Inflammation in Liver Tissue

TNF-α, IL-1β, NF-κB and TLR4 levels showed a significant increase between the Cd group and the control group. However, the BCP200+Cd group significantly suppressed this increase by significantly lowering levels.

Table 2. *Illustrates the levels of oksidan and antioksidant mediators MDA, SOD, GSH, and CAT in liver tissues (n = 10). One Way ANOVA and Tukey test (P<0.05, n=10) (the results are expressed as mean±SD)*

Groups	MDA	SOD	GSH	CAT
Control	17.45±1.37^{b}	28.51±1.76^{b}	391.15±13.56^{b}	34.25±2.05^{b}
CD	32.07±2.22^{a}	16.51±1.21^{a}	302.95±11.24^{a}	13.79±2.94^{a}
BCP100+CD	28.24±1.71^{a}	20.14±1.36^{a}	331.29±7.79^{c}	20.51±1.47^{c}
BCP200+CD	20.93±1.4^{c}	26.17±2.22^{b}	360.87±11.24^{d}	29.59±2.77^{b}
BCP200	16.8±1.05^{b}	30.01±0.93^{b}	395.04±10.34^{b}	34.64±2.95^{b}

a, b, c, d: *Values different from a indicate "a" statistically significant difference compared to the CD group*

Table 3. *Illustrates the levels of inflammatory mediators TNF-α, IL-1β, IL-10, NF-κB, and TLR4 in liver tissues. One Way ANOVA and Tukey test (P<0.05, n=10) (the results are expressed as mean±SD)*

Groups	TNF-α	IL-1β	IL-10	NF-κB	TLR4
Control	229.4±20.66^{b}	960.33±54.31^{b}	111.35±4.71^{b}	1.76±0.34^{b}	1.85±0.42^{b}
CD	457.4±31.88^{a}	1662.33±71.85^{a}	79.51±2.46^{a}	3.53±0.18^{a}	4.22±0.55^{a}
BCP100+CD	386.88±25.08^{c}	1383.66±87.12^{c}	91.78±3.83^{c}	3.05±0.2^{c}	3.27±0.26^{c}
BCP200+CD	304.17±32.61^{d}	1137±39.59^{d}	107.57±4.86^{b}	2.19±0.25^{b}	2.21±0.68^{d}
BCP200	216.57±24.12^{b}	997.66±82.49^{b}	115,24±4.78^{b}	1.85±0.15^{b}	1.81±0.35^{b}

a, b, c, d: *Values different from a indicate "a" statistically significant difference compared to the CD group*

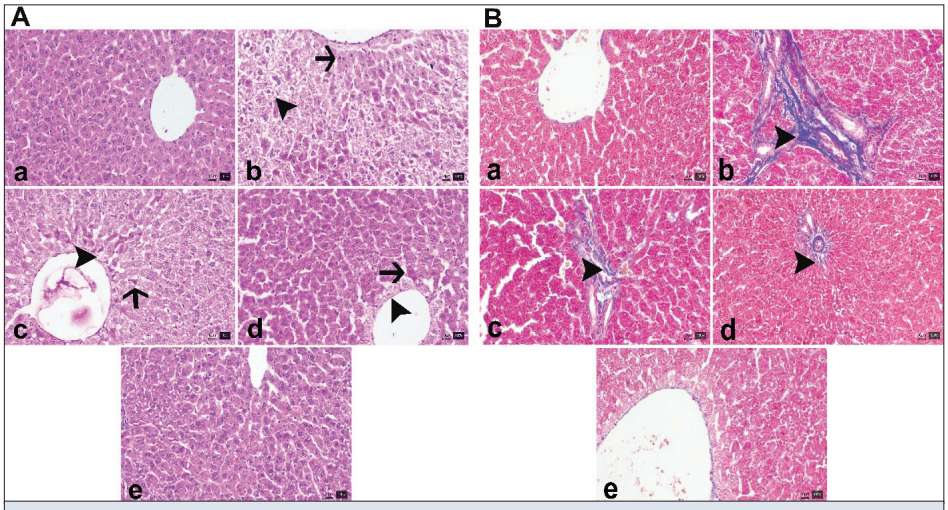

Fig 2. Liver tissue. **A)** control (a), Cd (b), BCP100+Cd (c), BCP200+Cd (d) and BCP200 (e). Degeneration in hepatocytes is shown by *arrowheads* and necrosis by *arrows* (H&E); **B)** Control (a), Cd (b), BCP100+Cd (c), BCP200+Cd (d) and BCP200 (e). Fibrosis is shown by arrowheads. MT, Bar:10μm, Objective:20X, Zoom:100%

Table 4. *Scoring of degeneration and necrosis observed in hepatocytes and statistical analysis data. Scoring of fibrosis determined in liver tissue and statistical analysis data. Kruskal-Wallis and Mann Whitney U test (P<0.05, n=10) (the results are expressed as mean±SD)*

Groups	Degeneration	Necrosis	Fibrosis
Control	0.4±0.48^{b}	0±0^{b}	0±0^{b}
CD	3±0^{a}	2.8±0.4^{a}	2.6±0.49^{a}
BCP100+CD	2.2±1.4^{ab}	1.6±0.49^{c}	1.2±0.4^{c}
BCP200+CD	1.4±0.49^{c}	0.4±0.49^{d}	0.6±0.49^{d}
BCP200	0.2±0.4^{b}	0±0^{b}	0±0^{b}

a, b, c, d: *Values different from a indicate "a" statistically significant difference compared to the CD group*

A significant decrease in IL-10 levels was observed in the Cd group compared to the control group, but BCP200+Cd administration significantly reversed this decrease and increased IL-10 levels *(Table 3)*.

Histopathological and Masson's Trichrome Findings

In histopathological and MT analysis of liver tissues, mild degeneration of hepatocytes was observed in Control and BCP200. In only Cd group, severe degeneration and necrosis in hepatocytes and severe fibrosis were detected in the centrilobular region of the liver. These findings were significantly decreased in BCP100+Cd and BCP200+Cd groups compared to Cd group at dose-dependent level. Histopathologic findings are shown in *Fig. 2-A*, MT findings in *Fig. 2-B* and scoring of these findings and statistical analysis data are presented in *Table 4*.

Immunohistochemical Findings

Immunohistochemical analysis revealed severe Bcl-2 and SIRT1 expression in hepatocytes of Control and BCP200 groups, while very mild Keap1 expression was detected. In Cd, Bcl-2 and SIRT1 expressions were observed at mild level, while Keap 1 expressions were detected at severe level. These values were found to be significantly closer to normal levels in BCP100+Cd and BCP200+Cd groups compared to Cd group. SIRT1 expression is shown in

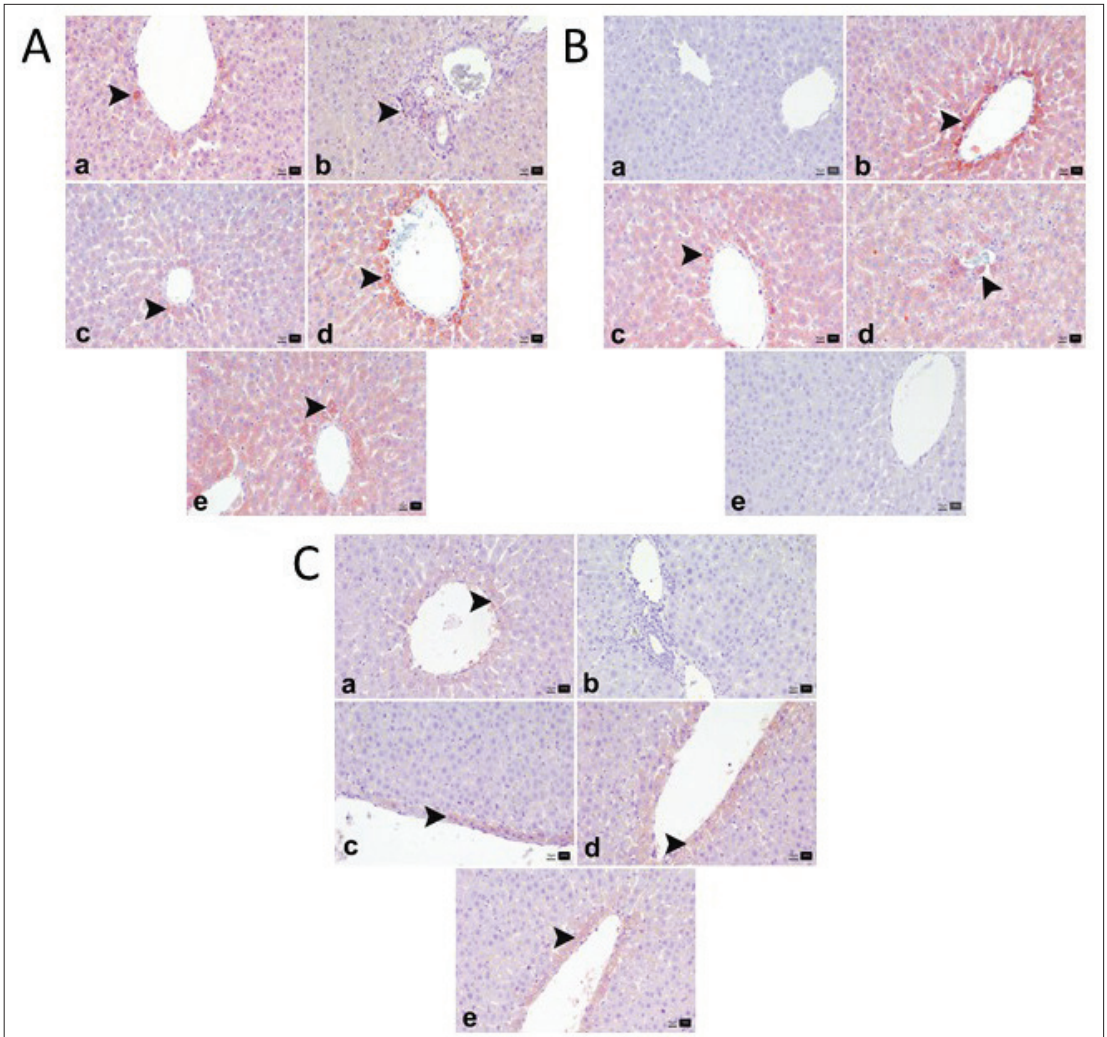

Fig 3. Liver tissue. **A)** control (a), Cd (b), BCP100+Cd (c), BCP200+Cd (d) and BCP200 (e). SIRT1 expressions are shown

Table 5. *Immunohistochemical staining results and statistical analysis data. One Way ANOVA and Tukey test (P<0.05, n=10) (the results are expressed as mean±SD)*

Groups	Bcl-2	Sırt1	Keap1
Control	8.37±0.84^{b}	6.48±0.42^{b}	0.38±0.12^{b}
CD	0.71±0.56^{a}	0.94±0.26^{a}	20.16±2.27^{a}
BCP100+ CD	2.92±0.35^{c}	2.75±0.25^{c}	10.9±1.46^{c}
BCP200+ CD	6.16±0.66^{d}	7±0.39^{d}	4.46±1.31^{d}
BCP200	8.39±0.58^{b}	10.12±0.55^{b}	0.41±0.17^{b}

a, b, c, d: *Values different from a indicate "a" statistically significant difference compared to the CD group*

Fig. 3-A, Keap1 expression in *Fig. 3-B* and Bcl2 expression in *Fig. 3C*. Immunohistochemical staining results and statistical analysis data are presented in *Table 5*.

Immunofluoresence Findings

In immunofluorescence analysis, severe Nrf2 and HO-1 expressions were observed in hepatocytes of Control and BCP200 groups, while very mild Bcl-2 and Caspase 3 expressions were detected. In Cd, mild Nrf2 and HO-1 expressions were observed, while severe Bcl-2 and Caspase 3 expressions were detected. These values were found to be significantly closer to normal levels in BCP100+Cd and BCP200+Cd groups compared to Cd group. Nrf2 expression is shown in *Fig. 4-A*, HO-1 expression in *Fig. 4-B*, BAX expression in *Fig. 5-A* and Caspase 3 expression in *Fig. 5-B*. Immunofluorescence staining results and statistical analysis data are presented in *Table 6*.

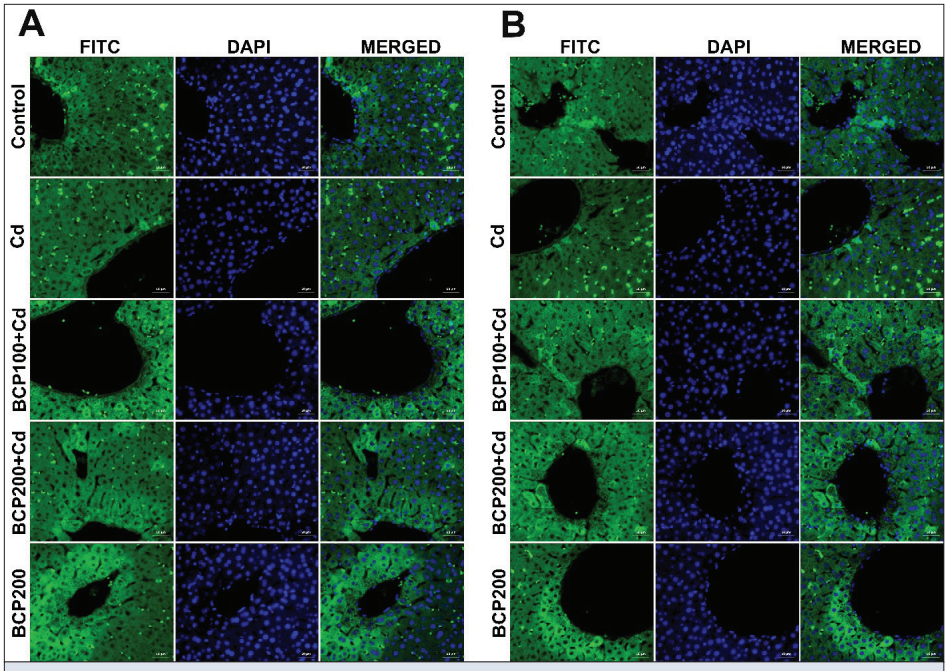

Fig 4. Liver tissue. **A)** Nrf2 expressions in hepatocytes (FITC); **B)** HO-1 expressions in hepatocytes (FITC). DAPI: nuclear staining. MERGED: Merge of images. IF, Bar: 50 µm

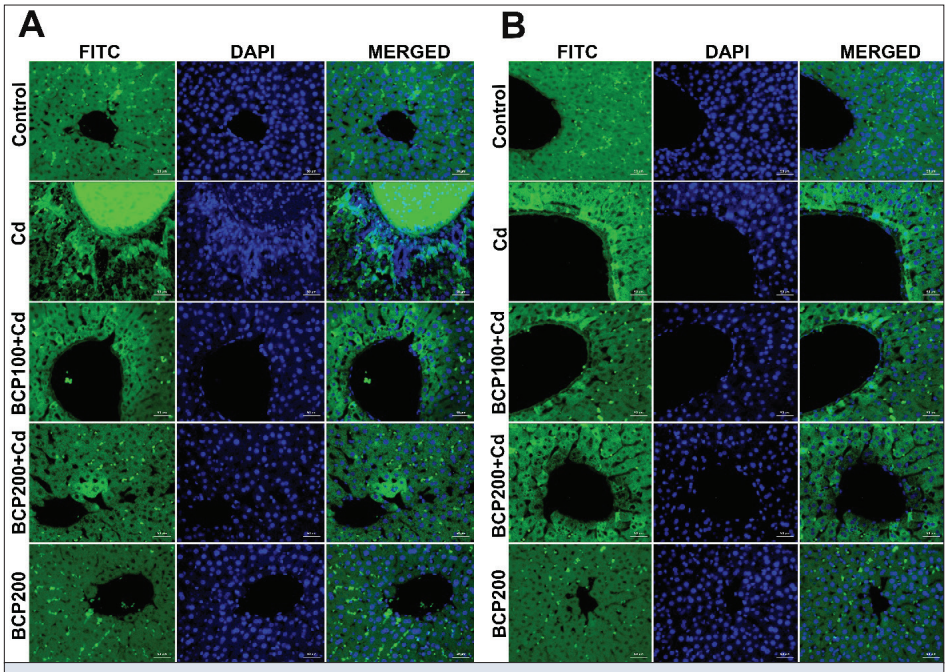

Fig 5. Liver tissue. **A)** BAX expressions in hepatocytes (FITC); **B)** Caspase 3 expressions in hepatocytes (FITC). DAPI: nuclear staining. MERGED: Merge of images. IF, Bar: 50 µm

Table 6. *Immunofluorescence staining results and statistical analysis data. One Way ANOVA and Tukey test (P<0.05, n=10) (the results are expressed as mean±SD)*

Groups	Nrf2	HO-1	BAX	Caspase 3
Control	38.18±2.86[b]	3.59±3.13[b]	1.84±0.32[b]	2.24±0.51[b]
CD	8.74±1.1[a]	6.27±1.63[a]	71.81±3.28[a]	61.5±3.65[a]
BCP100+ CD	22.06±2.66[c]	24.57±3.33[c]	59.76±5.16[c]	37.81±4.07[c]
BCP200+ CD	31.92±2.58[d]	33.75±2.33[d]	30.03±3.87[d]	16.3±2.02[d]
BCP200	51.51±2.1[e]	45.39±3.42[e]	1.89±0.22[b]	1.8±0.31[b]

a, b, c, d, e: *Values different from a indicate "a" statistically significant difference compared to the CD group*

Discussion

Cd an extremely toxic heavy metal, enters the body, primarily through inhalation and ingestion, leading to serious health problems [22]. Since the metabolism and accumulation of Cd occurs mainly in the liver and kidneys, these organs are directly affected by toxicity [23]. In this study, the protective effects of BCP, a biologically active compound, against Cd-induced liver toxicity were investigated.

Cd increases the production of reactive oxygen species (ROS) inside the cell, causing lipid peroxidation, protein oxidation, and DNA damage. These processes can disrupt the structural and functional integrity of hepatocytes, triggering cellular death and inflammation [24-28]. It has been shown by various studies that Cd disrupts the oxidative stress balance by increasing the production of ROS and causes cellular damage [29,30]. In addition, BCP has been reported to alleviate oxidative damage, reduce lipid peroxidation, and increase antioxidant enzyme activities (SOD, GSH, CAT) thanks to its strong antioxidant properties. It has been noted that these effects of BCP reduce oxidative stress and strengthen cellular defense mechanisms by activating Nrf2 signaling pathways [31]. In addition, it has been demonstrated by various studies that it exerts hepatoprotective effects by preserving mitochondrial functions and lowering inflammatory cytokine levels [32]. In our study, it was observed that lipid peroxidation (MDA levels) and oxidative stress increased with cadmium exposure. In addition, BCP has been found to reduce oxidative stress and provide significant improvements in antioxidant enzyme activities by alleviating these processes. Our findings support the potential protective effect of BCP against cadmium-induced oxidative stress and liver damage.

Cd initiates inflammatory processes in the liver by activating immune cells and increasing the production of pro-inflammatory cytokines [33]. In particular, when Cd enters the body, it activates the TLR4/NF-κB signaling pathway in the immune system, leading it to initiate inflammatory processes. Activation of NF-κB triggers the expression of cytokines such as IL-1β, TNF-α, and IL-6, which increase the inflammatory response, which exacerbates hepatic inflammation [34]. Previous studies show that BCP has the capacity to suppress inflammatory processes and plays a role in the regulation of the inflammatory response [35]. It has been reported that BCP may suppress oxidative stress-induced inflammatory responses by activating the Nrf2 signaling pathway, one of the important regulators of inflammation, and inhibiting overactivation of NF-κB [33,36-38]. In our study, we observed that BCP reduces inflammation by inhibiting the NF-κB/TLR4 pathway.

Cd exposure activates mitochondrial pathways to initiate the process of apoptosis in cells. This process is usually associated with a destabilization of the ratio between BAX/BCL-2 and caspase activation [38,39]. Increased BAX/BCL-2 ratio as a result of cadmium exposure increases mitochondrial cytochrome C release, causing the apoptotic pathway to progress. As a result, cellular lysis and necrotic changes in liver cells are observed with the activation of effector caspases such as Caspase3 [40]. Previous studies indicate that BCP has effects that improve BAX/BCL-2 balance and reduce the cellular death process by inhibiting caspase activation [41,42]. In our study, we found that BCP protects liver cells against the toxic effects of cadmium by regulating these apoptotic mechanisms. In particular, BCP administration was observed to lower the BAX/BCL-2 ratio and reduce cellular apoptosis by inhibiting Caspase3 activity.

Nrf2 is one of the key transcription factors that govern cellular antioxidant defenses, providing cellular protection by regulating the expression of HO-1 (Heme Oxygenase-1), SOD, GSH and other detoxification enzymes [43,44]. But heavy metals such as cadmium suppress this defense mechanism by increasing the ubiquitination and proteasomal degradation of Nrf2 via KEAP1, causing cells to become more vulnerable to toxic effects [45,46]. Previous studies show that BCP plays an important role in regulating oxidative stress by activating the Nrf2 signaling pathway and increases cellular antioxidant capacity by preventing the suppressive action of KEAP1 [31]. It has also been noted that SIRT1 eliminates the suppressive effect of KEAP1 by activating Nrf2 and provides cellular protection by increasing HO-1 levels [47,48]. In our study, we observed that BCP strengthens the antioxidant response by activating the Nrf2 signaling pathway. In particular, it was determined that BCP administration caused a significant increase in HO-1 levels by increasing the translocation of Nrf2 to the nucleus and activated cellular defense mechanisms.

The results of this study show that BCP has a significant hepatoprotective effect against cadmium-induced liver toxicity. BCP provides effective protection against cadmium-induced biological processes such as oxidative stress, inflammation and apoptosis, and reduces hepatotoxicity. These effects are related to the effects of BCP on important biochemical pathways TLR4/NF-κB, KEAP1/Nrf2/HO-1/SIRT1 and BAX/BCL-2/CASP3. The findings suggest that BCP can be used as a potential treatment option to prevent the toxic effects of cadmium on the liver.

Declarations

Availability of Data and Materials: Data and Materials are available from the corresponding author (İ. Bolat).

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Authors' Contributions: Conceptualization, MB and IB; methodology, MB, IB, ST, and TY; software, MB and IB; resources, IB; writing - original draft preparation, MB; writing - review and editing, IB.

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Research Article

Clinical and Paraclinical Assessment of the Efficacy of Calcium Carbonate, Vitamin D_3 and Chitosan in the Management of Chronic Renal Failure in Dogs

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Abstract

Chronic kidney disease (CKD) is the most commonly diagnosed renal condition in dogs, having a gradual and irreversible course. In this context, the present study aimed to evaluate the efficacy of a nutritional supplement containing calcium carbonate, vitamin D_3, and chitosan, administered over a 60-day period to a group of 20 mixed-breed dogs (10 females and 10 males), aged between 8 and 12 years, diagnosed with CKD stages 1, 2 and 3, according to the IRIS classification. Supplement administration resulted in significant changes in hematological and biochemical parameters**:** a 4.14% increase in red blood cell count, a 34.18% increase in total serum protein concentration and a 55.18% increase in albumin levels. Concurrently, marked decreases were observed in key renal dysfunction markers: phosphorus (-53.7%), creatinine (-75.54%), SDMA (-48.1%), and urea (-71.7%). Pulsed Doppler ultrasound revealed a 48.6% reduction in the renal resistive index (RRI), suggesting improved renal perfusion. In addition to these favorable biochemical effects, clinical improvements were also reported, including reduced polyuria and polydipsia, increased appetite, and enhanced general health status. These findings suggest that the tested supplement may play a beneficial role in slowing CKD progression and supporting the metabolic and clinical condition of affected dogs, indicating promising therapeutic potential as an adjuvant in the management of this disease.

Keywords: Calcium carbonate, Chitosan, Chronic kidney disease, Dogs, Doppler ultrasound, Vitamin D_3

Introduction

Chronic kidney disease (CKD) is the most frequently diagnosed renal disorder in dogs, characterized by its irreversible and progressive nature. The onset and progression of CKD are associated with a gradual decline in glomerular filtration rate (GFR), accumulation of metabolic waste products, and disruption of fluid, electrolyte, and acid–base homeostasis. This condition predominantly affects geriatric dogs, with an estimated prevalence ranging from 0.5% to 1.0% in the general canine population, increasing to approximately 7% among elderly animals [1]. Clinical manifestations commonly include polyuria, polydipsia, weight loss, muscle atrophy, and gastrointestinal signs such as vomiting, anorexia, and halitosis [2].

Therapeutic strategies are aimed at slowing disease progression, maintaining metabolic homeostasis, and improving the animal's quality of life through nutritional modifications, pharmacological interventions, and metabolic support [3]. Among the frequent complications of CKD, hyperphosphatemia plays a critical role, being directly associated with disease progression, increased risk of secondary hyperparathyroidism, and the development of renal osteodystrophy [4].

Calcium carbonate is widely used as a phosphate-binding agent, acting at the gastrointestinal level by forming insoluble complexes with dietary phosphorus, thereby preventing its absorption and promoting fecal excretion [4]. Calcium-based phosphate binders have demonstrated efficacy in maintaining serum phosphorus within normal limits, thereby reducing the risk of bone-related complications [5].

Another key component of CKD therapy is vitamin D_3, whose active metabolism is impaired due to reduced renal conversion to calcitriol. Supplementation with vitamin D_3 has been shown to enhance intestinal calcium absorption, suppress parathyroid hormone (PTH) secretion, and prevent the development of metabolic bone disorders [6]. However, careful monitoring is essential to avoid potential adverse effects such as hypercalcemia and ectopic calcification, which may worsen the clinical course of CKD.

Chitosan, a polysaccharide derived from chitin, has gained recent attention for its nephroprotective potential. Studies have reported that chitosan administration can lead to reductions in serum creatinine and urea levels, thereby exerting a favorable effect on renal function and disease progression [7]. Furthermore, chitosan exhibits phosphate-binding properties, contributing synergistically with calcium carbonate to control serum phosphorus concentrations [8].

An integrated nutritional approach that combines calcium carbonate, vitamin D_3, and chitosan may offer an effective strategy for managing early to intermediate stages of CKD. Such interventions could help delay the onset of severe complications and reduce the need for more aggressive treatments in advanced stages [9].

Accurate and early assessment of renal function is essential for the effective management of chronic kidney disease (CKD). Among the biochemical markers used for this purpose, dimethylarginines (DMA) -specifically asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA)- have emerged as important indicators. SDMA, predominantly eliminated via renal excretion and unaffected by muscle mass, offers a more precise reflection of glomerular filtration rate (GFR) compared to traditional markers such as serum creatinine [10]. Its capacity to reveal early renal impairment, prior to detectable changes in creatinine or urea levels, underscores its value in guiding clinical decision-making in CKD [10].

The present study investigates the therapeutic effects of a nutritional supplement (Renal Vet), composed of calcium carbonate, vitamin D_3, and chitosan, in dogs diagnosed with chronic kidney disease (CKD) at stages 1, 2, and 3 according to the IRIS classification system, which reflects the progressive severity of renal impairment. Biochemical and hematological parameters were evaluated with respect to the severity of the IRIS stages, focusing in the early stages (IRIS 1 and 2) on sensitive biomarkers of early renal function such as symmetric dimethylarginine (SDMA), alongside serum creatinine and urea monitoring, to enable early detection of renal dysfunction and timely therapeutic adjustment. In the intermediate stage (IRIS 3), emphasis was placed on parameters associated with metabolic imbalances secondary to progressive renal failure, including serum phosphorus levels, which influence the risk of secondary hyperparathyroidism and renal osteodystrophy, as well as relevant hematological indicators for assessing the overall clinical condition of the patient. The primary scientific objective was to determine the capacity of this combined nutritional regimen to effectively modulate stage-specific biomarkers, with the aim of slowing CKD progression, maintaining metabolic homeostasis, and improving clinical and functional parameters, thereby providing a differentiated and stage-adapted therapeutic approach according to disease severity.

Material and Methods

Ethical Statement

This study received ethical approval from the Local Ethics Committee for Animal Experimentation at the University of Life Sciences "King Mihai I" from Timişoara, under approval certificate no. 137/26.09.2022.

Study Design and Sample Collection

The study was conducted on a group of 20 mixed-breed dogs (n=20), consisting of 10 females and 10 males, aged between 8 and 12 years (mean age: 10.53 ±1.31 years). Clinically, the dogs included in the stud exhibited recurrent dyspeptic syndrome (n=13), characterized by episodes of vomiting (n=8), soft stools (n=5), decreased appetite (n=13), alopecia in the dorsolombar region (n=12), mild weight loss (n=20), and lethargy (n=11).

The research was conducted over a 60-day period. All subjects had been previously diagnosed with chronic kidney disease (CKD), and their disease severity was classified according to the staging system established by the International Renal Interest Society (IRIS), based on clinical assessments and laboratory findings.

Among the 20 dogs diagnosed with chronic kidney disease (CKD), 13 were classified as Stage 1, 4 as Stage 2, and 3 as Stage 3, according to the severity of the disease.

Upon reassessment at 30 and 60 days, all dogs included in the study remained alive.

The study population consisted of dogs from Timiş County, Romania, specifically from the commune of Cerneteaz, housed in an authorized veterinary shelter. Each dog was kept in an individual pen, providing approximately 4 m^2 of indoor space and 2.5 m^2 of outdoor space.

Water was provided *ad libitum* throughout the study period, and dogs were fed a renal-specific diet, Purina Pro Plan Veterinary Diets NF Renal Function, administered twice daily in individualized portions according to the manufacturer's guidelines. This veterinary diet

is specifically formulated for dogs with chronic kidney disease and comprises carefully selected ingredients, including corn, rice, corn gluten meal, animal fat, pea fiber, dehydrated salmon protein, dried egg powder, calcium carbonate, monocalcium phosphate, fish oil, potassium chloride, sodium chloride, as well as essential vitamins (Vit-E, Vit-C, and Vit-B-complex) and trace minerals (iron, zinc, and selenium). The formulation aims to support renal function, reduce metabolic burden on the kidneys, and maintain overall nutritional balance in affected animals.

A control group was not included due to the nature of the shelter, where healthy animals were periodically adopted. The exclusion of a control group aimed to prevent disruption to the adoption process and to ensure animal welfare. Paraclinical assessments were conducted at baseline, 30 days and 60 days, with the initial evaluation serving as a reference for comparison.

The nutritional and therapeutic management of dogs with chronic kidney disease (CKD) was performed following the IRIS treatment guidelines for stages 1, 2, and 3. The treatment regimen included dietary sodium (Na) restriction and administration of isotonic and polyionic fluids, such as lactated Ringer's solution, Ringer's solution, and 5% glucose, to maintain fluid and electrolyte balance. Antiemetic medications, including maropitant and ondansetron, were used to control nausea and vomiting. Gastric protection was provided through H2-receptor antagonists (e.g., famotidine) or proton pump inhibitors (e.g., pantoprazole, omeprazole). Vitamin supplementation included B-complex vitamins, specifically thiamine (B_1), pyridoxine (B_6), and cobalamin (B_{12}).

Throughout the study, the dogs received an oral nutritional supplement, Renal Vet, containing calcium carbonate, chitosan, and vitamin D_3, administered according to the manufacturer's instructions. Dogs weighing up to 10 kg received one capsule per day, while those over 10 kg were given one capsule per 10 kg of body weight daily.

The clinical presentation of the dogs included recurrent vomiting episodes, reduced appetite, soft stools, moderate weight loss, lethargy, muscle weakness and dorsolumbar alopecia. Given the inherent subjectivity of clinical evaluation, a series of hematological and biochemical tests were performed to support the diagnosis.

The assessed parameters included:

• *Erythrocyte profile:* total red blood cell count (RBC), hematocrit, hemoglobin, mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular volume (MCV).

• *Leukocyte profile:* total white blood cell count (WBC), neutrophils, lymphocytes, monocytes, eosinophils, and basophils.

• *Renal biochemical profile:* total protein, albumin, creatinine, symmetric dimethylarginine (SDMA), urea, phosphorus, and calcium.

The study was conducted in two phases, with clinical, hematological, and biochemical evaluations performed at baseline (before treatment initiation), after 30 days and after 60 days of supplement administration.

Blood samples were collected from the cephalic vein following standard veterinary procedures. Sampling was repeated on days 30 and 60. The blood samples were stored in BD Vacutainer® K2 EDTA tubes (Becton Dickinson, USA) for hematological analysis and transported to the Bioclinca SA laboratory for processing. Before analysis, the samples were homogenized for 3 min at 40 rpm using a roller mixer.

Hematological and biochemical analyses were performed using the automated Exigo EosVet analyzer (Boule Medical AB, Stockholm, Sweden).

For abdominal ultrasound evaluations, two imaging systems were used: a stationary device (My Lab XVet) and a portable unit (Chisson 2 Vet), both equipped with sector transducers operating at frequencies between 5.5 and 6.5 MHz. To enhance imaging quality, the hair over the examination area was clipped, and ultrasound gel was applied before scanning. Each kidney was assessed in both longitudinal (dorsal and sagittal) and transverse planes, with additional evaluation of the renal resistive index (RRI) using pulsed-wave Doppler ultrasound [11,12].

Statistical Analysis

Hematological and biochemical data were systematically recorded in Microsoft Excel and statistically analyzed using SPSS software, version 26.0 (SPSS Inc., Chicago, IL, USA). The normality of data distribution was assessed using the Shapiro-Wilk test, and results were expressed as mean ± standard deviation (SD). To evaluate differences across the three time points -T0 (baseline, before administration - BA), T1 (30 days after administration - AA_30d), and T2 (60 days after administration - AA_60d) - a Linear Mixed Model (LMM) was applied, allowing for the appropriate analysis of repeated measures and intra-subject variability. Statistical significance was set at $P<0.05$. Graphs were generated using GraphPad Prism, version 10.

Results

The graphical representation of the mean hematological parameter values, as determined according to the experimental protocol in dogs treated with calcium carbonate, vitamin D_3 and chitosan is presented in *(Fig. 1)*.

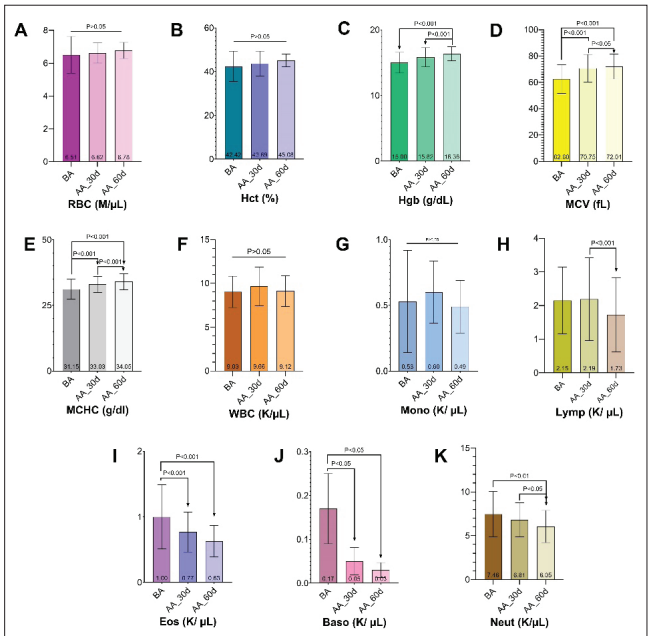

Fig 1. Graphical representation of hematological parameters (A-K) recorded in dogs before and after the administration of calcium carbonate, vitamin D_3, and chitosan. **A-** Red Blood Cells, **B-** Hematocrit, **C-** Hemoglobin, **D-** Mean Corpuscular Volume (MCV), **E-** Mean Corpuscular Hemoglobin Concentration (MCHC), **F-** White Blood Cells, **G-** Monocytes, **H-** Lymphocytes, **I-** Eosinophils, **J-** Basophils, **K-** Neutrophils. Normally distributed data are expressed as mean ± standard deviation (SD). Statistical comparisons between groups were performed using a Linear Mixed Model (LMM), with the significance threshold denoted on the corresponding graph BA: Before administration, AA_30d: After 30 days of administration, AA_60d: After 60 days of administration

The administration of a dietary supplement containing calcium carbonate, vitamin D_3, and chitosan (Renal Vet) to dogs diagnosed with stage 1-3 chronic kidney disease (CKD) was associated with moderate improvements in several hematological parameters [13].

The mean erythrocyte count (A) increased over the course of the study, from 6.51 M/µL at baseline to 6.62 M/µL after 30 days (1.68%) and 6.78 M/µL after 60 days (4.14%); however, these changes were not statistically significant (P>0.05). Erythrocyte indices remained within physiological limits during the study.

Hematocrit values (B) increased by a total of 6.27%, from an initial mean of 42.42 g/dL to 43.69 g/dL at 30 days and 45.08 g/dL at 60 days, with no statistically significant differences observed between time points (P>0.05). In contrast, the mean hemoglobin concentration (C) showed a statistically significant increase throughout the study, rising from 15.0% at baseline to 15.82% after 30 days and 16.36% after 60 days (P<0.001).

The mean corpuscular volume (MCV) (D) increased from 62.6 fL to 70.75 fL after 30 days and 72.01 fL after 60 days (15.03%), while the mean corpuscular hemoglobin concentration (MCHC) (E) rose from 31.15 g/dL to 33.03 g/dL and 34.05 g/dL, respectively. Both MCV and MCHC showed statistically significant differences across the evaluation points (P<0.001).

Leukocyte dynamics (F) revealed a non-significant increase of 6.97% at 30 days, followed by a slight decrease to 9.12 K/µL at 60 days (P>0.05). These variations were considered nonspecific and unlikely to impact disease progression.

Monocyte levels (G) showed a slight increase from an initial value of 0.53% to 0.60% at 30 days, followed by a decrease to 0.49% at 60 days. These changes were not statistically significant (P>0.05).

Lymphocytes (H) showed a slight increase of 1.86% after 30 days, followed by a significant reduction of 21% after 60 days compared to baseline (P<0.001).

Eosinophil counts (I) decreased by 23% at 30 days and by 37% at 60 days, while remaining within the physiological reference range; statistically significant differences were observed at each evaluation point (P<0.001).

Basophils (J) exhibited a marked decrease of 70.58% after 30 days and 82.35% after 60 days, with statistically significant differences between the two stages of the study (P<0.05).

Supplementation with calcium carbonate, chitosan, and vitamin D_3 resulted in a significant reduction in neutrophil counts (K): a 15.41% decrease at 30 days and 18.9% at 60 days compared to baseline (P<0.001), with an additional 4.12% reduction between days 30 and 60 (P<0.05), suggesting a potential systemic anti-inflammatory effect.

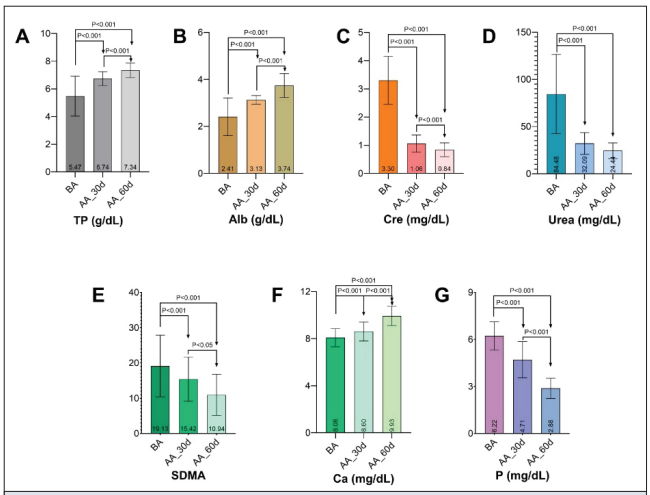

Fig 2. Graphical representation of the values of blood biochemical parameters **(A-G)** recorded in dogs before and after the administration of calcium carbonate, vitamin D_3, and chitosan. **A-** Total protein (TP), **B-** Albumine (Alb), **C-** Creatinin (Cre), **D-** Urea, **E-** Symmetric dimethylarginine (SDMA), **F-** Calcium (Ca), **G-** Phosphor (P). Normally distributed data are expressed as mean ± standard deviation (SD). Statistical comparisons between groups were performed using a Linear Mixed Model (LMM), with the significance threshold denoted on the corresponding graph BA: Before administration, AA_30d: After 30 days of administration, AA_60d: After 60 days of administration

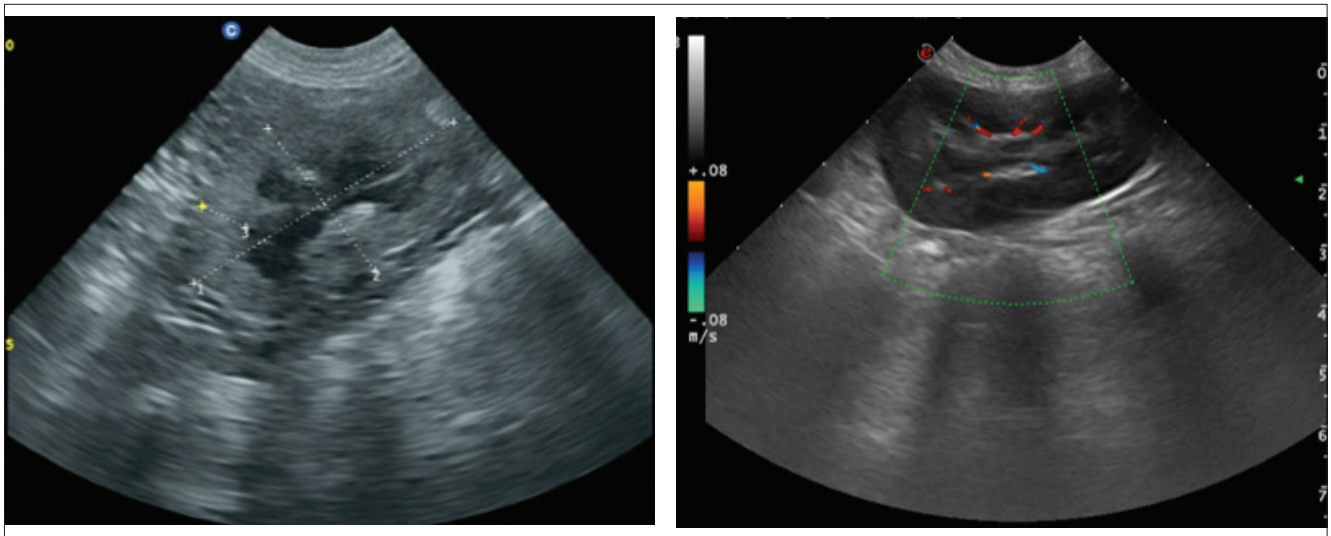

Fig 3. Ultrasound image at the level of the left kidney, in a dog. **A**- sagittal section, mode B and **B**- color Doppler at renal arcuate arteries' level, before treatment

The graphical representation of serum levels of total proteins, albumin, creatinine, symmetric dimethylarginine (SDMA), urea, calcium, and phosphorus in dogs with chronic kidney disease from the current study is presented *(Fig. 2)*.

The serum levels of total proteins (A) and albumin (B), initially below physiological limits (5.47 g/dL and 2.41 g/dL, respectively), increased significantly after 30 days of supplementation (total proteins: by 17.4%; albumin: by 6.99%; P<0.001). After 60 days, the mean total protein concentration reached 7.34 g/dL, reflecting a 34.18% increase compared to baseline, while albumin rose to 3.74 g/dL (an increase of 55.18%). Statistically significant differences were observed between all study phases (P<0.001).

Creatinine (C) and urea levels (D), initially elevated (3.3 mg/dL and 84.48 mg/dL, respectively), showed a marked decline following supplementation. After 30 days, creatinine decreased to 1.06 mg/dL (a 67.87% reduction), and urea to 32.09 mg/dL (a 62.01% reduction). By day 60, creatinine further declined to 0.84 mg/dL (a 74.54% decrease from baseline), while urea dropped to 24.44 mg/dL (a 71.7% reduction), both values approaching or falling within the physiological reference range. These reductions were statistically significant (P<0.001).

Symmetric dimethylarginine (SDMA) (E), a sensitive biomarker of early renal dysfunction, had an initial mean value of 19.13 µg/dL, exceeding the reference threshold (<18 µg/dL). After 30 days, the mean SDMA concentration decreased to 15.42 µg/dL (a 19.39% reduction), and by day 60 it further declined to 10.94 µg/dL, representing a 42.81% decrease from baseline. The changes were statistically significant (P<0.001).

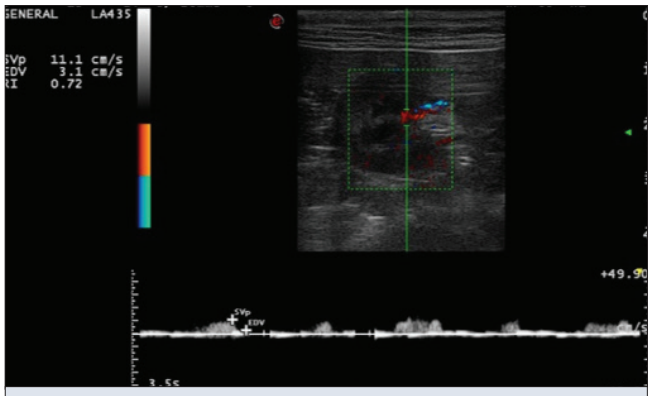

Fig 4. Ultrasound image at the level of the kidney, in a dog, sagittal section, pulsed Doppler at the level of the renal interlobar artery, before treatment

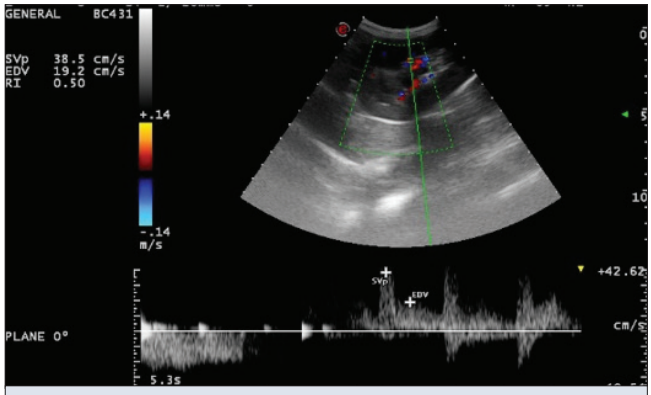

Fig 5. Ultrasound image at the level of the kidney, in a dog, sagittal section, pulsed Doppler at the level of the renal interlobar artery, after 60 days treatment

Serum calcium (F), initially below the physiological limit (8.08 mg/dL), increased to 8.6 mg/dL after 30 days, and normalized to 9.93 mg/dL by day 60, representing a 22.89% increase compared to baseline. These changes were statistically significant (P<0.001).

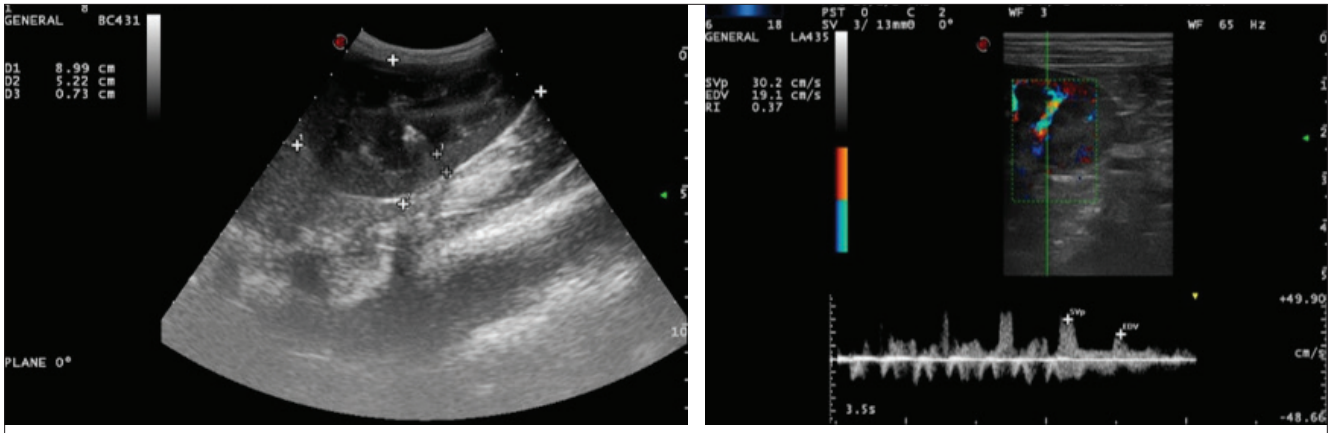

Fig 6. Ultrasound image of the left kidney, in a dog. **A**- sagittal section, B-mode and **B**- pulsed Doppler, 60 days after treatment

Phosphorus levels (G), initially elevated at 6.22 mg/dL, decreased to 4.71 mg/dL after 30 days (a 24.27% reduction), and further declined to 2.88 mg/dL at 60 days (a 53.7% reduction), returning to the physiological reference range. Statistically significant differences were recorded between all three study phases ($P<0.001$).

In the dogs diagnosed with stage 3 chronic kidney disease, ultrasonographic examination revealed degenerative changes in both the renal cortex and medulla, characterized by increased cortical echogenicity, reduced kidney size (longitudinal and transverse dimensions), and an elevated parenchymal index *(Fig. 3)*. Color Doppler ultrasound showed significantly decreased blood flow in the interlobar and interlobular arteries.

At baseline, the renal resistive index (RRI), measured using pulsed Doppler ultrasound, was elevated (0.72), indicating moderate vascular resistance and renal hypoperfusion *(Fig. 4)*. After 60 days of dietary supplementation, a notable improvement in renal hemodynamics was observed. The RI decreased significantly by 30.55% points at intermediate assessment and by 48.6% points by the end of the study, reaching 0.37 *(Fig. 5, Fig. 6)*. Concurrently, a reduction in cortical echogenicity was noted, suggesting improved renal perfusion and function.

Discussion

The clinical signs observed in the studied dogs were consistent with those reported in the literature, supporting the external validity of the findings [14]. Hematological analysis at treatment onset showed red blood cell counts within physiological limits, indicating that significant hematological changes are not typical in the early stages of chronic kidney disease (CKD).

By the end of the study, a slight increase in red blood cell count was noted, likely due to improved renal oxygenation. This may be attributed to the effects of calcium carbonate, vitamin D_3, and chitosan, which reduce oxidative stress and enhance erythropoietin bioavailability at the juxtaglomerular level.

Martello et al.[15] demonstrated that long-term supplementation with calcium carbonate, calcium lactate-gluconate, chitosan, and sodium bicarbonate in dogs with stage 3 CKD led to increased erythrocyte counts, reduced phosphorus levels, and improved urinary and biochemical markers. Similarly, a 90 days study using a comparable supplement with added *Lactobacillus acidophilus, Olea europaea* extract, and prebiotics showed effective control of uremia, phosphate, acid-base balance, inflammation, and oxidative stress, along with a mild increase in diuresis [16].

Literature emphasizes the role of renal microcirculation and juxtaglomerular function in chronic kidney disease (CKD) management, with supportive therapies proving essential in slowing disease progression [17]. Hematological changes such as non-regenerative anemia due to reduced erythropoietin and immune alterations like neutrophilia, lymphopenia, or monocytosis reflect CKD-related inflammation [18]. Early-stage lymphocytosis may represent a compensatory immune response, later evolving into immunosuppression, often exacerbated by chronic subclinical infections like pyelonephritis [19].

The progressive decrease in neutrophil levels during the administration of the supplement suggests a systemic anti-inflammatory effect, which is relevant in the context of chronic inflammation associated with renal disease. The active compounds -chitosan, vitamin D_3, and calcium carbonate- may influence immune response and mineral metabolism, thereby contributing to the normalization of neutrophil counts. These findings support the potential role of nutritional adjuvants as complementary therapy in the management of chronic kidney disease in dogs.

Monocytosis observed in the study, indicative of chronic inflammation, represents a significant hematological alteration in dogs with chronic kidney disease (CKD), being associated with macrophage activation and increased production of pro-inflammatory cytokines that contribute to progressive renal damage and systemic inflammation [13]. Monitoring these changes is essential for early detection and disease management. Correlating hematological data with biochemical and imaging analyses provides a more accurate understanding of disease progression and can guide therapeutic decisions, thereby impacting patient outcomes [20].

Lymphocytosis and monocytosis reflect the complex immune-inflammatory interplay characteristic of CKD. These parameters indicate immune activation and may inform therapeutic strategies aimed at modulating the immune response and improving prognosis [15,19]. The decrease in lymphocyte counts observed after 60 days of treatment may signal a relevant immunological shift, suggesting an adaptive or regulatory phase following at least two months of dietary supplement administration.

Continuous monitoring of hematological profiles is crucial for identifying complications associated with chronic kidney disease (CKD). Specifically, eosinopenia and basopenia require exclusion of iatrogenic causes such as glucocorticoid use, which may suppress bone marrow production and redistribute eosinophils to tissues via activation of the hypothalamic-pituitary-adrenal axis and increased cortisol secretion [19].

Dogs with CKD are predisposed to secondary infections and persistent inflammatory responses, contributing to decreased peripheral eosinophil counts. Basophils are also sensitive to elevated cortisol levels, and their reduction in circulation may be further influenced by oxidative stress and systemic inflammation [21]. Furthermore, secondary hyperparathyroidism, a common CKD complication, may adversely affect basophil numbers [22].

The decreased percentages of eosinophils and basophils may represent a compensatory mechanism aimed at limiting excessive inflammation and fibrotic progression in renal tissue. However, these changes require careful monitoring, as they may indicate underlying immune dysfunction and oxidative imbalances.

The observed increase in total protein and albumin levels in dogs with chronic kidney disease (CKD) may reflect an improvement in renal function or a reduction in proteinuria, suggesting a favorable response to the administered treatments. These fluctuations can also be modulated by systemic inflammation, with anti-inflammatory interventions potentially supporting the elevation of protein parameters. Supplements containing calcium and vitamin D_3 may enhance protein balance by reducing losses and supporting nephron function.

Recent studies highlight the efficacy of dietary supplementation with calcium carbonate, calcium lactate-gluconate, chitosan, and sodium bicarbonate in advanced CKD management, showing reductions in serum phosphorus, improved acid–base status, and better uremic control [15,16]. Notably, this supplement also demonstrated anti-inflammatory and antioxidant effects, aligning with findings that showed decreased serum creatinine at 30 days and reduced urea levels at 60 days, indicative of improved renal clearance [7,23].

These clinical and biochemical changes suggest effective with chronic kidney disease (CKD) management, marked by improvements in both hematologic and renal biomarkers. Early CKD diagnosis is crucial, as traditional markers like creatinine rise only after significant nephron loss. In contrast, support symmetric dimethylarginine (SDMA) is a more sensitive biomarker that increases earlier in the disease course, enabling prompt detection and monitoring of renal dysfunction.

Sargent et al.[24] support symmetric dimethylarginine (SDMA) as a screening tool in line with IRIS guidelines, although further studies are needed to clarify non-renal influences. Michael et al.[25] confirmed SDMA as a sensitive indicator of early GFR decline, often rising before creatinine. They recommend concurrent SDMA and creatinine evaluation for accurate disease staging and monitoring. Similarly, other studies underscore the value of tracking SDMA dynamics in with chronic kidney disease (CKD) progression, showing strong correlations with renal function and higher diagnostic relevance than creatinine alone [26].

Although SDMA levels are elevated in both acute kidney injury (AKI) and CKD, the SDMA/creatinine ratio is significantly higher in CKD, albeit with overlap between groups, indicating limited specificity for differentiating disease types. Therefore, SDMA should be interpreted alongside other diagnostic tools to achieve a comprehensive assessment [2].

Chronic kidney disease progression is closely linked to disturbances in calcium-phosphorus metabolism. As glomerular filtration rate (GFR) declines, phosphate excretion decreases, leading to hyperphosphatemia a key driver of renal osteodystrophy and secondary hyperparathyroidism (SHPT). At the end of the supplementation period, calcium levels normalized, with a progressive increase from baseline, reflecting restored calcium homeostasis. Statistically significant changes confirmed the supplement's impact on mineral regulation.

Initial hyperphosphatemia reflected impaired renal phosphate excretion. Post-supplementation, phosphorus

levels declined significantly, correlating with improved clinical status and restored phosphocalcic balance. These findings support the supplement's role in correcting mineral imbalances and slowing chronic kidney disease (CKD) progression.

Calcium and phosphorus homeostasis is regulated by kidney-bone-intestine interactions, modulated by parathyroid hormone (PTH), calcitriol, and fibroblast growth factor 23 (FGF-23) [27]. In chronic kidney disease (CKD), dysregulation of these mediators contributes to mineral and bone disorders. Managing phosphorus retention through dietary restriction and phosphate binders is essential. In SHPT, vitamin D receptor activators, phosphate binders, and calcimimetics are first-line; refractory cases may require parathyroidectomy, which has shown benefits in reducing symptoms and cardiovascular risks [28].

Supplementation with calcium carbonate, chitosan, and vitamin D_3 has demonstrated moderate increases in serum calcium and reductions in phosphorus after 30 days, indicating improved mineral balance and reduced calcification risk [29]. Hyperphosphatemia contributes to soft tissue calcification, impairing cardiovascular and renal functions.

Vitamin D_3 supplementation and calcium carbonate have proven effective in controlling hyperphosphatemia and enhancing renal function. Studies on calcifediol show improved vitamin D status, calcium homeostasis, and mineral metabolism in CKD, further supporting its therapeutic relevance [30,31].

A study assessing dietary supplements in advanced stages of CKD in dogs reported notable improvements in uremia control, phosphorus regulation, acid-base balance, blood pressure, and reductions in inflammation and oxidative stress [32]. These findings support the role of nutritional interventions in slowing CKD progression and enhancing clinical status.

Management of mineral imbalances in chronic kidney disease (CKD) relies on dietary phosphorus restriction, phosphate binders, and supplementation with vitamin D_3 and calcium. Monitoring serum calcium, phosphorus, parathyroid hormon (PTH), and the Ca/P ratio is essential for preventing complications and improving prognosis [33,34]. Calcium and vitamin D_3 supplementation can support calcium homeostasis and attenuate inflammation, though protocols must be individualized and monitored by veterinarians to ensure safety and efficacy [35].

Renal ultrasonography remains the gold standard for non-invasive morphological evaluation of kidney architecture and hemodynamics. In chronic kidney disease (CKD) typical sonographic findings include increased cortical echogenicity, diminished corticomedullary differentiation, reduced renal size, and irregular contours hallmarks of advanced disease [11,36].

The Renal Resistivity Index (RRI), derived from Doppler ultrasonography, provides insight into renal vascular resistance, especially within interlobar arteries. An RRI >0.8 suggests increased intrarenal vascular resistance, often associated with hypertension, renal artery stenosis, or CKD, while lower values may indicate vasodilation.

In our study, dogs with CKD exhibited cortical and medullary degeneration, evidenced by increased echogenicity and reduced renal dimensions. These alterations correlated with a higher parenchymal index, indicating structural damage. Color Doppler revealed diminished flow in interlobar and interlobular arteries, consistent with renal hypoperfusion.

Following 60 days of treatment with the dietary supplement, ultrasonographic re-evaluation showed reduced cortical echogenicity and improved perfusion, alongside a significant decrease in RRI, suggesting enhanced renal circulation and reduced vascular resistance. These findings imply a beneficial effect of the supplement on renal hemodynamics, with improved function and diuresis.

The reduction in both echogenicity and RRI post-treatment highlights improved renal perfusion and function, supporting the supplement's therapeutic potential in CKD management.

Chetboul et al.[10] emphasized the utility of renal resistivity index (RRI) in detecting subclinical renal lesions in dogs with MMVD, showing strong correlation with renal hemodynamic alterations. Compared to biochemical markers such as Symmetric Dimethylarginine (SDMA) and Cystatin C, Renal Resistivity Index (RRI) provides the advantage of a non-invasive, repeatable assessment of renal circulation and serves as an early indicator of renal dysfunction. Integrating RRI into clinical monitoring may thus enhance early detection and management of renal complications in Myxomatous Mitral Valve Disease (MMVD).

In human nephrology, renal venous blood flow assessment has gained relevance, while in dogs, predictive use of arterial indices such as RRI and PI remains standard. However, renal venous flow velocity remains unexplored in canine medicine [37].

Age-related variations in RRI were highlighted by a recent study [38], which observed a trend toward lower values in older dogs and a weak correlation between RRI and plasma renin activity in juveniles. These findings underscore the influence of hormonal modulation on renal hemodynamics and the necessity of age-adjusted

interpretation when using RRI in clinical settings.

The Renal Resistivity Index (RRI) is a valuable parameter in the evaluation of Acute Kidney Injury (AKI) in dogs. An increased RRI correlates with the severity of renal impairment, while a reduction in this index following treatment indicates an improvement in renal function and a favorable prognosis. Therefore, RRI can be employed as a reliable, non-invasive tool for monitoring disease progression and therapeutic response, although further studies are required to validate its routine use in AKI diagnosis and prognosis [39]. Data obtained through pulsed Doppler ultrasonography further support its role in establishing accurate renal prognosis.

Imaging analyses revealed improvements in cortical echogenicity and renal perfusion, suggesting a partial restoration of renal structure and function. The progressive decrease in Symmetric Dimethylarginine (SDMA) confirms the efficacy of supplementation in slowing the progression of chronic kidney disease (CKD). These findings are consistent with the literature, which links chronic renal hypoperfusion with characteristic ultrasonographic changes, such as increased cortical echogenicity, and supports the use of supplements that improve renal circulation through vasodilation or enhancement of renal hemodynamics [40].

In conclusion, calcium carbonate, vitamin D_3, and chitosan show promising therapeutic potential in CKD management. However, larger cohort studies and longer follow-up periods are necessary to confirm the long-term clinical benefits of this supplementation strategy.

Declarations

Availability of Data and Materials: The authors declare that the results of the investigations and other data related to the article are available at the correspondent author and at first author.

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Ethical Approval: This study received ethical approval from the Local Ethics Committee for Animal Experimentation at the University of Life Sciences "King Mihai I" from Timișoara, under approval certificate no. 137/26.09.2022

Conflict of Interest: The authors declared that there is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The authors declare that the article, tables and figures were not written/created by AI and AI-assisted Technologies.

Author Contributions: All authors have read and approved the final version of the manuscript. FSD, EDB, and CV conceived the experimental design. FSD and CV conducted the experiment and carried out the analyses. Data analysis was performed by FSD and EDB, with statistical analysis conducted by FSD. The initial draft of the manuscript was written by FSD and CV. All authors reviewed the manuscript and provided their final approval for publication.

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Research Article

The Effect of Pulsed UV Light on Biochemical Changes: Quail Egg Model for *Salmonella* Typhimurium Inactivation

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Abstract

Foodborne Salmonella infections pose a critical threat to public health and the disinfection potential of non-thermal technologies such as pulsed UV light is becoming increasingly important. The aim of this study was to determine the biochemical changes in quail eggs at varying process parameters in the model application of pulsed UV light for *Salmonella* Typhimurium inactivation. In this context, pulsed UV light was applied to quail eggs at distances of 5, 8, and 13 cm and durations of 20, 40, and 60 s. Salmonella Typhimurium inactivation was observed in all quail eggs depending on the treatment distance and duration and biochemical changes in quail eggs were evaluated in terms of thiobarbituric acid, phenolic and antioxidant content, fatty acid composition and color change. Salmonella inactivation was observed in all treatments. However, phenolic and antioxidant contents decreased for the longest time and at the closest distance in the application processes, while thiobarbituric acid and linoleic acid content (control:13.35%→5D60S:25.51%) increased. The increase in the amount of linoleic acid in 5D60S and 5D40S was higher than that in other samples. Although pulsed UV light is effective in Salmonella inactivation, long duration/close distance applications may adversely affect product quality. These findings emphasize the importance of parameter optimization in the food industry and reveal the need to develop protocols that are safe but minimize quality loss.

Keywords: Pulsed UV light, Quail eggs, *Salmonella* Typhimurium, Biochemical change, Fatty acid

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Introduction

Non-thermal methods have recently been studied as thermal disinfection methods for preserving the color, texture, and nutritional properties of foods [1]. Non-thermal technologies such as high-voltage processes, pulsed electric fields, ultrasound, cold plasma, high-pressure processes, ionizing radiation, ultraviolet (UV) light and pulsed light typically generate minimal heat during the food processing process [2]. Pulsed light effectively kills microorganisms on food surfaces, offering both advantages and disadvantages [3]. It effectively decontaminates surfaces by inactivating spores and vegetative cells of various bacteria, and a higher inactivation is achieved at higher input voltages [4]. Pulsed light also effectively decontaminates solid foods and food contact materials, prevents recontamination, and improves product quality and safety [5]. On the other hand, pulsed light applications can improve food safety and extend shelf life, but appropriate application conditions should be evaluated for each food and microorganism to minimize adverse effects on photosensitive compounds and sensory properties [6].

Quail eggs, which are frequently found in people's tables, are rich in vitamins, minerals (calcium, phosphorus), and antioxidants, and can serve as a dietary supplement to enhance bone healing [7]. Quail eggs are a useful source of nutrients that provide high-quality proteins and a variety of nutrients that support growth and development [8]. Studies on changes in the nutritional value of quail eggs

are generally based on quail nutrition. In a study on chemical changes in quail eggs, microalgae added to quail diets were found to improve egg quality and consumer health by reducing saturated fatty acids, increasing monounsaturated fatty acids, and increasing antioxidant [9]. In another study, curcumin nanocapsules added to quail feed enhanced the antioxidant effect and increased unsaturated fatty acid levels [10].

Salmonella Typhimurium is a well-recognized foodborne pathogen responsible for significant illness in both humans and animals globally. It ranks among the most prevalent causing foodborne disease, typically leading to gastroenteritis and, in severe cases, progressing to systemic infections. Its widespread occurrence and notable antibiotic resistance render it a critical concern for both food safety and public health. *S.* Typhimurium is frequently identified in a diverse array of food products, particularly raw and undercooked animal-derived items, including meat and poultry. It stands as a primary contributor to foodborne outbreaks worldwide, with high detection rates observed in retail and ready-to-eat foods. In certain regions, it represents the dominant serotype found in human infections and is closely linked to considerable morbidity and mortality [11,12].

Egg varieties are important sources of pathogenic microorganisms and nutritionally valuable. Therefore, it is important to develop methods for microbial decontamination of shell surfaces. Thermal and non-thermal decontamination methods can extend the shelf life of eggs and reduce consumer risk from foodborne pathogens [13]. In this context, research has been conducted using different techniques. Pulsed UV light treatment is an effective method for reducing microbial pathogens on the surface of egg shells. The study showed that higher energy levels resulted in greater reductions in microbial load. In particular, treatments with energy levels of 1.0, 2.4, 3.1 and 4.9 J/cm² resulted in a significant reduction of *Escherichia coli* and *Enterococcus faecium* bacteria on the egg surface [14]. Another study, using new technologies, investigated the effectiveness of pulsed UV light for the inactivation of *E. coli* K12 in hard-cooked eggs. In a study where distance and time were examined as variables, temperature increase, color, and texture of eggs were investigated in addition to microbial inactivation. In a study in which microbial inactivation was found to be important, no significant changes were observed in other parameters [15]. In a study in which pulsed light was applied to egg whites, the chemical and physical properties of the product were examined. Pulsed light caused deterioration in the protein structure and an increase in brown color formation, but did not change the viscosity. On the other hand, egg white foam obtained as a result of this process is more stable [16]. Pulsed UV light application on eggshells is an effective method for inactivating pathogens on eggshell surfaces, and no change in albumen height and eggshell strength has been observed [17]. In this study, it's hypothesized that the applied pulsed UV light will effectively inactivate *S.* Typhimurium contamination on the quail egg surface, and this application will lead to acceptable levels of changes in the egg's biochemical properties (e.g., fatty acid composition, TBARS, phenolic and antioxidant contents, and color values).

The aim of this study was to determine how pulsed UV light application affects the biochemical properties of quail eggs, which are small in size and generally used as a supplement in pediatric nutrition. In this context, *S.* Typhimurium inactivation was selected as a model application, and pulsed UV light was applied to quail eggs by changing the application parameters. All samples were examined for microbial decontamination and biochemical analysis of fatty acid composition, TBARS, phenolic and antioxidant contents, and color values.

Material and Methods

Ethical Statement

This study does not require ethical approval.

Material

Quail eggs were obtained from a local producer using the same batch of eggs for each replicate, using the same group of quails. Fresh quail eggs with an average weight of 10-10.5 g were used in this study. Eggs were transported to the laboratory in a cold chain and stored at +4°C. On the day of the experiment, the eggs were removed from the cold chain and transferred to the laboratory at an ambient temperature of approximately 20°C.

Activation and Inoculation of *Salmonella* Typhimurium

The target microorganism for the model inactivation media trial was determined to be *Salmonella* Typhimurium. In this context, lyophilized *Salmonella* Typhimurium strain (RSKK 11020-Türkiye) was activated. In the first step of the activation process, cells were incubated in Tryptic Soy Broth (Merck, 105459) agar at 37°C for 18-24 h. After the completion of this period, the strain was again transferred to a medium containing Tryptic Soy Broth and incubated at 37°C for 18-24 h. After 24 h, the medium prepared with Tryptic Soy Agar (TSA) (Merck, 105458) was inoculated by the smear method and incubated at 37°C for 18-24 h. Single colonies from the Petri dishes were transferred to tubes containing Tryptic Soy Broth and incubated at 37°C for 18-24 h. After incubation, the tubes were removed at +4°C. To the glass tubes, 5 mL of Tryptic Soy Broth and 300 µL of cultures in which growth was observed were added and incubated. Then, 300 µL of the previous

culture was added to tubes containing 10 mL of Tryptic Soy Broth and incubated. The tube with the most intense incubation was taken, the optical density was measured using a spectrophotometer, and it was determined that the measurement result was within the reference range [1]. Eight tubes were diluted to 10^{-8} with peptone water and inoculated on Tryptic Soy Agar, and at the end of incubation, uniform growth was observed [18].

Before starting the study, each egg was kept in 10 mL 68% ethyl alcohol for 10 min, and the egg surface was washed. *Salmonella* Typhimurium inoculation liquid was prepared at 10^5 CFU/mL, and quail eggs were kept in this liquid for 20 min. Samples removed from the inoculation liquid under aseptic conditions were kept for an average of 30 min (until dry), and after reaching room temperature, they were prepared for pulsed UV application.

Pulsed UV Light Application

The inactivation process was carried out using the Steri-Pulse XL 3000 Pulsed UV-Light Sterilization System (Xenon Corporation, MA, USA). This system comprised a stainless-steel sterilization chamber, a control unit, a lamp assembly, and a cooling system. The xenon lamp emitted light energy of 1.27 J/cm² at a distance of 1.5 cm below the lamp surface, operating with 3800 V energy at a frequency of 3 Hz, and a pulse duration of 360 µs (pulse time). The UV lamp produced 3 pulses per second. Samples were placed inside a chamber (measuring 0.64 m x 0.15 m x 0.19 m) and exposed at three different shelf distances (5, 8, and 13 cm) and three different exposure durations (20, 40, and 60 sec). Pulsed UV light treatment was applied to both the control group quails and those inoculated with *S.* Typhimurium according to the experimental conditions.

Salmonella Typhimurium Count Analysis

The eggshell surface in each bag was rubbed with the maximum recovery diluent for two minutes. After the scrubbing process, a five-minute break was taken, and the scrubbing process was repeated for the same duration. Therefore, the microbial load around and above the shell was completely transferred to the added liquid. After this process, the dilution liquids to be used in the study from 10^{-1} to 10^{-8} were prepared by consecutively transferring 1 mL each consecutively from the first dilution (10^{-1}) of the microbial-loaded washing water obtained from the first dilution (10^{-1}) culture liquid to tubes containing 9 mL of ringer solution. Salmonella counts were obtained from the samples. For *S.* Typhimurium enumeration in experimental samples, Xylose Lysine Deoxycholate (Merck, 105287) agar was inoculated and counts were performed after incubation at 35°C for 24-48 h [18].

Temperature and Energy Measurements in Pulsed UV Light Application

The definitions of "severe," "moderate" and "mild" for the pulse UV light applications planned within the scope of the study were determined as 60 sec at a distance of 5 cm, 40 sec at a distance of 8 cm and 20 sec at a distance of 13 cm, respectively. The surface temperature of the quail eggs was measured before and after treatment with an infrared thermometer (Extech Instrument, USA) adapted to the device under these working conditions.

The amount of energy released during the pulses was measured using an energy measurement sensor (PE50-DIF-C, Ophir Optronics, Israel) placed on the quail eggs at different treatment distances (5, 8, and 13 cm) with a reader (Nova II, Ophir Optronics, Israel) [17].

Color Values

The brightness L^*, redness-greenness a^*, and yellowness-blueness b^* color values of quail egg whites were measured using a color analyzer (Minolta Co., Osaka, Japan).

Thiobarbituric Acid Number

Thiobarbituric acid (TBA) count values of pulsed UV-treated quail egg samples were determined by modifying the spectroscopic method [19,20]. After the quail eggs were beaten, 10 g of sample was taken, and 10 mL of 15% trichloroacetic acid and 50 mL of distilled water were added and homogenized for 60 sec. After mixing, 8 mL of the sample was filtered through a filter paper and placed in a test tube. Two mL of 0.06 N TBA was added to the tube and kept in a water bath at 80°C for 90 min. When the time was increased, the samples in the tubes were quickly cooled to room temperature and analyzed against the control sample using a spectrophotometer (Genesys 10S) at 520 nm absorbance. The results obtained were multiplied by 7.8 and the TBA values of quail eggs were determined

Total Phenolic and Antioxidant Content

The antioxidant activity value for determining the physicochemical changes in quail eggs caused by the applied pulsed UV light process and changing operating parameters was determined by DPPH radical scavenging activity analysis. Eggs were extracted with 80% methanol. 0.1 mL of extract taken from the sample obtained after extraction was mixed with 3.9 mL of DPPH solution, vortexed, and kept in the dark for 30 min. Analysis was performed using a spectrophotometer (Genesys 10S) at 517 nm. Methanol was used as a control during the analysis, and the spectrophotometer was zeroed. The results were expressed as % inhibition [21].

The total phenolic content of quail eggs was determined using the Folin-Ciocalteu method. The prepared 0.5 mL sample solution was mixed with 0.5 mL of Folin-Ciocalteu reagent diluted three times. After adding 1 mL of supersaturated 35% sodium carbonate solution

(prepared the night before), 1 mL of pure water was added and the mixture was kept in the dark for 30 min. After this period, the absorbance at 760 nm was measured using a spectrophotometer. Pure water was used instead of the control sample and the total amount of phenolic substances was expressed as gallic acid equivalents [22].

Fatty Acid Composition Content

To analyze the fatty acid profile of quail eggs, both the eggs treated with Pulsed UV light and the control group were degreased using hexane. The fatty acid composition was evaluated using a gas chromatograph (Agilent 7890 GC/FID, USA) fitted with a flame ionization detector and a silica capillary column. The initial oven temperature was set to 165°C for 15 min, followed by an increase to 200°C at a rate of 5°C/min. Both the injector and detector temperatures were maintained at 250°C. A 1 µL sample volume was used for the injection. The fatty acid composition was expressed as the percentage of total fatty acids [23].

Statistical Analysis

At the end of the study, one-way variance analysis (ANOVA) and Tukey test were performed using SPSS Statistics 17.0, with the support of the advisor to evaluate the data.

Results

Microbiological Analysis

Quail eggs contaminated with *S.* Typhimurium were formed into 10 different experimental groups by taking into account the pulse duration and intensity applied. No pulsed light was applied to the control group samples. The number of microorganisms in the control group samples was determined as 4.13 $\log_{10}$ CFU/g. The most effective results were obtained with 40 and 60 s application at 5 cm distance and 60 s application at 8 cm distance *(Table 1)*.

Temperature and Energy Levels Occurring in Pulsed UV Light Application

The temperature increase and changes in energy levels of quail eggs resulting from pulsed UV light application are given in *Table 2*. The initial temperatures of quail eggs are 20.5±0.4°C. Measurements were made in 3 working parameters selected as the model for temperature increase. The temperature increase in 13D20S (mild), 8D40S (medium) and 5D60S (severe) samples was determined as 0.33, 14.25 and 25.11°C, respectively.

The energy amounts released in 1 sec during the pulsed UV light treatment applied to quail eggs at different distances were calculated as 1.57, 1.13 and 0.79 $J.cm^{-2}.s^{-1}$ for the application parameters of 5, 8, and 13 cm, respectively.

Color Values

The color changes in egg white as a result of the application of pulsed light at different distances and times to quail egg samples are given in *Table 3*. The images of quail eggs after pulsed UV light application were given in *Fig. 1*. The L^*, a^* and b^* color values were 57.65, 7.88 and 19.32, respectively, in the control sample without pulsed light. While the brightness value expressed by L^* color value decreased in all quail eggs analyzed, a^* and b^* values increased with the effect of the light. The highest decrease and increase in these values were observed in the sample coded 5D60S and it was determined that the color values changed as the distance of the applied beam decreased. In general, effective color changes were detected in the 5D sample groups compared to other distance trials. Again, the effect of the increase in the application time in the 5D group, especially in the L^* value, is clearly seen.

Thiobarbituric Acid Number

As a result of pulsed light treatment of quail eggs at different distances (5, 8, and 13 cm) and durations (20, 40 and 60 s), TBA values of eggs varied between 0.79-3.41 mg malondialdehyde/kg eggs *(Fig. 2)*. The lowest TBA value of 0.79 mg malondialdehyde/kg egg was determined in the control sample without pulsed UV light.

Table 1. *S. Typhimurium count on the surface of quail eggs ($\log_{10}$ cfu/g)*

Samples	Control	5D20S	5D40S	5D60S	8D20S	8D40S	8D60S	13D20S	13D40S	13D60S
S. Typhimurium ($\log_{10}$ cfu/g)	4.13^a	1.92^c	<10	<10	1.57^c	0.38^d	<10	3.01^b	1.15^c	0.36^d

Different letters in the same row indicate statistically significant differences in the results (P<0.05)

Table 2. *Temperature and energy levels in pulsed UV light application*

Energy Level ($J.cm^{-2}.s^{-1}$)		Temperature Increase (ΔC°)	
Distance (cm)	Value	Sample Code	Value
5	1.57±0.04^a	13D20S	0.33±1.19^c
8	1.13±0.03^b	8D40S	14.25±1.16^b
13	0.79±0.02^c	5D60S	25.11±1.31^a

Different letters in the same column indicate statistically significant differences in the results (P<0.05)

Table 3. Color values in quail eggs after pulsed UV light treatment

Samples	*L**	*a**	*b**
Control	57.65±1.67^{a}	7.88±1.38^{d}	19.32±0.94^{c}
5D20S	51.20±0.13^{b}	14.45±0.62^{bc}	30.02±0.40^{a}
5D40S	49.10±0.76^{c}	14.05±0.41^{c}	27.87±2.72^{ab}
5D60S	46.18±0.68^{d}	13.78±0.94^{c}	23.56±0.85^{bc}
8D20S	50.01±0.09^{c}	15.51±0.62^{b}	29.69±0.18^{a}
8D40S	49.46±0.76^{bc}	14.75±0.45^{bc}	27.97±1.38^{ab}
8D60S	50.97±2.18^{bc}	15.49±0.58^{b}	27.88±2.14^{ab}
13D20S	51.70±0.12^{bc}	16.11±0.56^{a}	30.51±0.36^{a}
13D40S	49.12±0.19^{c}	14.33±0.11^{bc}	28.16±0.12^{ab}
13D60S	50.91±0.08^{bc}	16.68±0.28^{a}	31.22±0.29^{a}

Different letters in the same column indicate statistically significant differences in the results (P<0.05)

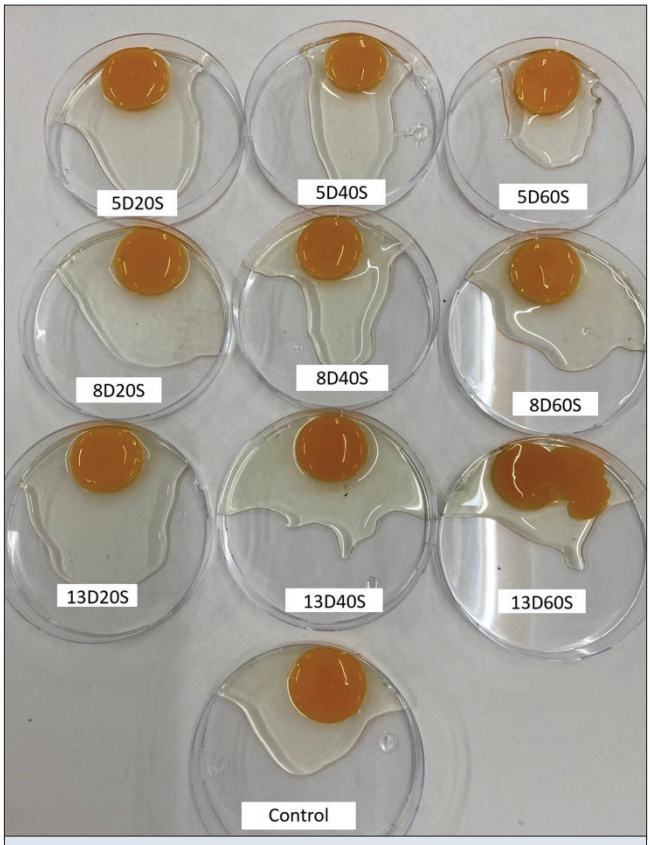

Fig 1. Images of quail eggs after pulsed UV light treatment

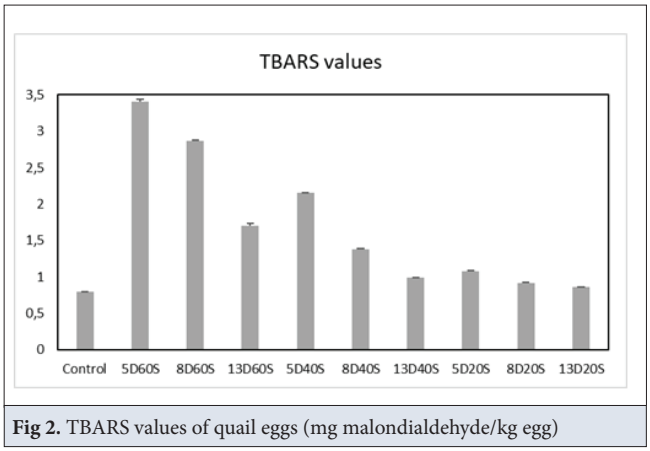

Fig 2. TBARS values of quail eggs (mg malondialdehyde/kg egg)

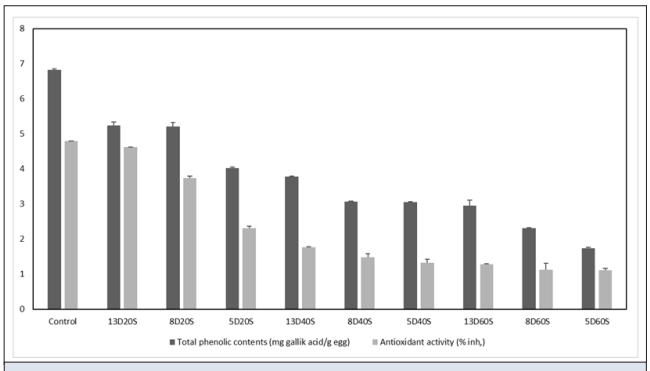

Fig 3. Total phenolic content and antioxidant activity level of quail eggs

Total Phenolic Content and Antioxidant Activity Level

The total phenolic content and antioxidant activity values of quail eggs exposed to pulsed UV light at different distances and times are shown in *Fig. 3*. The total phenolic content and antioxidant activity of the control sample without pulsed light were 6.822 mg gallic acid/g egg and 4.793%, respectively. The total phenolic matter content and antioxidant activity of quail egg samples decreased with the application of scattered light. While the highest decrease in total phenolic matter and antioxidant activity value was observed in the sample coded 5D60S, the lowest decrease was observed in sample 13D20S. This decrease is thought to be due to the breakdown of phenolic compounds in the egg because of the increase in temperature as a result of the applied beam being closer and the applied time being longer.

Fatty Acid Composition of Quail Eggs

Table 4 shows the fatty acid composition of quail egg samples irradiated with pulsed UV at different distances

Table 4. *Fatty acid composition values of quail eggs*

Samples	Fatty Acid Composition					
	Palmitic (C16:0)	Palmitoleic (C16:1)	Stearic (C18:0)	Cis-Oleic (C18:1)	Trans-Oleic (C18:1)	Linoleic (C18:2)
Control	28.92	1.11	18.00	41.87	2.34	13.35
5D20S	24.31	1.45	12.37	32.34	1.38	21.55
5D40S	23.23	1.43	11.95	30.21	1.36	25.35
5D60S	22.14	1.64	10.68	25.19	1.26	25.51
8D20S	26.56	1.25	14.41	34.62	1.53	17.59
8D40S	26.09	1.37	14.00	32.82	1.42	18.84
8D60S	24.44	1.34	13.87	31.35	1.30	20.07
13D20S	27.87	1.15	16.85	40.63	1.74	14.45
13D40S	27.31	1.18	15.97	40.10	1.69	15.25
13D60S	26.87	1.20	15.10	38.41	1.48	16.32

and times. The predominant fatty acids in the unirradiated control samples were palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), cis-oleic acid (C18:1), trans-oleic acid (C18:1), and linoleic acid (C18:2), which were determined as 28.92%, 18%, 41.87%, 2.34% and 13.35%, respectively. As the amount and proximity of irradiation increased, the amounts of cis-oleic and trans-oleic acids decreased, and the amount of linoleic acid increased. The increase in the amount of linoleic acid in 5D60S and 5D40S quail eggs was higher than that in the other samples. This increase is thought to be due to the breakage of carbonyl groups and double bonds in unsaturated fatty acids under UV light.

Discussion

Surface decontamination treatments, particularly non-thermal treatments, have been widely researched and used for microbial inactivation. However, while microbial inactivation has been evaluated in products after surface inactivation, other product properties, especially changes in biochemical structures, have not been examined. In this context, our study is considered to be very innovative in terms of its application and contains very effective results. Pulsed UV light application for surface decontamination of quail eggs, which are small in size, was studied using different distance and light intensity variables. A concentration of 4.13 $\log_{10}$ CFU/g of *S.* Typhimurium was observed in control eggs. Conversely, the application of pulsed UV light resulted in a reduction in microorganism counts in all samples. The most favorable application conditions were identified when the applied time was extended and the distance was minimized. *Salmonella* Typhimurium inactivation was targeted for surface decontamination, and inactivation was successfully achieved in each model. In a study; It was reported that there was a 7.9 log decrease in eggs contaminated with *Salmonella* Enteritidis with 4 J/cm^2 pulsed light application [24]. In a study in which 2.1 J/cm^2 pulsed light was applied to eggs contaminated with *S.* Typhimurium, a 5 log decrease was reported and no significant temperature increase was reported in the eggs [25]. On the other hand, in the study in which it was stated that *Salmonella* Enteritidis was inactivated by Pulse UV light application on the egg surface, no visual change was observed on the egg surface [17].

Energy is generated during pulsed UV light application, and this energy is absorbed by the egg shell, causing a temperature increase on the surface. The mentioned temperature increase is affected by the application parameters of time and distance [26]. The temperature increase increases more as the proximity to the lamp and the application time increase. For example, a 30-sec application at a distance of 9.5 cm from the lamp resulted in a temperature increase of up to 16.3°C [17]. The energy density released decreases as the distance from the light source increases due to the inverse square law [27]. In accordance with the literatures, an increase in temperature was observed as the application time increased and the distance decreased from the pulsed UV light process parameters. On the other hand, the energy levels decreased as the pulsed UV light application distance increased.

In most of the studies in which pulsed UV light application was performed on eggs, color changes were observed in egg white. It is stated that these changes are related to the process parameters and intensity [28,29]. The degree of color changes that occur in pulsed UV light applications is related to the intensity and duration of application. Higher energy doses and longer exposure times are more likely to cause noticeable changes in color [30]. On the other hand, this change is thought to be caused by enzymatic browning of the egg white due to the heat generated by the

effect of light [31,32]. In pulsed UV light application, color changes were observed in egg white, although the color changes varied according to the process conditions. These changes should be taken into consideration when making applications for food safety purposes.

TBARS are indicative of malondialdehyde levels and provide insight into the degree of lipid oxidation. In eggs, oxidative damage to cholesterol and unsaturated fatty acids significantly contributes to lipid peroxidation, leading to quality degradation [31,33]. The TBA value increased as the distance of the applied beam became closer and the applied time increased. This increase is thought to be due to the breakdown of unsaturated fatty acids in the egg. In a similar study conducted by Quyang et al.[30], it was determined that the TBA value increased as the distance decreased in pulsed light application applied to commercial and fresh liquid egg whites. In a study conducted on bacon, it was observed that TBARS and lipid oxidation values increased as the distance and application time increased [34]. High temperature is known to accelerate many chemical reactions, including lipid oxidation. The increase in lipid oxidation also affects TBARS formation [35]. In addition, it can also be explained by the decrease in the antioxidative effects in the egg with increasing temperature and the inability to resist lipid oxidation, which also increases with increasing temperature [36].

The total phenolic content and antioxidant activity of quail egg samples decreased with the application of scattered light. In a study conducted by Manzocco et al.[32], it was reported that antioxidant activity decreased as a result of egg paste irradiation. The high energy levels created by light pulses in pulse light applications may cause degradation by breaking down phenolic components and reducing the amount of phenolic substances and antioxidant effects. The decrease in the amount of phenolic substances and structural damage to phenolic components affect the antioxidant activity values of the products because of the antioxidative properties of phenolic components [37]. However, it has been stated that the decrease in the amount of phenolic and antioxidant substances caused by pulsed UV light application can be explained by the disruption of the secondary tertiary structures of enzymes in the metabolism of phenolic components by the intense energy generated during the process [38].

Changes in the fatty acid composition are important because of their significant impact on health, disease risk, and biological processes. These changes can affect obesity, cancer, metabolic health, and the quality of food sources [39,40]. Pulsed UV treatment caused changes in fatty acid composition were observed. Fragmentation of chain structures in saturated fatty acids changes the fatty acid composition and causes the formation of radicals, accelerating lipid oxidation. Soro et al.[41] examined the effects of two different UV sources, conventional UV lamp and UV-LED, on fresh chicken breast meat. At the end of the study, it was reported that lipid oxidation was the most affected quality characteristic in meat treated with UV lamp. Wang et al.[1] found in a different study that UV light exposure can trigger lipid oxidation by breaking carbonyl groups and double bonds in unsaturated fatty acids, leading to the formation of free radicals that promote further lipid oxidation through auto-oxidative chain reactions. Meanwhile, a study investigating the impact of pulsed UV light on microalgal growth revealed an increase in saturated and monounsaturated fatty acids, alongside a reduction in polyunsaturated fatty acids following treatment [42].

Among the pulsed UV light application parameters, the small application distance and prolonged application time affected and increased all biochemical transformations. Our results revealed the necessity of evaluating such applications preferred for microbial safety in terms of biochemical changes. In light of these findings, it's clear that while pulsed UV light application effectively ensures microbial safety for surface decontamination of quail eggs, it can also lead to significant alterations in the product's biochemical properties. Crucially, parameters such as application distance and duration directly impact critical biochemical metrics including temperature increase, color changes, lipid oxidation (TBARS), and phenolic content/antioxidant activity. Furthermore, observed changes in fatty acid composition indicate that such non-thermal treatments have the potential to profoundly affect not only microbial load but also the nutritional value and overall quality of the product. These results lay an important foundation for future studies aiming to determine optimized application conditions and develop balanced solutions that preserve both microbial safety and nutritional quality in food products.

Declarations

Availability of Data and Materials: The data that findings of this study are available from the corresponding author (MGS) upon reasonable request.

Conflict of interest: The authors declare that they do not have any conflict of interest.

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Research Article

The Potential of *Spirulina platensis* to Substitute Antibiotics in Broiler Chickens Diets: Influences on Growth Performance, Serum Biochemical Profiles, Meat Quality, and Gut Microbiota

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Abstract

The rise of antibiotic-resistant microbes has prompted a search for effective alternatives to antibiotics. This study evaluated the effects of *Spirulina platensis* extract (SPE) as a dietary supplement and a potential alternative to antibiotics for broiler chickens, focusing on growth performance, antioxidant activity, blood parameters, and cecal microbiota. SPE contained antimicrobial active compounds, including heptadecane and geosmin. A total of 300 broilers were divided into five groups (T1-T5). T1 received a basal diet (control), while T2-T5 were supplemented with 0.5, 1, 2, and 3 mg SPE/kg of diet, respectively, for 35 days. Results showed that including SPE (3 mg/kg) in the broiler diet significantly enhanced growth, decreased the feed conversion ratio (FCR), and improved body weight gain (BWG, 9%), while maintaining optimal carcass quality and intestinal pH at 6.8. Liver enzymes remained stable, with a 13-45% reduction in kidney markers based on the SPE concentration. SPE (3 mg/kg) also reduced oxidative stress by decreasing malondialdehyde (MDA) levels while sustaining antioxidant enzyme levels. The cecal microbiota showed an increase in lactic acid bacteria and exhibited enhanced immunity compared to the control. Additionally, SPE improved meat quality by boosting protein and moisture content, enhancing juiciness and tenderness. In conclusion, supplementing broiler diets with SPE (3 mg/kg) enhances growth performance, productivity, overall health, and disease resistance, making it a potential viable alternative to antibiotics.

Keywords: Eco-friendly antibiotic, Broiler chickens, *Spirulina platensis,* Immune response, Gut microbiota

Introduction

Different pathogens can significantly impact the health and productivity of poultry, which in turn affects their welfare and production efficiency. This can lead to the formation of antimicrobial or multidrug-resistant strains of pathogens [1], which increases the risk of poultry products being contaminated with pathogens that can be transmitted to humans. Additionally, consumers are increasingly demanding organic poultry [2]. Some pathogens, such as *Salmonella* and *Campylobacter* species, exacerbate the situation by forming biofilms, which contribute to the severity of poultry diseases and promote resistance to antimicrobial drugs. These biofilms are complex structures of bacterial cells and the substances they produce, creating a protective barrier that makes eradication efforts more challenging [3].

The poultry industry relies heavily on the use of synthetic antimicrobial agents, which are commonly administered through feed or drinking water. While this practice has contributed to the industry's success, a significant risk is associated with the prolonged use of antibiotics at low levels. This can lead to the emergence of drug-resistant pathogens, which can have negative consequences for human, animal, and environmental health [4].

Additionally, the overreliance on antimicrobials for controlling diseases in the poultry industry poses financial sustainability risks, as it promotes the growth of bacterial reservoirs that are resistant to treatment [1]. Thus, it is essential to practice proper antimicrobial stewardship that reduces the use of antimicrobials in animal feeds, particularly for preventive purposes, to mitigate the impact of antimicrobial resistance on human health [5].

The rise of antibiotic-resistant bacteria in food, including *Campylobacter jejuni, Bacillus cereus, Escherichia coli,* and *Staphylococcus aureus,* necessitates a deeper understanding of their pathogenesis [6]. It is imperative to investigate alternatives to the use of preventive antibiotics to prioritize public health and ensure revenue in livestock production. One potential solution is the use of phytogenic additives, which are plant-derived and classified as non-antibiotic antimicrobials [7,8]. These additives show promise as feed supplements for promoting growth and preventing diseases in poultry. Plant-derived natural antimicrobial compounds can control both susceptible and resistant pathogens, thereby minimizing their presence in the food chain and enhancing microbial food safety [9].

Spirulina is a nutritious and widely used ingredient in broiler feed worldwide [10]. *Spirulina* is a single-celled cyanobacterium, often referred to as "blue-green algae." *Spirulina* can thrive and proliferate in saline and freshwater environments [11]. *Spirulina* is commonly used as a dietary supplement and growth promoter for animals due to its high concentrations of Fe, protein, P, and all essential and non-essential amino acids [12]. Antibiotics are used in poultry production due to ongoing efforts to decrease illnesses and improve the quality of meat and eggs [13]. The widespread use of antibiotic alternatives in the diet has led to the disappearance of drug-resistant microbes, antibiotic residues, and the development of natural microflora [14,15].

Spirulina is a safe, non-toxic, and nutritious organism that promotes growth, reproduction, and immune function in poultry and animals [16]. Broilers supplemented with *Spirulina* as a growth enhancer exhibit improved performance, as evidenced by a higher feed conversion ratio (FCR) and enhanced live body weight gain (BWG) [17]. Feeding chicken diets containing *Spirulina platensis* resulted in a substantial rise in meat production compared to the control group [18]. *Spirulina platensis* improves nutrient digestion and mineral absorption and reduces diarrhea [19].

Spirulina platensis is a powerful natural supplement that enhances reproductive function and strengthens the immune system [20]. *Spirulina platensis* in the diet may significantly improve the immune system's ability to process antigens (T-cells) and reduce the presence of harmful microbes [21]. Despite the promising preliminary findings on *Spirulina*'s benefits in poultry, a comprehensive study is still needed that integrates various aspects of broiler health and performance. Specifically, research is limited on the combined impact of *Spirulina* supplementation on immune responses, oxidative stress markers, detailed lipid profiles, and the modulation of the caecal microbiome in broilers, particularly as a complete replacement for conventional antibiotics. Further investigation into these interconnected physiological parameters will provide a more holistic understanding of *Spirulina*'s potential as a sustainable and effective alternative to antibiotic growth promoters in poultry production. Therefore, this research aims to examine the effects of replacing antibiotics with *Spirulina platensis* extract (SPE) in broiler chickens' diets, specifically investigating the potential benefits of this supplementation on the birds' immunity, oxidation status, lipid profile, caecal bacterial content, blood parameters, liver and kidney functions, productivity, and carcass and meat quality.

Material and Methods

Ethical Approval

The animal study was reviewed and approved by the ZU-IACUC Committee. It was conducted in accordance with the guidelines of the Egyptian Research Ethics Committee and the Guide for the Care and Use of Laboratory Animals (2024). The ethical approval code is ZU-IACUC/2/F/394/2024. Written informed consent was obtained from the owners for the participation of their animals in this study.

Spirulina platensis Extract Preparation

A pure culture of *Spirulina platensis* was recovered and grown using the Zarrouk medium, which was created by Zarrouk in 1966. The composition of the Zarrouk medium included the following consistunants dissolved in 1L of water: 1 g of NaCl, 16.8 g of $NaHCO_3$, 2.5 g of $NaNO_3$, 0.5 g of K_2HPO_4, 1 g of K_2SO_4, 0.2 g of $MgSO_4.7H_2O$, 0.04 g of $CaCl_2.2H_2O$, 0.01 g of $FeSO^4.7H_2O$, and 0.08 g of EDTA. Using a 1 M KOH solution, the medium's pH was adjusted to 9.5. To initiate a fresh *Spirulina platensis* culture, 10 mL of a 5-day-old culture was added to a 250 mL amount of Zarrouk's media in 500 mL screw bottles. The bottles were placed in an environment with a constant temperature of 25±2°C and exposed to continuous light from a 36W white fluorescent lamp with an intensity of 600-800 lux for ten days. The *Spirulina platensis* pure culture was successfully obtained through the effective streaking method on Zarrouk's media. We isolated a single culture from this strain using the streaking technique on Zarrouk's medium to produce a pure culture of *Spirulina platensis* [22]. The plates were carefully stored in an environment with a temperature of 25°C and a constant light exposure of 600 lux. Once the colonies were obtained, they were carefully collected and inspected under a microscope. Zarrouk's medium was then used to preserve the *Spirulina platensis* cells on slants.

Identification of *Spirulina platensis* Isolates

The morphological identification, as determined by microscopic examination of *Spirulina platensis* isolates, was applied at various phases of development in Zarrouk's

medium. In the process of cold-water extraction of *Spirulina platensis*, as follows: 10 g of *Spirulina platensis* powder was homogenized in 90 mL of distilled water (1:9, *w/v)* for two hours. To produce a solid mass, the resultant supernatant was quickly frozen at a temperature of -20°C. This mass was then thawed at 4°C and subjected to centrifugation (10.000 rpm for 10 min) to separate the supernatant, which was subsequently freeze-dried (Heto PowerDry lyophilizer) until it reached a powdered state.

Identification of Volatile Compounds in SE by GC-MS Spectroscopy

One g of SPE was dissolved in 10 mL of Hexane (1:10, w/v), then sonicated for 10-30 min at room temperature. The obtained extract was filtered through centrifugation, and the supernatant was obtained. The solvent was removed under reduced pressure (rotary evaporator) to obtain the crude extract. An extract volume of 1 µL was injected into the GC-MS system (Agilent 6890, Foster City, CA), which had an HP-5 MS column and an Agilent mass spectrometer detector. The carrier gas was helium, with a flow rate of 1.0 mL/min. After adding 1 µL of volume to the sample, the solvent was left in place for three minutes. The rate of temperature increase was 8°C/min, starting at 40°C and reaching 260°C. The detector temperature was set to 280°C, while the injector temperature was maintained at 250°C. Following Saad et al.[23] Wiley 9 datasets were used to determine peaks.

Experimental Design

Five groups were carefully assigned to a total of 300 broiler chicks, ensuring a fair and balanced distribution for the study, with each group consisting of 3 replicates of 20 chicks. The standard basal diet was given to the control negative group (T1). For 35 consecutive days, the other four groups (T2, T3, T4, and T5) received a basic diet supplemented with 0.5 mg SPE/kg, 1 mg SPE/kg, 2 mg SPE/kg, and 3 mg SPE/kg, respectively. Using a randomized methodology, every chick in the study was grown on a litter model. In a shed with adequate ventilation, rice husk was employed as litter. All broiler chicks were provided with standard management conditions and access to water throughout the experiment. We tracked the weekly weights of each bird and recorded the daily feed intake for all groups. Once the experiment was over, we extracted blood from the veins in the wings and stored it in EDTA vials for further analysis.

Growth Performance

Broiler chickens were assessed for their live body weights (LBW) and feed consumption. Body weight gain (BWG) was calculated by deducting the initial live body weight (7 days old) from the final live body weight (35 days old). The feed conversion ratio (FCR) was calculated by dividing feed consumption by body weight gain, as explained in Saad et al.[24]. The performance index (PI) and the growth Rate (GR) were estimated.

$$\text{Body weight gain (BWG)} = \text{FBW} - \text{IBW} \quad (1)$$

$$\text{GR} = (\text{LBW35} - \text{LBW7})/0.5 \times (\text{LBW7} + \text{LBW35}) \quad (2)$$

$$\text{PI} = \text{BWG/FCR} \quad (3)$$

Carcass Traits

After the experiment, three birds were randomly selected from each replication, and their weights were measured. After collecting body measures, the birds were butchered to evaluate carcass features, including carcass weight, dressing percentage, and the weight of the visceral organs, which included giblets, heart, liver, and gizzard. Intestinal pH was also estimated.

Digestive Enzyme Activities

The concentrations of digestive enzymes, including amylase, protease, and lipase, in the intestine were evaluated during the investigation. The chicken ileum was dissected, and then the contents of the intestine (ileum) were carefully gathered and placed into sterile containers equipped with screw closures to prevent contamination. The activity of the enzymes in the ileum was evaluated using the methodology established by Najafi et al.[25].

Hematology

Plasma samples were obtained using gauge needles from the broiler chickens' wing veins (3 birds per replicate). The samples included 200 µL of EDTA, which was applied as an anticoagulant. To provide a comprehensive analysis of essential blood parameters, including red blood cells (RBCs), packed cell volume (PCV), haemoglobin, and white blood cells (WBCs), plasma samples were obtained in labeled screw-top tubes.

Liver and Kidney Function

Blood samples were meticulously extracted from slaughtered chicks that were 35 days old and promptly preserved in an anticoagulant-containing tube for efficient plasma extraction using high-speed centrifugation at 4000 rpm for 10 min. After the plasma was collected, it was securely sealed in a sterile tube and stored at a temperature of -20°C till it was needed. Spectrophotometers (Apel 310 Spectrophotometer, Japan) were used to measure photometric biological processes. Calorimetric analysis was conducted using specific commercial kits to evaluate the biochemical characteristics of blood components. The biochemical profiles including alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate amino-transferase (AST), creatinine, urea, total protein (TP), total globulin (TG), albumin/globulin (A/G) ratio,

Lipid Profile

The lipid profile, including total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and very low-density lipoprotein (VLDL), was assessed using a spectrophotometer and commercial kits according to the manufacturer's instructions

Immunological Parameters

The colorimetric estimation of IgA and IgG immunoglobulin isotypes was tested using a spectrophotometer with respective kits [26].

Antioxidant Status

At slaughter, nine birds from each group were blood sampled and centrifuged for twenty minutes at 4500 rpm. Subsequently, the plasma was stored at -20°C to preserve its integrity. The antioxidant key biomarkers, including SOD, CAT, glutathione, malondialdehyde, and antioxidant enzymes, were measured using top-quality commercial kits provided by Biodiagnostic company.

Estimation of Caecal Bacterial Load

For the collection of caecal content, three birds from each replicate (a total of 5 birds/group) were sacrificed at 35 days of age. The careful dissection of the caeca and collection of contents in sterile cups was performed using aseptic techniques to ensure the integrity of the samples. 1 mL of cecal content was homogenized in 9 mL of sterilized saline solution to obtain 10-1 diluation, serial diluations were conducted up to 10^{-6}. Then, 0.1 mL of each dilution was spread on specific media for counting total bacterial *Salmonella*, coliforms, *E. coli*, *Salmonella, Enterococcus,* total fungal on Nutrient, McConkey's agar, Eosin-Methylene blue agar, XLD, and *Enterococcus* agar, sabaroud dextrose agar media, respectively. The samples were applied to the agar surface using a sterilized glass spreader while the Petri dish was rotated beneath. For the total bacterial count, the nutrient broth is employed. The Petri plates were incubated at a temperature of 37°C for 24 h, and individual colonies were counted with a colony counter and quantified as:

CFU/mL = (No. of colonies ×dilution factor)/volume of the culture plate

Meat Quality

The color of the chicken breasts (2 cm in diameter) was measured using a Hunter spectrophotometer. Lipid and protein oxidation were determined according to Sayed-Ahmed et al.[27]. The chemical composition of the chicken breast was determined according to the AOAC [28]. Sensory evaluations were measured using a 9-hedonic scale [29].

Statistical Analysis

The statistical analysis was conducted using one-way ANOVA in the SPSS program (SPSS, 2021). The LSD test was used to compare all tested means (treatments) at a significance level of $P<0.05$. The sample size was calculated using the following equation: $n = (\frac{ZSD}{E})^2$.

PCA was performed using R (FactoMineR and factoextra packages) or equivalent statistical software, following standard procedures for multivariate analysis.

Results

Table 1 highlights the active components in SPE, where the main compounds in the SPE profile, detected by GC/MS, were identified as the active compounds of SPE, including heptadecane (63.21%), pentadecane (6.32%), and β-ionone (5.21%), which were found to be the primary components. These components are followed by β-cyclocitral and 2-methylisoborneol (0.77%). The SPE contains geosmin (0.06%), a critical component of cyanobacterial odor.

As mentioned in *Table 2,* supplementating the broiler diets with *Spirulina platensis* extract (SPE) produced clear, dose-dependent improvements in growth performance parameters. As the concentration of SPE increased from T1 (control) to T5 (highest dose), both live body weight (LBW) and body weight gain (BWG) showed progressive

Table 1. *Active compounds in Spirulina platensis extract (SPE) detected by GC-MS*

Retention Time	Volatile Compounds	% Area
10.21	2-Pentylfuran	1.55±0.2d
19.99	Geosmin	0.06±0.001f
20.53	β-cyclocitral	0.45±0.05e
27.05	2-Methylisoborneol	0.32±0.08e
31.51	β-ionone	5.21±0.3bc
32.10	Pentadecane	6.32±0.8b
34.74	Hexadecane	4.94±0.4c
37.79	Heptadecane	63.21±1.9a

Data are presented mean ±SE, n=3

Table 2. *The influence of dietary Spirulina platensis extract (SPE) at four concentrations on the growth performance parameters of broiler chickens*

Growth performance		SPE Treatments (mg/kg)					P-value
		T1	T2	T3	T4	T5	
LBW (g)	1d	44.9±0.1	45.1±0.0	45±0.0	45.7±0.0	45.5±0.0	0.89
	35d	2312±11.2e	2339±10.9d	2370±12.3c	2412±11.1b	2450±10.2a	<0.0001
BWG (g)	1-35d	2267±9.8e	2294±8.7d	2325±11.2c	2366±11.3b	2405±8.9a	<0.0001
FI (g)	1-35d	3878±12.7a	3812±11.2b	3778±10.8c	3768±9.9cd	3754±8.3d	<0.0001
FCR	1-35d	1.71±0.5a	1.65±0.2b	1.62±0.1bc	1.59±0.1c	1.56±0.0c	<0.0001
GR	1-35d	196±3.2b	199±3.8a	202±2.9a	206±4.2a	210±5.2a	<0.0001
PI	1-35d	226±6.8c	231±7.5b	233±6.9b	237±5.3ab	240±6.5a	<0.0001

n=5, data are presented as mean ±SD. Different lowercase letters in the same raw indicate significant variation at P<0.05. ***T1*** *= basal diet + 0 mg/kg SPE;* ***T2****, SPE 0.5 = basal diet + 0.5 mg/kg SPE,* ***T3****, SPE 1 = basal diet + 1 mg/kg SPE,* ***T4****, SPE 2 = basal diet + 2 mg/kg SPE;* ***T5****, SPE 3 = basal diet + 3 mg/kg SPE*

Table 3. *The impact of dietary Spirulina platensis on Carcass traits and intestinal pH of broiler chickens*

Traits (%)	SPE Treatments, mg/kg					P-value
	T1	T2	T3	T4	T5	
Carcass	70.0±2.5c	73.11±3.6b	74.36±2.6a	74.9±4.1a	75.8±3.9a	0.001
Liver	2.20±0.1	2.23±0.2	2.22±0.0	2.17±0.0	2.20±0.2	0.9
Gizzard	1.9±0.2b	2.2±0.3a	2.25±0.5a	2.26±0.5a	2.25±0.6a	0.05
Heart	0.84±0.01b	0.93±0.02ab	0.95±0.01a	0.96±0.03a	0.95±0.04a	0.04
Dressing	73.23±4.5d	75.11±5.1c	77.24±5.5b	78.88±4.9b	80.89±4.5a	0.001
Intestinal pH	6.8±0.6b	6.5±0.7b	6.7±0.4b	6.8±0.6b	7.2±0.7a	0.05

n = 5; data are presented as mean ± SD. Different lowercase letters in the same row indicate significant variation at P<0.05. ***T1*** *= basal diet + 0 mg/kg SPE;* ***T2****, SPE 0.5 = basal diet + 0.5 mg/kg SPE,* ***T3****, SPE 1 = basal diet + 1 mg/kg SPE,* ***T4****, SPE 2 = basal diet + 2 mg/kg SPE;* ***T5****, SPE 3 = basal diet + 3 mg/kg SPE*

Table 4. *The influence of dietary Spirulina platensis extract (SPE) at four concentrations on the serum digestive enzymes of broiler chickens*

Digestive Enzymes Activity	SPE Treatments, mg/kg					P-value
	T1	T1	T1	T1	T1	
Amylase	310±12.3d	420±11.3c	480±13.1b	500±13.6ab	510±14.6a	<0.0001
Lipase	12±1.1e	17±0.9d	22±0.9c	26±0.6b	29±1.1a	<0.0001
Trypsin	25±0.9d	30±1.2c	36±1.3b	42±1.0ab	45±1.1a	<0.0001

n = 5; data are presented as mean ± SD. Different lowercase letters in the same row indicate significant variation at P<0.05. ***T1*** *= basal diet + 0 mg/kg SPE;* ***T2****, SPE 0.5 = basal diet + 0.5 mg/kg SPE,* ***T3****, SPE 1 = basal diet + 1 mg/kg SPE,* ***T4****, SPE 2 = basal diet + 2 mg/kg SPE;* ***T5****, SPE 3 = basal diet + 3 mg/kg SPE*

increases, with the highest dose (T5) resulting in a 5.97% increase in LBW and a 6.09% increase in BWG compared to the control. This indicates that higher levels of SPE can effectively enhance the growth rate of broiler chickens.

Feed intake (FI) demonstrated a gradual reduction as SPE concentration increased, with T5 showing a 3.20% decrease relative to T1. This reduction in feed intake, coupled with increased weight gain, led to a marked improvement in feed conversion ratio (FCR), which decreased by 8.77% at the highest SPE level. A lower FCR reflects more efficient conversion of feed into body mass, highlighting the positive impact of SPE on feed efficiency.

Additionally, both growth rate (GR) and performance index (PI) improved with higher SPE supplementation. The greatest enhancements were observed at T5, where GR increased by 7.14% and PI by 6.19% over the control. These findings collectively demonstrate that dietary inclusion of *Spirulina platensis* extract not only boosts growth and performance metrics but also optimizes feed utilization, making it a valuable additive for improving broiler production outcomes.

As presented in *Table 3*, dietary supplementation with *Spirulina platensis* extract (SPE) led to notable improvements in carcass traits and intestinal pH in broiler chickens. As the level of SPE increased from the control (T1) to the highest dose (T5), both carcass yield and dressing percentage showed significant enhancements, with the highest values observed at the greatest SPE

inclusion. This indicates that SPE can effectively boost meat yield in broilers. Additionally, there were modest but statistically significant increases in gizzard and heart percentages at higher SPE levels, suggesting a potential influence on organ development. Liver weight, however, remained unaffected across all treatments, indicating that SPE does not notably impact liver size. Intestinal pH exhibited a slight but significant increase at the highest SPE dose, which may have implications for gut health and digestive processes. Overall, the results demonstrate that increasing dietary SPE improves key carcass characteristics and dressing percentage, with minimal effect on liver weight and a modest rise in intestinal pH at higher supplementation levels.

Table 4 demonstrated that the digestive enzyme levels, such as the lipase enzyme level, showed that lipase level exhibited a substantial elevation in T5 as opposed to T1 (P-value <0.0001) while the level of protease exhibited a significant elevation of all groups supplied with *Spirulina platensis* in contrast with unsupplied birds (G1). The amylase level was substantially elevated in T4 and T5 compared to T1, T2, and T3 birds.

Fig. 1 revealed that the blood parameters of birds supplied with dietary *Spirulina platensis* were significantly improved compared to those of the birds (T1). The haemoglobin (Hb) level demonstrated a substantial rise in groups (T2, T3, T4, and T5), contrary to group (T1). When comparing T5 (4.5) to T1 (2.4) birds fed a 3 mg SPE/kg diet, the RBC count revealed a considerable rise. The WBC count significantly increased at T5 (17.33) compared to T1 (13.41) birds.

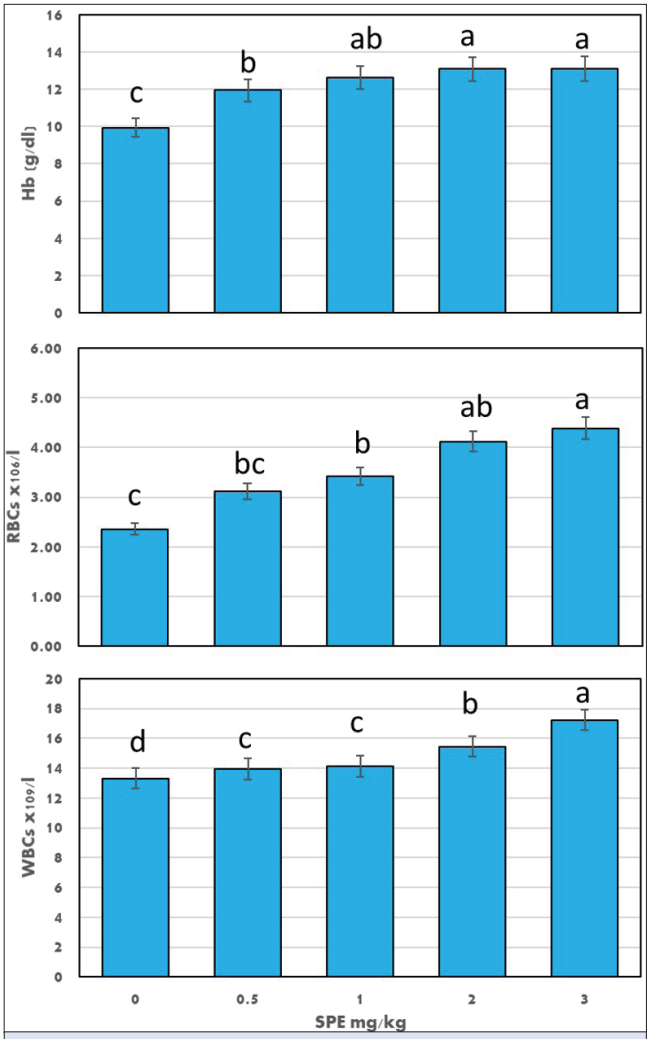

Fig 1. The effect of dietary *Spirulina platensis* extract (SPE) at four concentrations on the hematology of broiler chickens

The results in *Table 5* showed that liver and kidney functions indicated a significant increase in total protein (TP) in birds fed a diet of 3 mg/kg in T5, compared to those in T1. Albumin (ALB) levels appear to have a significant elevation in T3 birds as opposed to birds in T1, T2, and T4. Globulin (GLOB) levels showed a substantial increase in birds in T5 compared to T1, T2, T3, and T4. A notable decrease in the A/G ratio in T5 in contrast with T1. Regarding liver functions, ALT and AST levels revealed a substantial reduction in T5 compared to birds in T1. Regarding renal function, urea and creatinine levels exhibited a considerable decrease in birds supplied with different *Spirulina platensis* extracts from T2 to T5, as opposed to control birds (T1).

In *Table 5*, the birds in treatments (T2, T3, T4, and T5), treated with varied quantities of SE, showed a substantial drop in total cholesterol (TC) compared to those in group G1 that were not supplied with SE. Birds exhibiting the most significant stress-induced sympathetic excitation (SE) showed a notable reduction in total cholesterol (TC) levels, measured at 74 mg/dL. This drop was shown to be statistically significant compared to other groups (P-value <0.0001). Triglyceride levels (TG) showed a substantial reduction in all experimental groups supplied with SE, as opposed to birds in T1 (P-value <0.0001). The birds in T5 showed a significant increase in high-density lipoprotein (HDL), demonstrating that the highest quantity of SPE enhanced the lipid profile with a P-value of <0.0001. Low-density lipoprotein (LDL) exhibited a substantial drop in the birds supplied with different SE, while birds in T5 revealed a significant drop in LDL level (15 mg/dL) in contrast with its level in T1 (48 mg/dL) (P-value <0.0001). Very low-density lipoprotein (VLDL) appeared to have a significant decline in birds in T5 (18 mg/dL) when compared with birds in T1 (P-value <0.0001). The findings suggest that adding SPE to the birds' food improved their lipid profile compared to birds that did not receive the supplement.

The data in *Table 5* demonstrate that the birds in T5 exhibited a noteworthy increase in IgG and IgM levels (17-19%) compared to the control birds (T1). Additionally, the

Table 5. *The influence of dietary Spirulina platensis extract (SPE) at four concentrations at the serum biochemical parameters of broiler chickens*

Parameter	Serum Biochemistry	SPE Treatments (mg/kg)					P-value
		T1	T2	T3	T4	T5	
Liver and kidney functions	AST (U/L)	255±12.3a	220±11.3b	208±10.5c	189±9.5d	177±8.8e	<0.0001
	ALT (U/L)	5.9±0.5a	5.2±0.3b	4.7±0.6c	3.9±0.2d	3.1±0.2e	<0.0001
	Uric acid (mg/dL)	5.5±0.6a	4.8±0.2b	4.5±0.1c	3.8±0.5d	3.0±0.5e	<0.0001
	Creatinine (mg/dL)	0.36±0.01a	0.35±0.02a	0.32±0.05ab	0.28±0.02b	0.27±0.03b	0.05
	Total protein (g/dL)	2.8±0.6c	3.6±0.3bc	3.9±0.3b	4.2±0.4ab	4.6±0.1a	0.00123
	Albumin (g/dL)	1.8±0.2d	1.92±0.1c	2.1±0.4b	2.4±0.2ab	2.7±0.2a	<0.0001
	Globulin (g/dL)	1.2±0.3c	1.4±0.2bc	1.7±0.6b	1.9±0.3ab	2.1±0.3a	0.0011
	Albumin/Globulin (%)	1.5±0.5a	1.35±0.3b	1.23±0.2d	1.26±0.1c	1.28±0.5c	0.0023
Lipid profile	Total cholesterol (mg/dL)	235±9.2a	196±6.7b	141±7.1c	125±3.2d	111±4.5e	<0.0001
	Triglycerides (mg/dL)	192±6.8a	187±7.1b	168±6.6c	100±6.1d	74±4.3e	<0.0001
	HDL (mg/dL)	85±2.3d	92±1.9c	96±3.5c	100±3.3b	110±4.0a	<0.0001
	LDL (mg/dL)	48±0.8a	33±1.1b	25±0.7c	18±0.5d	15±0.7e	<0.0001
	VLDL (mg/dL)	46±0.9a	35±0.5b	28±0.6c	20±0.4d	18±0.2e	<0.0001
	Abdominal fat	1.33±0.2a	1.21±0.2b	0.91±0.1c	0.85±0.1d	0.69±0.01e	<0.0001
Oxidative status	GSH (ng/mL)	0.35±0.02c	0.52±0.01bc	0.59±0.03b	0.67±0.01ab	0.69±0.02a	<0.0001
	SOD (U/mL)	0.51±0.03e	0.68±0.03d	0.72±0.03c	0.83±0.01b	1.01±0.7a	<0.0001
	CAT (ng/mL)	0.33±0.02d	0.51±0.02c	0.62±0.01bc	0.68±0.02b	0.77±0.01a	<0.0001
	MDA (nmol/mL)	0.55±0.01a	0.41±0.01b	0.35±0.02c	0.30±0.03c	0.21±0.02d	<0.0001
	TAC (ng/mL)	0.35±0.02c	0.42±0.02c	0.55±0.04c	0.68±0.01b	0.85±0.03a	<0.0001
Immunity	IgG (mg/dL)	960±13.2e	1050±14.3d	1071±14.8c	1099±15.0b	1120±13.8a	<0.0001
	IgA (mg/dL)	177.8±3.5e	188.2±3.6d	191.3±4.1c	205.6±4.5b	211±4.3a	<0.0001

n = 5; data are presented as mean ± SD. Different lowercase letters in the same row indicate significant variation at P<0.05. ***T1*** *= basal diet + 0 mg/kg SPE;* ***T2****, SPE 0.5 = basal diet + 0.5 mg/kg SPE,* ***T3****, SPE 1 = basal diet + 1 mg/kg SPE,* ***T4****, SPE 2 = basal diet + 2 mg/kg SPE;* ***T5****, SPE 3 = basal diet + 3 mg/kg SPE*

Table 6. *Effect of Spirulina platensis dietary treatments on meat quality of broiler chickens*

Parameters		SPE Treatments (mg/kg)					P-value
		T1	T2	T3	T4	T5	
Chemical composition	Moisture	60.3±3.5d	62.3±4.1c	63.4±3.6b	65.9±4.2ab	66.7±5.2a	<0.0001
	Protein	20.9±1.1c	21.5±0.9bc	22.0±1.0b	23.2±1.1ab	24.0±1.3a	<0.0001
	Fat	14.1±0.4a	11.2±0.5b	10.6±0.7c	8.7±0.5d	7.1±0.3e	<0.0001
	Ash	0.88±0.01a	0.74±0.02b	0.68±0.02c	0.61±0.01c	0.60±0.02c	<0.0001
	pH	5.9±0.5c	6.1±0.3b	6.3±0.2b	6.6±0.5ab	6.7±0.3a	<0.0001
	TVBN	7.1±0.4a	5.5±0.2b	4.8±0.2c	4.4±0.1c	4.2±0.0c	<0.0001
	TBA	0.79±0.02a	0.59±0.01b	0.41±0.03c	0.28±0.01d	0.25±0.01d	<0.0001
Sensory properties	Juiciness	9±0.0a	8.8±0.1b	8.5±0.3b	8.6±0.3b	9.0±0.0a	0.025
	Tenderness	8.7±0.2ab	8.6±0.1b	8.7±0.2ab	8.7±0.2ab	8.8±0.1a	0.031
	Aroma	8.5±0.1a	8.3±0.2b	8.1±0.5b	8.2±0.4b	8.5±0.3a	0.032
	Taste	8.8±0.1a	8.5±0.2b	8.5±0.2b	8.4±0.3b	8.8±0.1a	0.034
Color properties	*L**	60±2.3d	61.2±2.5c	62.1±2.6b	62.9±2.9ab	63.1±2.1a	0.031
	*a**	6.1±0.2	5.9±0.5	6.0±0.5	6.1±0.5	6.2±0.2	0.6
	*b**	14.9±0.9	14.8±1.1	15.2±0.6	15.3±0.8	15.1±0.9	0.7

n = 5; data are presented as mean ± SD. Different lowercase letters in the same row indicate significant variation at P<0.05. ***T1*** *= basal diet + 0 mg/kg SPE;* ***T2****, SPE 0.5 = basal diet + 0.5 mg/kg SPE,* ***T3****, SPE 1 = basal diet + 1 mg/kg SPE,* ***T4****, SPE 2 = basal diet + 2 mg/kg SPE;* ***T5****, SPE 3 = basal diet + 3 mg/kg SPE*

antioxidant status in birds fed a diet containing 3 mg/kg of T5 revealed a significant improvement in MDA, SOD, CAT, GSH, and TAC levels compared to control birds (T1). The birds fed with varying levels of SE significantly improved immunity and antioxidant status compared to control birds (T1).

Table 6 illustrates that the chemical composition of chicken breast meat varied in response to different levels of SPE supplementation in the broiler diet. Notably, the group receiving 3 mg/kg of SPE exhibited the most favorable results compared to the other treatments. In this group, the moisture content increased by 25% and the protein content rose by 12% compared to the control group. These improvements in moisture and protein were reflected in the sensory evaluation, with panelists awarding the highest scores for juiciness (9) and tenderness (8.8) to the 3 mg/kg SPE group. Both the control and the 3 mg/kg SPE groups were noted for their superior taste.

Additionally, increasing SPE levels led to reductions in both fat and ash content in the breast meat. Regarding meat quality parameters, the pH value rose to 6.7, while nitrogen compounds and TBA values decreased to 4.4 and 0.25, respectively. The addition of BP significantly influenced the color characteristics of the meat, enhancing its lightness, although the a* (redness) and b* (yellowness) values remained unchanged.

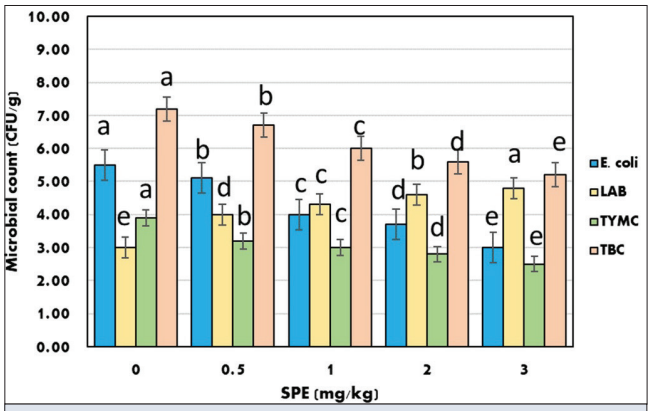

Fig 2. The influence of dietary *Spirulina platensis* extract (SPE) at four concentrations on the intestinal microbiota of broiler chickens

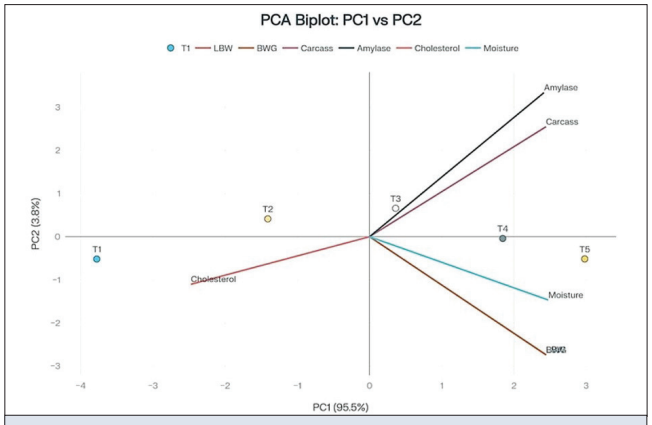

Fig 3. PCA biplot showing parameter vectors for PC1 and PC2. PC1 (95.5% variance) and PC2 (3.8% variance). Treatments (T1-T5) and measured properties (growth, blood biochemistry, enzymes, carcass, meat quality)

Additionally, the total yeast and fungal count showed a substantial decrease in T2, T3, T4, and T5, in contrast to T1. Regarding the *E. coli* count, the birds fed a diet with elevated SPE exhibited a substantial drop compared to their counts in control birds (T1). Furthermore, compared to control birds (T1), the SPE treatments showed a significant decline in TBC counts, accompanied by a considerable rise in LAB counts *(Fig. 2)*.

The PCA biplot visualizes the relationships between five treatments (T1-T5) and six measured properties: live body weight (LBW), body weight gain (BWG), carcass percentage, amylase activity, cholesterol, and meat moisture (*Fig. 3*). The first two principal components (PC1 and PC2) collectively account for over 99% of the total variance, with PC1 contributing 95.5% and PC2 contributing 3.8%. This means the biplot provides an accurate and comprehensive summary of the multivariate data structure. The results showed that T1 and T2 are positioned on the left side of the biplot, indicating lower values for most performance and quality traits, but higher cholesterol. However, T3, T4, and T5 are distributed to the right, aligning with higher values for LBW, BWG, carcass percentage, amylase activity, and moisture, and lower cholesterol levels.

LBW, BWG, carcass, amylase, and Moisture vectors all point in a similar direction, indicating these properties are positively correlated. Treatments in this direction (T3-T5) exhibit higher values for these traits. While cholesterol vector points in the opposite direction, showing a strong negative correlation with the other properties. Treatments closer to this vector (T1, T2) have higher cholesterol levels.

Fig. 3 shows that T5 is closest to the vectors for LBW, BWG, carcass, amylase, and moisture, suggesting it yields the best overall performance and meat quality, with the lowest cholesterol levels. T1 is positioned near the cholesterol vector, indicating the least favorable profile in terms of growth, carcass, and enzyme activity, but the highest cholesterol. meanwhile T3 and T4 show intermediate profiles, with improvements over T1 and T2 but not as pronounced as T5.

Discussion

The global poultry industry faces a critical challenge: how to maintain high productivity and animal health in broiler chickens while reducing or eliminating the use of antibiotics, which have been linked to the rise of antimicrobial resistance in both animals and humans. In

this context, *Spirulina platensis*, a blue-green microalga renowned for its rich nutritional and bioactive profile, has emerged as a promising natural alternative to antibiotic growth promoters (AGPs).

Multiple recent studies have consistently demonstrated that dietary supplementation with *Spirulina platensis* can significantly improve growth performance in broiler chickens. Supplementation levels ranging from 0.1% to 1% in feed have been shown to increase body weight gain, enhance feed intake, and improve feed conversion ratios (FCR) [11,30-32]. For example, Abdelfatah et al.[31] found that broilers fed 0.3% and 0.5% *Spirulina* had higher body weight gains and better FCR compared to controls. Similarly, Khan et al.[33] reported that a 1% inclusion of *Spirulina* resulted in significantly higher body weight gain and improved FCR, with optimal results at this level, while higher inclusion (2%) did not yield further benefits.

The mechanisms behind these improvements are multifaceted. *Spirulina* is rich in high-quality protein, essential amino acids, vitamins (such as B-complex and vitamin E), minerals (including iron and selenium), and bioactive compounds like phycocyanin and beta-carotene [34]. These nutrients support efficient nutrient utilization, promote muscle growth, and enhance overall metabolic activity in broilers [11,35]. Additionally, the presence of polyunsaturated fatty acids and antioxidants in *Spirulina* may help reduce oxidative stress, further supporting growth under intensive rearing conditions [36,37].

The inclusion of *Spirulina platensis* in broiler diets has been shown to beneficially modulate serum biochemical parameters, which are key indicators of animal health and metabolic status [38,39]. Several studies have reported that *Spirulina* supplementation can lowered total cholesterol, triglycerides, and LDL-cholesterol levels, while increasing HDL-cholesterol, contributing to better cardiovascular health in broilers [40,41]. Additionally, increased the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), as well as total antioxidant capacity (TAC) in serum, thereby reducing oxidative damage [42,43].

Furthermore *Spirulina* can mitigate the elevation of liver enzymes (ALT, AST) and renal markers (uric acid, creatinine) that are often associated with dietary or environmental stressors, such as ochratoxin A exposure [36]. They found that broilers supplemented with *Spirulina* exhibited significant decreases in abdominal fat percentage and improved liver and kidney function test results, even under toxin-induced stress. These effects are attributed to the bioactive compounds in *Spirulina*, including phycocyanin and phenolic acids, which possess hepatoprotective and nephroprotective properties [44,45].

Meat quality is a critical determinant of consumer acceptance and market value. Recent research indicates that *Spirulina platensis* supplementation can positively influence several aspects of broiler meat quality, where broilers fed *Spirulina* exhibit increased redness (a*) and yellowness (b*) in their meat, likely due to the deposition of natural pigments, such as beta-carotene and phycocyanin, from the microalga [30,32]. This can improve the visual appeal of poultry products. Additionally, the improved water-holding capacity results in reduced cooking and thawing losses, leading to juicier and more tender meat [32]. Meat from *Spirulina*-fed broilers tends to have higher protein levels, reflecting improved muscle accretion [30,32].

On the other hand, the antioxidant properties of *Spirulina* help maintain meat freshness and extend shelf life by reducing oxidative rancidity [31,35]. Sensory evaluations have also shown that meat from *Spirulina*-supplemented broilers is more tender, juicy, and generally preferred by panelists [30]. These improvements are particularly valuable in markets where meat quality attributes drive consumer choice.

A healthy gut microbiota is essential for nutrient absorption, immune function, and disease resistance in broilers. Antibiotics, while effective in controlling pathogenic bacteria, can disrupt the balance of gut microbiota and contribute to the development of resistance issues. In contrast, *Spirulina platensis* acts as a prebiotic and immunomodulatory agent, promoting beneficial bacteria. Studies have shown that *Spirulina* supplementation increases the population of *Lactobacillus* species in the cecum, which are associated with improved gut health and pathogen exclusion [11,46]. On the other hand, there is a concurrent decrease in *Escherichia coli* and other potential pathogens, reducing the risk of enteric infections [11].

Additionally, supplementation improves villus height, the villus-to-crypt ratio, and the villus surface area, thereby facilitating better nutrient absorption and gut barrier function [46]. It is found that when combined with native probiotics, *Spirulina* further enhances gut health and immune response, providing a robust alternative to antibiotics [37]. These effects are attributed to the oligosaccharide-rich content and bioactive molecules in *Spirulina*, which serve as substrates for beneficial microbes and modulate the gut environment [30].

One of the most compelling arguments for using *Spirulina platensis* as an alternative to antibiotics is its ability to support the immune system. *Spirulina* supplementation leads to larger bursa, thymus, and spleen, indicating enhanced development of these immune organs [47]. Also, increased WBC counts and improved phagocytic activity suggest a more robust innate immune response [11], which enhances both humoral (antibody-mediated) and cellular immune responses, making broilers more resilient to

infections [35]. Under stress conditions, such as heat or mycotoxin exposure, *Spirulina* helps maintain immune function and reduces pathological changes in lymphoid tissues [36]. These immune-enhancing effects are crucial for maintaining flock health in the absence of antibiotic protection, especially in intensive production systems.

Several studies have directly compared *Spirulina platensis* with conventional antibiotics such as enrofloxacin and zinc bacitracin. The results indicate that *Spirulina* can match or even surpass antibiotics in promoting growth, optimizing feed conversion, supporting egg and meat quality, and maintaining liver health [48,49]. For instance, a 2024 study on laying hens found that 1% *Spirulina* was as effective as zinc bacitracin in improving performance and protecting liver health, without the risk of antibiotic residues or resistance [49].

The safety profile of *Spirulina platensis* is well-established, with no reports of adverse effects at recommended inclusion levels (typically up to 1% of the diet for broilers) [31,32]. Higher inclusion rates (e.g., 15%) may impair growth, suggesting that moderation is key [30]. The optimal dosage appears to be between 0.3% and 1% of the diet, balancing efficacy and cost-effectiveness [50].

While the evidence supporting *Spirulina platensis* as an antibiotic substitute is robust, some limitations remain: Large-scale production and consistent supply of high-quality *Spirulina* are necessary for widespread adoption in the poultry industry [36]. The nutrient and bioactive content of *Spirulina* can vary depending on cultivation conditions, affecting its efficacy [30]. Long-term effects, where most studies are short-term; long-term impacts on productivity, health, and resistance development warrant further investigation.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Research Article

Risk Factors Associated with Prolapsed Nictitating Membrane Gland in Cats with Conjunctivitis: Analysis of 65 Cases

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Abstract

This study aims to evaluate animal-related and environmental risk factors influencing the development of prolapsed nictitating membrane gland (PNMG) in cats presented with complaints of conjunctivitis. A retrospective review of 65 cats diagnosed with PNMG, selected from a cohort of 318 cats presenting with conjunctivitis between 2019 and 2024. Data recorded included signalment, case history, ophthalmic examinations, intraoperative findings and outcomes. Direct ophthalmoscopy and slit-lamp biomicroscopy revealed no ocular abnormalities in cats with PNMG. Notably, 81.5% of cats with gland prolapse were identified as brachycephalic. Univariate logistic regression analysis indicated that breed, season, and history had a statistically significant effect on the occurrence of PNMG ($p<0.05$). Classification and regression tree analysis identified season as the most influential factor affecting PNMG, with the highest prevalence (40.5%) observed in brachycephalic breeds during spring season. The brachycephalic head structure was confirmed as a significant contributing factor to the development of PNMG in cats, consistent with findings in dogs. However, the seasonal variation and the role of ocular irritants in gland prolapse suggest that allergens may play a primary role in the etiology, as proposed by the authors.

Keywords: Brachycephalic, Cherry eye, Conjunctivitis, Hyperplasia, Ocular irritants, Third eyelid

Introduction

The third eyelid, also nictitating membrane, is a conjunctival fold located at the medial canthus of the eye, positioned between the lower eyelid and the globe in animals. Anatomically, in many animal species, including cats, it consists of a T-shaped cartilage and a prominent lacrimal gland situated at the base of this cartilage. This gland is anchored to the periorbital region by the fascia retinaculum. Known by various terms such as the glandula nictitans and tarsal gland, it is responsible for producing approximately 30-57% of the aqueous component of tears [1,2]. Its secretions play a crucial role in maintaining ocular health by supplying oxygen and nutrients to the eye. The T-shaped cartilage, with its horizontal segment running parallel to the eyelid margin and a vertical segment extending perpendicularly, forms the structural framework of the third eyelid. In cats, this cartilage exhibits elastic properties [2].

Prolapsed nictitating membrane gland (PNMG), predominantly observed in dogs [3], also occurs in cats [4], rabbits [5], wild animals [6], and bird species [7]. This condition involves the displacement of the lacrimal gland within the third eyelid from its anatomical position, accompanied by hyperplasia, which manifests as a visible red mass at the medial canthus. The condition, referred to as Harderian gland prolapse in rabbits and birds and as nictitating membrane gland prolapse in other species, is commonly known as cherry eye [1]. PNMG is significantly less common in cats than in dogs, and a review of the literature reveals that many studies on this condition are presented as case reports. In these studies, the most affected cat breeds are Burmese, British shorthair and Persian cats [8-11].

The prolapsed gland should be promptly surgically repositioned to its anatomical location to maintain essential lacrimal function and prevent desiccation and infection of the overlying conjunctival tissue. Neither the

removal of the third eyelid nor the gland is recommended, as this may lead to keratoconjunctivitis sicca later in life [2].

The aim of this study is to evaluate animal-related and environmental risk factors influencing the development of PNMG in cats presented with complaints of conjunctivitis.

Material and Methods

Ethical Statement

This study was approved by the Ankara University Animal Experiments Local Ethics Committee (Approval no: 2024-08-64). An "Informed Consent Form" was obtained from the animal owners before examination of animals.

Study Design

A retrospective review of a cohort of 318 cats of various breeds, ages, and genders presenting with conjunctivitis to the Ankara University Faculty of Veterinary Medicine Animal Hospital Ophthalmology Clinic between July 2019 and May 2024 identified 65 cases diagnosed with prolapsed nictitating membrane gland (PNMG). Statistical analyses primarily focused on these 65 cases, with additional comparisons involving the broader cohort to provide further context. Detailed records were maintained for all animals, encompassing signalment, history, ophthalmic examinations, the duration and localization of gland prolapse, the presence of third eyelid cartilage eversion, and postoperative outcomes. The ophthalmic examinations, including direct ophthalmoscopy, slit-lamp biomicroscopy, intraocular pressure measurements, Schirmer test type I and fluorescein staining were performed on all cats.

Specifically, antigen detection was conducted on tear samples from animals suspected of herpesvirus infection. Based on the anamnesis obtained from the owners, recommendations were made to eliminate environmental factors that could contribute to ocular irritation. Throughout the procedure, all animals were equipped with Elizabethan collars, and hyaluronic acid eye lubricants were prescribed for application to the prolapsed gland. Prophylactic treatment with topical tobramycin eye drops twice daily was started in all eyes a few days before surgery.

General anesthesia was induced with propofol and maintained with isoflurane. Periocular preparation was performed using diluted 10% povidone-iodine, and the conjunctival sac was irrigated. All cats underwent gland repositioning via a modified Morgan pocket technique [12]. Two parallel conjunctival incisions were made over the prolapsed gland to create a subconjunctival pocket. The intervening tissue was excised, and the gland was secured using a continuous Schmieden suture pattern with 5-0 absorbable monofilament suture. Knots were placed on the palpebral surface, and suturing was completed 3-4 mm from the incision margins. Light pressure was applied post-suturing. In cases of persistent cartilage eversion, a small horizontal conjunctival incision was made, the folded cartilage was dissected, and a strip was excised. All surgeries were performed by the same clinicians.

Postoperative treatment included topical tobramycin and hyaluronic acid eye drops twice daily, along with oral amoxicillin suspension (20 mg/kg) for one week. An Elizabethan collar was recommended for two weeks. Patients were monitored periodically for up to three years, during which no recurrences or complications were observed.

Statistical Analysis

The proportional distributions of conjunctivitis-affected cats were calculated based on breed, gender, neuter status, age, season, and medical history. The impact of these variables on the likelihood of gland prolapse occurrence was evaluated using univariate logistic regression analysis. To determine and classify the outcomes related to gland prolapse occurrence, including season, breed, and other historical factors, the classification and regression tree method was employed. A criterion of $P<0.05$ was used for all statistical comparisons. Data analyses were performed using the SPSS 30 software package.

Results

In the study, 318 cats complaining of conjunctivitis were categorized as brachycephalic (n=128) and non-brachycephalic (n=190) cats. Prolapsed nictitating membrane gland (PNMG) was diagnosed in 65 cats (20.4%). Of the 65 cats diagnosed with PNMG, 53 cats (81.5%) were identified as being of brachycephalic breeds. It was observed that cats under 2 years of age were most affected by PNMG (0-6 months: n=21, 6.1 months-2 years: n=29, 2.1-6 years: n=11, 6.1-10 years: n=2, 10.1-14 years: n=1, <14.1 years: n=1).

In the ophthalmic examinations of cats presenting with PNMG, fluorescein staining yielded negative results. Direct ophthalmoscopy and slit-lamp biomicroscopy revealed no ocular abnormalities or evidence of disease. Intraocular pressure was within normal limits for all animals (mean 20.60±2.02 mmHg). The mean Schirmer tear test value was 25.44±1.65 mm in the preoperative period, and measured 20.18±2.63 mm at the end of the first postoperative week.

Gland prolapse was bilateral in only one cat, while it was unilateral in all other animals (Left eye = 47.7%; Right eye = 50.8%) *(Fig. 1)*. The prevalence of cartilage eversion accompanying gland prolapse was determined to be 43.11% *(Fig. 2)*. In all animals affected by eversion,

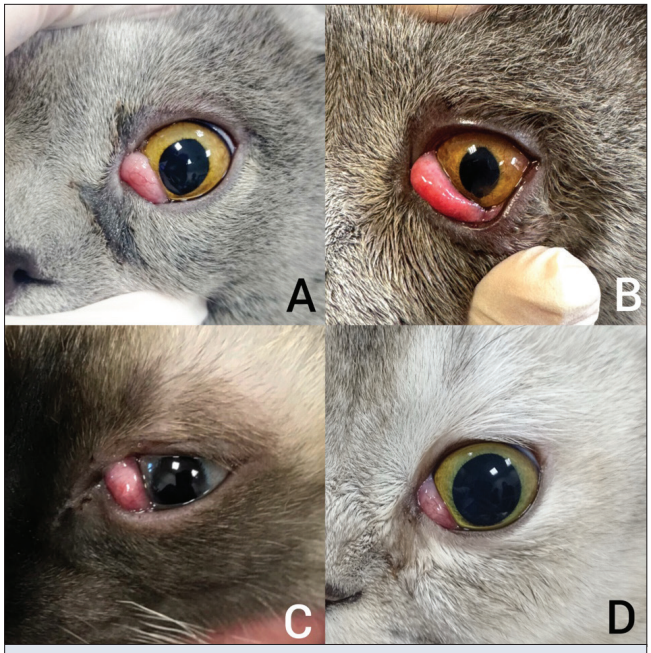

Fig 1. Preoperative appearance of prolapsed nictitating membrane gland with varying severity at the medial canthus

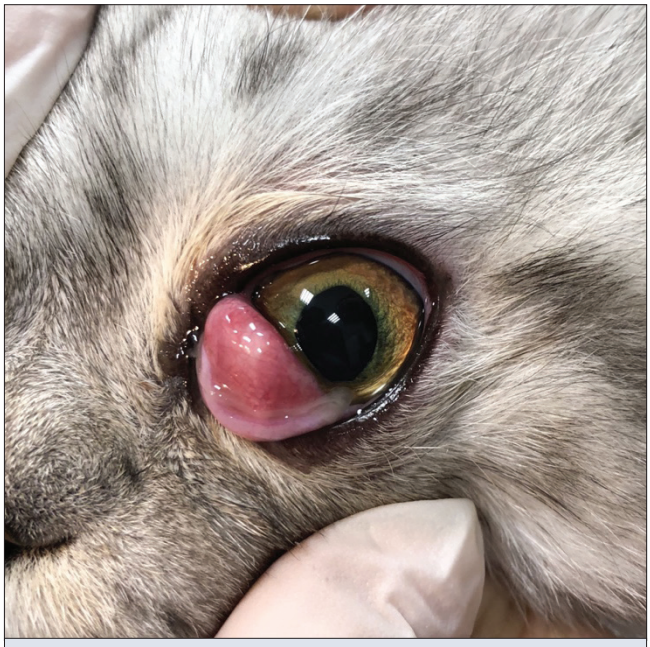

Fig 2. Cartilage eversion accompanied by prolapsed nictitating membrane gland in the left eye of a 3-year-old female Scottish Fold cat

the duration of prolapse development was observed to be two weeks or longer. In contrast, cases without cartilage eversion exhibited a variable duration, ranging from a few days to one week. Operative intervention was performed in 9 cats with persistent cartilage eversion following prolapse surgery. Throughout the entire follow-up period after the surgery, no complications or recurrence were observed in any of the cats *(Fig. 3; Fig. 4)*.

In the study, the effects of risk factors on the likelihood of gland prolapse in cats with conjunctivitis were evaluated

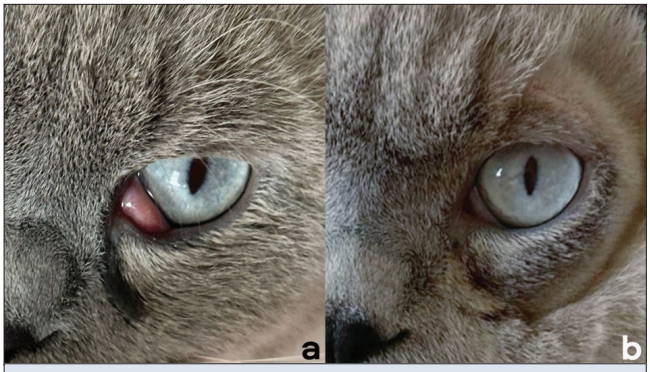

Fig 3. Prolapsed nictitating membrane gland in the left eye (a) and its appearance on postoperative day 5 (b) in an 8-month-old intact male Scottish Fold cat

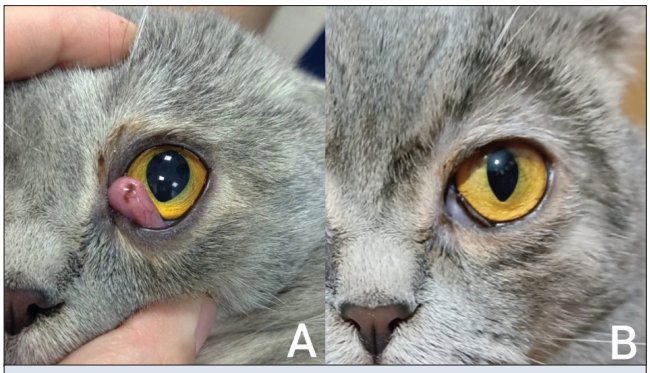

Fig 4. Preoperative (A) and postoperative 1st week (B) appearance of the prolapsed nictitating membrane gland in the left eye of a 1-year-old neutered male British Shorthair cat

using univariate logistic regression analysis. Based on the results of the univariate logistic regression model, breed, season, and history were found to have a statistically significant effect on the occurrence of PNMG in cats ($P<0.05$). Accordingly, the prevalence of PNMG was 6.556 times higher in brachycephalic breeds compared to non-brachycephalic breeds. As for the season of occurrence, the incidence during spring was noted to be 2.993 times higher compared to winter. Regarding animal history, the presence of ocular irritations and viral infections was observed. Notably, the incidence of the condition was found to be 3.113 times higher in cats exposed to ocular irritants compared to those with a history of infections *(Table 1)*. In conjunctivitis cases presented with complaints of herpesvirus infections, virus antigen (n=92) positivity was detected. Cats with PNMG were found to have the highest prevalence of herpesvirus antigen (12/17).

The study also utilized the classification and regression tree (CRT) method to classify cats with conjunctivitis based on factors influencing the occurrence of gland prolapse. According to CRT, the most influential factor affecting the occurrence of PNMG was found to be the season. The overall prevalence of PNMG was 17.0%, which increased to 27.4% in the spring season and was determined to be 40.5% in brachycephalic breeds. The

Table 1. *Univariate logistic regression analysis evaluating the effects of risk factors on the likelihood of gland prolapse in cats with conjunctivitis*

Variable	Variable Range	β	SE (β)	Wald	P	OR	95% Confidence Interval of OR	
Breed	Non-brachycephalic (Ref)	-	-	-	-	-	-	-
	Brachycephalic	1.88	0.34	30.672	<0.001	6.556	3.37	12.754
	Constant	-1.822	0.17	115.181	<0.001	0.162	-	-
Gender	Female (Ref)	-	-	-	-	-	-	-
	Male	0.514	0.283	3.308	0.069	1.672	0.961	2.91
	Constant	-	0.141	133.247	<0.001	0.196	-	-
Neutering status	Non-neutered (Ref)	-1.631	-	-	-	-	-	-
	Neutered	0.263	0.448	0.345	0.557	1.301	0.541	3.129
	Constant	-1.481	0.224	43.766	<0.001	0.227	-	-
Age	0-6 months	-	-	0.407	0.816	-	-	-
	6-24 months	0.2	0.314	0.407	0.524	1.222	0.66	2.26
	>24 months	0.118	0.37	0.102	0.75	1.125	0.545	2.322
	Constant	-1.594	0.141	127.912	<0.001	0.203	-	-
Season	Winter (Ref)	-	-	7.247	0.064	-	-	-
	Spring	1.096	0.409	7.168	0.007	2.993	1.341	6.679
	Summer	0.496	0.397	1.562	0.211	1.643	0.754	3.577
	Autumn	0.483	0.392	1.518	0.218	1.621	0.752	3.493
	Constant	-1.551	0.138	125.929	<0.001	0.212	-	-
History	FHV-I	-	-	13.987	0.003	-	-	-
	N/A	0.232	0.396	0.344	0.558	1.261	0.581	2.739
	Trauma	-19.192	7338.199	0.00	0.998	0.00	0.00	.
	Irritation	1.136	0.327	12.031	<0.001	3.113	1.639	5.913
	Constant	-6.467	1834.55	0.00	0.997	0.002	-	-

Categories indicated as 'Reference' are used as the baseline
Ref: *Reference;* ***β:*** *Estimated slope coefficient;* ***SE (β):*** *Standard error of the estimated slope coefficient;* ***Wald:*** *Wald statistic testing whether the slope coefficients are equal to zero for the model;* ***P:*** *P value associated with the Wald statistic;* ***OR:*** *Odds ratio*

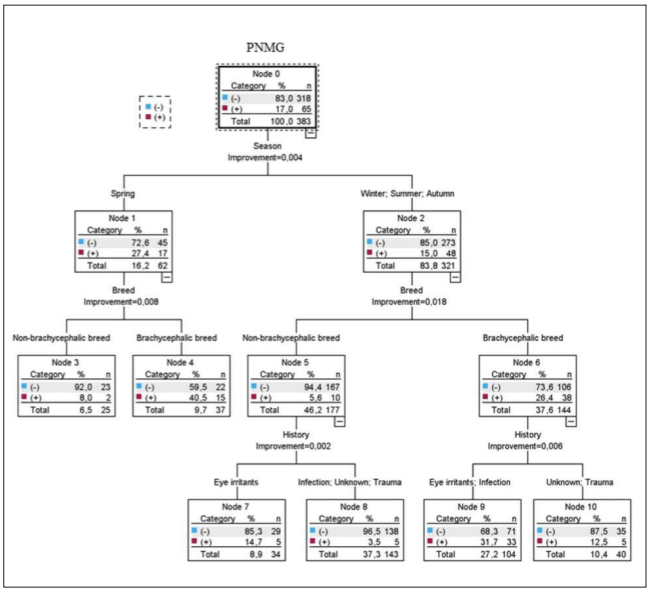

Fig 5. Classification and regression tree (CRT) used to classify cats with conjunctivitis based on factors influencing the occurrence of gland prolapse

prevalence of the condition was calculated as 15.0% during other seasons, increasing to 26.4% in brachycephalic breeds. Additionally, it was determined to be 31.7% in animals with a history of ocular irritation and infections. Upon classification results, the highest prevalence (40.5%) of PNMG was found to occur in brachycephalic breeds during the spring season *(Fig. 5)*.

Discussion

The conjunctival mucosa plays a crucial role in protecting the ocular surface through its anatomical, physiological, and immunological mechanisms. It supports ocular health while detecting and eliminating environmental irritants and pathogens. The nictitating membrane, a key component of the conjunctival surface, serves as a protective structure for the corneal surface due to its mobility across various species. Additionally, its glandular structure contributes significantly to normal tear production [2].

The exact etiology of prolapsed nictitating membrane gland (PNMG) remains unclear. Research has primarily focused on the deformation of the fascia retinaculum, which serves to anchor the gland in its anatomical position. It is hypothesized that the weakening of the gland's attachment to surrounding tissues also compromises its connection to the cartilage, thereby predisposing it to prolapse. Genetic and congenital factors are also suggested to play a role in the development of this condition. Notably, breeds such as the English Bulldog, Cocker Spaniel, Cane Corso, Neapolitan Mastiff, Lhasa Apso, Pekingese, and Shih Tzu have been reported to be predisposed to gland prolapse in dogs. A common characteristic among many of these breeds is the presence of disproportionately long eyelids relative to the size of the eyeball and orbit, resulting in a wide palpebral fissure. This condition is often associated with laxity of the lateral canthal ligament and supporting structures, with gland prolapse being particularly frequent in giant breeds [13-15]. Furthermore, skull morphology has been identified as a significant predisposing factor for PNMG. Numerous studies have established a strong correlation between brachycephalism and gland prolapse [13,16,17]. The narrow inferior orbital region in brachycephalic skulls has been proposed as a contributing factor to this predisposition [16,18]. Furthermore, the atypical positioning of the zygomatic salivary gland in the periorbital region, particularly in small and brachycephalic dog breeds, has been documented. The smaller size and steeper angle of the nictitating membrane gland compared to the zygomatic salivary gland suggest that this unusual placement, along with the resulting orbital congestion, may contribute to the development of prolapse [19].

However, the limited studies conducted in cats have not sufficiently established a breed-specific association with PNMG. An examination of the available literature, predominantly consisting of case reports, highlights brachycephalic breeds -characterized by their large, wide eyes, short noses, and disproportionate cheek widths- which have recently gained popularity [4,8,11]. The potential role of periorbital tissue laxity, a factor implicated in the etiopathogenesis of PNMG in brachycephalic dog breeds, should also be considered in brachycephalic cats. Notably, these cats are frequently reported to exhibit a predisposition to eyelid abnormalities such as entropion. While one previous study hypothesized that this may be related to increased laxity of the palpebral musculature, this remains speculative and lacks direct empirical confirmation [20]. In the present study, PNMG was most frequently observed in brachycephalic cats presenting with conjunctivitis. It is hypothesized that the development of this condition in brachycephalic cats may involve an abnormality characterized by the loosening of the nictitating membrane gland's attachment to surrounding tissues and cartilage, analogous to the palpebral laxity proposed in prior literature. The significant representation of brachycephalic cats within the study population further suggests a potential predisposition to PNMG. However, the condition is likely multifactorial in origin, underscoring the need for further investigative studies to clarify the underlying mechanisms.

The significant representation of brachycephalic cats within the study population further suggests a potential predisposition to PNMG. However, the condition is likely multifactorial in origin, underscoring the need for further investigative studies to clarify the underlying mechanisms. In this context, environmental contributors such as seasonal variation and ocular irritants, both of which showed statistically significant associations with PNMG development in the present study, warrant particular attention. The conjunctival mucosa, especially that of the third eyelid, contains conjunctiva-associated lymphoid tissue (CALT), a structured form of mucosa-associated lymphoid tissue (MALT) involved in local immune responses. In cats, CALT is most prominently located on the bulbar surface of the nictitating membrane and consists of lymphoid follicles lined by specialized epithelium containing cells with morphological characteristics of M cells [2,21]. These cells are known to transport antigens to underlying lymphoid structures, initiating mucosal immune cascades under inflammatory or allergenic conditions [22,23].

Given the observed seasonal and environmental correlations with PNMG in this study, it is plausible that conjunctival immune activation, potentially mediated by M cells, may contribute to inflammatory changes and glandular instability in predisposed individuals. Chronic antigenic stimulation due to allergens or irritants could exacerbate tissue remodeling processes in the third eyelid, particularly in cats with existing anatomic predispositions. While the present study was not designed to elucidate these immunopathogenic pathways, the findings underscore a plausible link between environmental immune triggers and gland prolapse. Future investigations incorporating immunohistochemical and molecular assessments of conjunctival immune activity under environmental stressors may provide deeper insights into the multi-factorial pathogenesis of PNMG.

In dogs, third eyelid gland prolapse is most commonly observed in young individuals [12,15]. While case reports in cats have referenced individuals under 2 years of age, no studies have specifically investigated the age-related incidence or prevalence of this condition. In the present study, age was found to be statistically insignificant in the development of gland prolapse in cats presenting with conjunctivitis. However, it was noted that cats with PNMG were numerically more likely to be under 2 years of age.

PNMG may occur independently or concurrently with the eversion of the T-shaped cartilage that forms the skeleton of the third eyelid [4,24]. In cats, the cartilage is elastic, with elastic fibers originating from connective tissue and extending into the perichondrium. In this region, the fibers form an extensive network that deepens, while the central cartilage is composed of similarly dense, finer elastic fibers. Notably, it has been observed that these elastic fibers form a regular network around chondrocytes exclusively in the cartilage of cats and horses. Additionally, the spade-like anatomical structure at the base of the cat's cartilage provides an advantage over species such as dogs and small ruminants, offering a more robust base for the cartilage [25,26]. In the present study, cartilage eversion was observed in nearly half of the cases alongside gland prolapse. Given the anatomical structure of this region in cats, the prevailing hypothesis is that gland prolapse contributes to the occurrence of eversion. In a small number of affected cases, surgical intervention was performed; however, in the majority of cases, gentle pressure applied for a few minutes was sufficient to correct the cartilage structure, either partially or completely. For mild cases of eversion, surgical intervention was generally avoided to prevent unnecessary manipulation of the cartilage, which forms the structural framework of the third eyelid and supports the gland. Notably, during the follow-up period, complete resolution of the eversion was observed in these cases.

Numerous surgical techniques have been developed for repositioning the third eyelid gland. These techniques primarily involve either repositioning the gland to its anatomical position using specialized sutures [27] or placing the prolapsed gland into a pocket created in the surrounding tissue [12]. Among these methods, the Morgan pocket technique remains the most commonly preferred approach due to its advantages, such as minimizing damage to the gland's ducts and preserving tear production [3,28,29]. In the present study, the modified Morgan pocket technique was utilized for the treatment of PNMG in cats. All cats exhibited excellent tolerance to the procedure, which resulted in a highly successful, complication-free outcome. Long-term follow-up findings confirmed complete success, supporting the conclusion that the modified Morgan pocket technique is a safe and effective method for managing PNMG in cats. Postoperative normalization of tear production was observed, likely due to the repositioning of the prolapsed gland and the resolution of inflammation achieved through medical therapy. This resolution appears to have alleviated conjunctival irritation, thereby restoring normal tear production levels.

Total or partial excision of the nictitating membrane gland was deliberately avoided, given the well-documented risk of severe complications -particularly keratoconjunctivitis sicca- associated with gland removal [30,31]. Additionally, the improper selection of suture materials or surgical techniques during gland repositioning may result in secondary complications, such as cyst formation, persistent inflammation, or recurrence of prolapse [13,16]. These findings underscore the critical importance of preserving the gland and adopting meticulous surgical practices to achieve favorable outcomes. Notably, the careful selection of suture materials and the precise application of the pocket technique in this study successfully prevented such complications, leading to complete and uneventful healing.

In conclusion, the inclusion of cats diagnosed with conjunctivitis in this study was based on clinical observations indicating that PNMG is frequently detected during evaluations of ocular surface irritation or inflammation. This case selection enabled a focused analysis of animal-related and environmental risk factors potentially contributing to PNMG development. Our findings confirmed a strong association between brachycephalic head conformation and PNMG, consistent with previous reports in dogs. Additionally, the pronounced impact of seasonal variation and ocular irritants on gland prolapse supports the hypothesis that environmental allergens may play a significant etiological role. While immunological mechanisms were not directly examined in this study, the dense lymphoid architecture of the third eyelid mucosa -together with the observed seasonal and environmental influences- raises the plausible involvement of mucosa-associated lymphoid tissue (MALT) and M cells in the pathogenesis of this condition. Future immunopathological investigations focused on antigenic stimulation and mucosal immune responses may further elucidate these mechanisms. Although the symptomatic nature of the study population may limit the generalizability of our results, large-scale studies incorporating asymptomatic individuals will be essential for identifying independent risk factors beyond clinical presentation.

Declarations

Availability of Data and Materials: The data that support the findings of this study are available on request from the corresponding author.

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Conflict of Interest: The authors declare that there is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The authors declare that the article, tables and figures were not written/created by AI and AI-assisted Technologies.

Authors' Contributions: İE: Conceptualization, data curation,

investigation, methodology, writing-original draft, writing-review and editing. AA: Data curation, formal analysis, writing-review and editing. SS: Data curation, investigation, writing-review and editing. OOŞ: Conceptualization, writing-review and editing, supervision.

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Research Article

Oxidative Stress in Neurological Feline Infectious Peritonitis: Cerebrospinal Fluid 8-Hydroxy-2'-deoxyguanosine and Superoxide Dismutase Levels

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Abstract

Oxidative stress plays a key role in the pathogenesis of neurological disorders and viral infections affecting the central nervous system. 8-Hydroxy-2'-deoxyguanosine (8-OHdG) is a marker of oxidative DNA damage, while superoxide dismutase (SOD) reflects antioxidant defense. This study aimed to evaluate SOD and 8-OHdG levels in the cerebrospinal fluid (CSF) of cats with neurological feline infectious peritonitis (FIP) to assess oxidative stress and antioxidant response. Twelve cats with neurological FIP and 10 age-matched control cats euthanized for non-neurological conditions were included. FIP diagnosis was confirmed by detecting feline coronavirus (FCoV) RNA in the CSF using real-time RT-PCR and by histopathological examination. CSF samples were analyzed for total protein, glucose, SOD, and 8-OHdG. Cats with FIP showed significantly higher CSF protein (740±230 mg/dL) than controls (17±7 mg/dL). The CSF/serum glucose ratio was lower in FIP cats (0.39±0.18) than in controls (0.66±0.06). 8-OHdG levels were elevated in FIP cats (6.88 ng/ml) compared to controls (1.09 ng/ml; $P<0.05$). SOD levels were reduced in FIP cats (0.034±0.026 U/mg protein) versus controls (0.312±0.136 U/mg protein; $P<0.001$). These findings highlight a pronounced oxidative stress condition in neurological FIP, characterized by elevated 8-OHdG levels and reduced SOD concentrations in the CSF. This concurrent pattern may not only serve as a valuable biomarker of disease activity but also represent a potential therapeutic target for antioxidant-based strategies in affected cats.

Keywords: 8-OHdG, Cat, Cerebrospinal fluid, Feline infectious peritonitis, Oxidative stress, Superoxide dismutase

Introduction

Feline infectious peritonitis (FIP) is a progressive disease caused by a mutant biotype of feline coronavirus (FCoV) in domestic cats. It presents with a wide spectrum of clinical manifestations, ranging from effusive to no effusive forms, with neurological involvement occurring in approximately one-third of affected cats. The neurological form of FIP is the most common infectious disease affecting the feline central nervous system (CNS) and is characterized by multifocal inflammatory lesions involving the brain and spinal cord. These lesions frequently manifest as meningitis, choroid plexitis, ependymitis, and periventriculitis, potentially leading to obstructive hydrocephalus or hydromyelia. Clinically, neurological FIP can present with seizures, compulsive behaviors, cognitive impairment, and urinary or fecal incontinence [1-3].

The CNS is particularly vulnerable to oxidative stress due to its high metabolic rate, lipid-rich composition, and limited antioxidant defenses. Reactive oxygen species (ROS), generated as byproducts of cellular metabolism and viral infections, contribute to neuronal injury and neurodegeneration. While enzymatic and non-enzymatic antioxidant systems protect against ROS-induced damage, these defenses may be insufficient to prevent oxidative injury in pathological conditions. Superoxide dismutase (SOD), a key endogenous antioxidant enzyme, plays a

critical role in mitigating oxidative stress by catalyzing the dismutation of superoxide radicals. Conversely, oxidative DNA damage is frequently assessed using 8-hydroxy-2'-deoxyguanosine (8-OHdG), a well-established biomarker of oxidative stress and DNA repair processes [4,5].

Oxidative stress and antioxidant imbalance have been extensively investigated in human and experimental CNS diseases, where they are known to contribute to neuronal damage and have been explored as potential therapeutic targets [4,5]. In feline medicine, however, the involvement of oxidative mechanisms in neurological conditions such as FIP has not been fully elucidated. In this study, CSF concentrations of 8-OHdG, a marker of oxidative DNA damage, and SOD, a key antioxidant enzyme, were evaluated in cats with neurological FIP. By assessing oxidative stress and antioxidant responses in this context, the study aims to contribute to the current understanding of the disease's pathophysiology and explore the potential relevance of these markers in clinical evaluation and future research.

Material and Methods

Ethical Statement

This study was carried out after the animal experiment was approved by Ankara University Local Ethics Committee of Animal Experiments (Decision number: 2024-19-156; Date: 11/12/2024). Written informed consent was obtained from the owners of each animal prior to their inclusion in the study.

Study Population and Sample Processing

This study included 12 cats diagnosed with the neurological form of FIP and 10 age-matched control cats without any CNS pathology. All cats included in the study were presented to the Ankara University Veterinary Faculty Hospital from October 2021 to October 2023 and euthanized because of poor prognosis. Euthanasia recommendations were independently made by two veterinarians who were not involved in the study, and informed consent was obtained from the owners. Cats showing neurological signs suggestive of FIP and a poor prognosis were included in the FIP group. Control cats had similarly poor prognoses but no neurological signs.

A complete clinical history was recorded for all cats, followed by thorough physical and neurological examinations. Cerebrospinal fluid samples were collected from the cerebellomedullary cistern, and full necropsies with histopathological examination were performed after euthanasia.

Blood samples were collected from all cats prior to euthanasia. After clotting, serum was separated by centrifugation and used for biochemical analysis. The analyses were performed immediately after sample collection. Serum concentrations of total protein, albumin, and glucose were measured using an automated biochemistry analyzer (Randox RX Monaco, Randox Laboratories) at the Diagnostic Laboratory of the Faculty of Veterinary Medicine, Ankara University. Standard colorimetric and enzymatic assay methods were used, following the manufacturer's protocols. For CSF collection, cats were anesthetized via intramuscular administration of medetomidine HCl (Domitor® 1 mg/mL; Orion Pharma, Espoo, Finland) and ketamine HCl (Ketasol® 100 mg/mL; Interhas, Ankara, Türkiye) to ensure adequate immobilization. The dorsal cervical region was shaved and aseptically prepared for cerebellomedullary cisternal puncture. CSF was collected using a 19G spinal needle, and a minimum of 2 mL was transferred into sterile Eppendorf tubes. Each CSF sample was divided into three aliquots. One of the two aliquots was immediately frozen at –80°C for subsequent biomarker analysis, while the second was stored as a backup sample under the same conditions. The third aliquot was kept under cold chain conditions (4°C) and promptly delivered to the laboratory for the measurement of total protein, glucose concentration, and real-time RT-PCR analysis. Euthanasia was performed using T61® (MSD; containing 200 mg embutramide, 50 mg mebezonium iodide, and 5 mg tetracaine hydrochloride per mL).

CSF total protein concentration was measured using the biuret method, based on the reaction of copper ions with peptide bonds in the protein molecule, forming a complex that produces a violet color [6]. Glucose concentration was measured using a UV test method based on the enzymatic trinder's method. Glucose in the sample is oxidised to yield gluconic acid and hydrogen peroxide in the presence of Glucose oxidase. The enzyme peroxidase catalyses the oxidative coupling of 4-aminoantipyrine with phenol to yield a coloured quinonemine complex, with absorbance proportional to the concentration of glucose in sample [7].

All cats were screened for feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) using a commercial antibody test kit (GenBody FeLV-FIV®, GenBody Inc., South Korea). In addition, Toxoplasma gondii serology was performed using a semi-quantitative immunoassay (ImmunoComb® Toxoplasma, Biogal, Israel).

Inclusion in the FIP group required the presence of neurological signs, a low serum albumin-to-globulin (A/G) ratio (<0.6), detection of FCoV RNA in the CSF via RT-PCR, and histopathological confirmation of FIP. Control cats showed no evidence of CNS involvement. Cats from the control group were excluded if CNS lesions were identified at necropsy. In both groups, cats that tested positive for feline leukemia virus, feline immunodeficiency virus, or *Toxoplasma gondii*, or those showing signs of

multisystemic disease, were excluded from the study. RT-PCR analysis confirmed the absence of FCoV RNA in the CSF of control cats. None of the cats received antioxidants, nonsteroidal anti-inflammatory drugs, or corticosteroids following the onset of clinical signs.

Histopathological Assessment

Following euthanasia, necropsies were performed on all cats in both the FIP and control groups. Brain and spinal cord tissues were carefully taken and fixed in 10% neutral-buffered formalin for at least 48 h. After fixation, samples were routinely processed, embedded in paraffin, and sectioned at 4 µm thickness. Histological sections were stained with hematoxylin and eosin (HE), evaluated under light microscopy (Leica DM 4000) and photographed (Leica DFC-280). Central nervous system tissues were examined for histopathological lesions consistent with FIP, including perivascular inflammation, pyogranulomatous areas, and meningoencephalitis/meningoencephalomyelitis. Tissues from the control group were also examined to confirm the absence of CNS pathology.

RNA Extraction

Viral RNA was extracted from the cell-free fraction of CSF samples using the QIAamp Viral RNA Mini Kit® (Qiagen, Hilden, Germany), following the manufacturer's recommended protocol. In brief, 140 µL of each CSF sample was mixed with lysis buffer under highly denaturing conditions to ensure the complete inactivation of RNases. The lysed mixture was then applied to silica spin columns, where viral RNA selectively bound to the membrane. After sequential washes to remove impurities, the RNA was eluted in 60 µL of RNase-free elution buffer and stored at −80°C until further analysis.

Real-Time RT-PCR

The presence of FCoV RNA in CSF was assessed using a one-step real-time reverse transcription PCR (RT-PCR) [8]. Reactions were set up using the QuantiTect Probe RT-PCR Kit® (Qiagen, Germany) in a final volume of 25 µL. Each reaction included 5 µL of extracted RNA, 12.5 µL of 2X Master Mix, 0.25 µL of reverse transcriptase mix, 2 µL of a primer–probe mix specific for the FCoV 3′ untranslated region (UTR), and 5.25 µL of RNase-free water.

Primers were used at a final concentration of 0.8 µM, and a TaqMan hydrolysis probe labeled with 5′-FAM and 3′-BHQ-1 was used at 0.3 µM. The thermal cycling protocol consisted of reverse transcription at 50°C for 30 min, followed by enzyme inactivation and initial denaturation at 95°C for 15 min. This was followed by 42 amplification cycles of denaturation at 95°C for 30 sec and combined annealing and extension at 60°C for 60 sec. Amplification and real-time fluorescence detection were performed using a Stratagene Mx3005P® (Thermo Scientific, USA) instrument. Samples were considered FCoV-positive when a specific amplification curve crossed the threshold level before cycle 42.

8-Hydroxy-2'-deoxyguanosine (8-OHdG) Analysis

This test was done according to the steps of the ELISA kit instructions. The kit principle uses the competitive ELISA method (Elabscience®, cat no: E-EL-0028). The intra and inter assay CV of the kit was <8% and <10%. The sensitivity of the test was 0.94 ng/mL and the detection range was between 1.56 and 100 ng/mL. The micro-ELISA plate provided in this kit has been precoated with 8-OHdG. During the reaction, 8-OHdG in the sample or standard competes with a fixed amount of 8-OHdG on the solid phase supporter for sites on the Biotinylated Detection Ab specific to 8-OHdG. Excess conjugate and unbound sample or standard are washed from the plate and Avidin conjugated to Horseradish Peroxidase (HRP) are added to each well and incubated. Then A TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of stop solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of 8-OHdG in the samples is then determined by comparing the OD of the samples to the standard curve.

Superoxide Dismutase (SOD) Analysis

Analysis was carried out according to the method developed by Sun et al.[9]. For superoxide dismutase activity, it involves the inhibition of nitro blue tetrazolium reduction by xanthine - xanthine oxidase used as a superoxide generator. The OD values were read at 560 nm and first the % of the inhibition was calculated with the formula given; % inhibition = (Blank OD. – Test OD)/Blank OD) x 100. A Unit of SOD has an inhibition activity for 50% so this calculation can give the Unit of SOD in 1 mL. By dividing Unit to protein, the result is given as U/mg protein.

Statistical Analysis

SPSS 21.00 packet programme was used for the statistical analysis. According to the Normality contribution (Shapiro wilk), independent samples T test (Me±SD) or Mann Whitney U test (Median (Q1-Q3)) was chosen and $P<0.05$ were accepted as important. The results were given as Me±SD and Median (Q1-Q3) in *Table 1*. The statistical test according to the normality contribution was Mann Whitney U test for CSF glucose/serum glucose, 8-OHdG and SOD measurements, while independent samples t-test was for age, CSF total protein and serum Alb/ serum glb measurements. In the result section Median (Q1-Q3) concentrations were used for 8-OHdG because of the large SD value.

Results

FCoV RNA was detected in all 12 CSF samples from FIP cats, while all 10 samples from the control group tested negative.

Of the 12 cats in the study group, 5 (41.7%) were female, and 7 (58.3%) were male. In the control group, 4 out of 10 cats (40%) were female, and 6 (60%) were male. There was no statistically significant difference in age between the study and control groups (P>0.05) *(Table 1)*. In the control group, four cats were euthanized due to atrial thromboembolism, while six were euthanized as a result of pneumothorax and pulmonary contusion secondary to high-rise syndrome.

Neurological examination findings of 12 cats diagnosed with neurological FIP included anisocoria, blindness, nystagmus, fascial paralysis, decreased gag reflex, paresis, paralysis, cross-extension reflex, ataxia, decerebellar rigidity, hyperesthesia, decreased to absent menace response, proprioceptive deficits, urinary incontinence, fecal incontinence *(Table 2)*.

Cats with FIP had higher CSF Total Protein (740±230 mg/dL) compared to control group (17±7 mg/dL). CSF glucose/serum glucose (mg/dL divided by mg/dL) ratio was significantly lower in cats with neurological FIP compared to the control group (0.39±0.18, 0.66±0.06, respectively). The median (Q1-Q3) concentration of 8-OHdG in CSF samples of cats with FIP (6.88 [2.13-48.20] ng/mL) was significantly higher than in control subjects (1.09 [0.33-3.56] ng/ml; P<0.05). Cerebrospinal fluid levels of SOD were significantly decreased in cats with FIP (0.034±0.026 U/mg) compared to controls (0.312±0.136, P<0.001) *(Table 2)*.

Histopathological examination of the brains of cats with FIP revealed hyperemia in vessels, perivascular mononuclear cell infiltration, edema, and necrosis in meninges *(Fig. 1-a)* and choroid plexus *(Fig. 1-b)*.

Internal hydrocephalus occurred in some cats. Gliosis and demyelination were noted, especially in the substantia alba.

Table 1. *Comparison of age, CSF biochemical parameters, oxidative stress markers, and serum albumin/globulin ratio between cats with feline infectious peritonitis (FIP) and healthy controls*

Parameters	Groups				P
	Cats with FIP		Control		
	Mean ± SD	Median (Q1-Q3)	Mean ± SD	Median (Q1-Q3)	
Age (Year)	2.35±0.57	2.25 (2.00-3.00)	2.24±0.48	2.45 (1.75-2.60)	>0.05
CSF Total Protein (mg/dL)	740±230	780 (510-920)	17±7	14 (11-22)	<0.001
CSF Glu/Serum Glu (mg/dL divided by mg/dL)	0.39±0.18	0.41 (0.25-0.45)	0.66±0.06	0.65 (0.60-0.72)	<0.001
8-OHdG (ng/mL)	24.38±34.32	6.88 (2.13-48.20)	5.73±13.26	1.09 (0.33-3.56)	<0.05
SOD (U/mg protein)	0.034±0.026	0.027 (0.017-0.041)	0.312±0.136	0.285 (0.238-0.350)	<0.001
Serum ALB/GLB	0.35±0.15	0.30 (0.23-0.50)	0.87±0.06	0.86 (0.82-0.90)	<0.001

P-values <0.05 were considered statistically significant

Table 2. *Neurologic examination findings of cats with FIP*

Neurologic Examination Findings	Number of Cats with Clinical Signs	Percentage of Cats with Clinical Signs
Anisocoria	4	33.3%
Blindness	2	16.6%
Nystagmus	5	25.0%
Fascial paralysis	2	16.6%
Decreased gag reflex	2	16.6%
Paresis	5	41.6%
Paralysis	1	8.3%
Cross-extension reflex	10	83.3%
Ataxia	5	41.6%
Decerebellar rigidity	3	25.0%
Hyperesthesia	5	25.0%
Decreased to absent menace response	4	33.3%
Proprioceptive deficits	10	83.3%
Urinary incontinence	8	66.6%
Fecal incontinence	8	66.6%

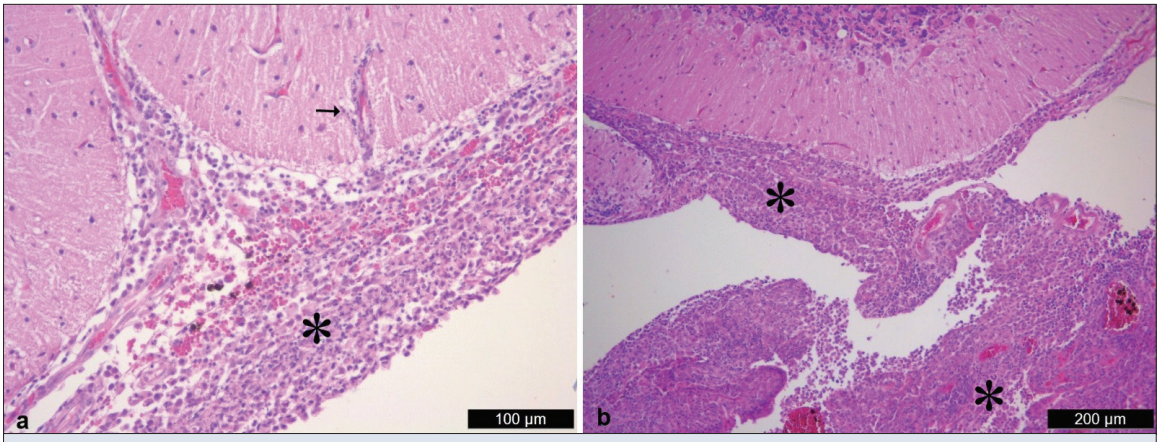

Fig 1. (a) Meningitis *(asterisk)* composed of mononuclear cells and perivascular cell infiltration *(black arrow)*, cerebellum, HE, **(b)** Inflammation characterized by mononuclear cells *(asterisk)*, choroid plexus, HE

Discussion

In this study, CSF total protein concentration was significantly elevated, whereas CSF glucose concentration were markedly reduced in cats with neurological FIP compared to the control group. The inflammatory response, including cytokine and nitric oxide metabolite production, likely disrupt mitochondrial function, increasing glucose consumption through anaerobic glycolysis and resulting in reduced CSF glucose levels and impaired brain function [10,11]. Cerebrospinal fluid proteins are derived from serum and local intra-cranial synthesis. Disruption of the blood-brain barrier increases permeability, allowing increased levels of serum proteins to access the CSF [12]. Inflammation, viral replication, and altered vascular permeability associated with neurological feline infectious peritonitis (FIP) contribute to elevated CSF protein and albumin levels [13-17]. These findings are consistent with previous reports, including those by Chrisman [13], Deisenhammer et al.[14], DiTerlizzi and Platt [15], Regeniter et al.[16] and at least Singh et al.[17] suggesting that severe inflammation and immune-mediated damage in neurological FIP contribute to elevated CSF protein levels and reduced glucose concentrations.

8-Hydroxy-2'-deoxyguanosine is recognized as the most prevalent oxidatively damaged product resulting from DNA exposure to free radicals, and it serves as a reliable biomarker for assessing the extent of oxidative DNA damage [18-20]. In the present study, histopathological examination of the CNS of cats with FIP revealed inflammatory lesions consistent with previous descriptions of neurological FIP [21-24]. These inflammatory changes, including perivascular infiltration of macrophages, lymphocytes, and neutrophils, are known to contribute to oxidative stress within the CNS microenvironment [25]. The inflammation-associated oxidative burden observed in our study is in agreement with the findings of Tecles et al.[26], who demonstrated the presence of oxidative stress in the serum of cats with FIP. In our study, significantly increased levels of 8-OHdG were detected in the cerebrospinal fluid of cats with neurological FIP. This observation may suggest the involvement of localized oxidative DNA damage within the central nervous system. These combined findings support the role of inflammation-induced oxidative damage as a contributing factor in the neuropathogenesis of feline infectious peritonitis. Oxidative stress of DNA contributes to early neuronal damage [27]. Increased levels of oxidative stress have been reported in the CSF of patients with various neurodegenerative disorders including Alzheimer's disease [28], Parkinson's disease [29], Amyotrophic Lateral Sclerosis (ALS) [30] and Multiple Sclerosis (MS) [31], as well as infectious diseases such as bacterial and aseptic meningitis [32], encephalitis associated with Influenza A [33] and Herpes Simplex Virus [34]. In the present study, we demonstrated that CSF-8-OHdG levels in cats with neurologic FIP were significantly higher than in controls, suggesting that DNA oxidative stress is induced by neurological FIP infection.

Recent studies have identified oxidative damage as a fundamental mechanism underlying central nervous system injury induced by viral infections such as herpes simplex virus type 1 in murine [35,36], human herpesvirus-6 (HHV-6) [37]. Oxidative injury is a significant component of acute encephalitis induced by herpes HSV-1 [38]. In neurological FIP, several cytokines are consistently elevated, indicating a strong inflammatory response, which contributes to oxidative stress within the CNS [23]. The increase in ROS may occur due to a mitochondrial dysfunction caused by penetration of the virus into the cell or by signaling exacerbated by the "cytokine storm" with release of IL-2, IL-6, IL-7, and TNF-α [39]. Similarly, immune cells such as macrophages and neutrophils play a potential pathological role by producing and secreting high pro-inflammatory cytokines and ROS levels [40]. Cells utilize antioxidant defense mechanisms, including

the activation of enzymes such as SOD to counteract ROS-induced damage. Notably, increased activities of both SOD and catalase in CSF have been observed in patients with chronic neurodegenerative diseases [41,42], bacterial meningitis [32] and malaria [43]. Under normal physiological conditions, a dynamic equilibrium is maintained between ROS production and the antioxidant enzyme system. When the balance between ROS production and antioxidant defense is disrupted, excessive ROS can induce oxidative modifications in lipids, proteins, and polysaccharides, ultimately resulting in DNA and RNA damage [44,45]. In the present study, CSF 8-OHdG levels were significantly elevated in cats with FIP compared to controls, whereas CSF SOD concentrations were markedly reduced. The regulation of ROS activity is maintained by a complex antioxidant system that modulates intracellular ROS levels. However, under prolonged oxidative stress, ROS concentrations surpass the scavenging capacity of the antioxidant defense system, leading to extensive cellular damage and necrosis [46]. The decreased CSF-SOD activity observed in FCoV-infected cats may reflect depletion resulting from excessive oxidative burden within the CSF. Specifically, the heightened oxidative stress and inflammation associated with FIP may have resulted in the substantial consumption of SOD as it attempted to counteract ROS-mediated damage in CNS. Alternatively, the observed reduction in SOD levels may not only be due to enzymatic depletion caused by excessive oxidative stress but also reflect coronavirus-mediated immuno-suppression or direct viral interference with antioxidant gene expression. Previous studies have shown that several viruses, including coronaviruses can suppress antioxidant defense by downregulating SOD transcription or disrupting the nuclear factor erythroid 2-related factor 2 pathway, a key regulator of cellular antioxidant responses [47-49].

Elevated levels of free radicals, in conjunction with diminished antioxidant defenses, have been implicated in the pathogenesis of severe neurological and systemic manifestations in COVID-19 patients, largely through mitochondrial dysfunction and cytokine-mediated oxidative injury [50]. By analogy, the severe neurological signs observed in the cats in our study may reflect a similar imbalance, wherein excessive ROS production overwhelms depleted antioxidant systems, including SOD. This oxidative dysregulation could potentially exacerbate neuronal damage and clinical deterioration in feline infectious peritonitis. Further studies involving larger populations are warranted to elucidate this relationship and validate the role of oxidative stress in the neuropathogenesis of FIP.

Oxidative stress and damage to cellular components may be mitigated through antioxidant therapy. Various antioxidants and their supplements have been shown to be effective against different neurological diseases [51]. Future studies in cats with FIP could assess the potential benefits of antioxidant therapy in disease management.

In our study, cerebrospinal fluid (CSF) total protein concentrations in cats with neurological FIP were found to be elevated compared to established reference intervals. This finding is consistent with the results of Crawford et al.[52], who reported markedly increased CSF total protein levels in all 11 affected cats, with a mean of 940 mg/dL. These data support the presence of a strong inflammatory response within the central nervous system in feline neurological FIP. Similarly, Rand et al.[53] reported a mean CSF total protein concentration of 368 mg/dL, which is still markedly elevated when compared to healthy cats. On the other hand, Steinberg et al.[54] reported substantially lower protein concentrations, with a mean of only 18 mg/dL. These variations among studies may be attributed to differences in disease stage at the time of sampling, the degree or distribution of CNS lesions (focal vs. diffuse) [55], or the site of CSF collection [56]. It is also known that lumbar CSF samples tend to have higher protein concentrations and lower white blood cell counts compared to cerebellomedullary cistern samples [56]. Therefore, both biological and methodological factors may account for the discrepancies observed across different studies.

This study has several limitations. First, CSF 8-OHdG and SOD levels were measured at only a single time point during the disease. Conducting a longitudinal study that assesses these biomarkers at multiple stages following FIP infection would be essential to better understand their temporal dynamics and evaluate their prognostic value in later stages of the disease. Furthermore, whether this oxidative perturbation in DNA metabolism plays a role in initiating or sustaining neuronal cell death in neurological FIP remains to be determined. If so, pharmacological interventions targeting DNA modifications could potentially offer a novel therapeutic approach for affected cats.

Another limitation of this study is that it focused solely on cerebrospinal fluid (CSF) concentrations of 8-OHdG. However, numerous previous studies -both in human and veterinary medicine- have evaluated 8-OHdG levels in serum or plasma across a variety of diseases [57-61]. These studies have demonstrated that elevated circulating 8-OHdG levels are associated with oxidative stress-related pathologies, such as neurodegenerative diseases [60], cancers [59], and chronic inflammatory conditions [57,62]. Particularly in clinical settings where CSF collection is not feasible, measuring serum or plasma 8-OHdG levels could offer a less invasive and more accessible diagnostic alternative.

Additionally, a notable limitation of this study is the relatively small sample size. However, enrolling cats with confirmed neurological FIP that were also negative for both FeLV and FIV proved particularly difficult. Obtaining informed consent for necropsy procedures was also challenging. Further constraints included the narrow postmortem time window required for cerebrospinal fluid collection, rapid clinical deterioration in neurological cases, and logistical limitations encountered during sample collection. Despite these difficulties, strict diagnostic criteria and careful case selection were employed to ensure the scientific reliability of the results. Although limited in number, the data provide valuable preliminary insights into oxidative stress biomarkers in feline neurological FIP. In feline infectious peritonitis, significantly elevated CSF levels of 8-OHdG, coupled with markedly reduced concentrations of SOD, indicate an increased state of oxidative damage in DNA and a compromised antioxidant defense mechanism. This study highlights the role of oxidative stress and inflammatory processes in the pathophysiology of neurological FIP, wherein augmented oxidative damage and metabolic disturbances may contribute to neuronal injury.

In conclusion, this study demonstrated significantly increased CSF levels of 8-OHdG and decreased concentrations of superoxide dismutase (SOD) in cats with neurological FIP. These findings suggest that oxidative stress and impaired antioxidant defense mechanisms may be involved in the neuropathogenesis of the disease. Within the scope of this study, the measurement of 8-OHdG and SOD in CSF contributes to the understanding of the oxidative processes associated with neurological FIP. Further research is warranted to better define the relevance of these biomarkers in the clinical evaluation of affected cats.

Declarations

Availability of Data and Materials: The data supporting this study's findings are available from the corresponding author (İ. Baştan) upon reasonable request.

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RESEARCH ARTICLE

Neuroprotective Role of Dexpanthenol and Butafosfan-Vitamin B_{12} Against Brain Damage Induced by Circadian Rhythm Disorder

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Abstract

This study investigates the neuroprotective effects of dexpanthenol (DEX) and butafosfan-vitmain B_{12} combination (BUT) in a circadian rhythm disorder (CRD)-induced brain damage model in mice. Control, CRD, DEX, BUT, and BUT + DEX groups were subjected to a 19-day experimental period during which CRD was induced by repeated phase shifts in the light/dark cycle. The CRD group experienced circadian rhythm disorder, while the DEX and BUT groups received intraperitoneal DEX (1000 mg/kg/day) and subcutaneous BUT (200 mg/kg/day) treatments, respectively, for the same duration. Serum cortisol and *creatine kinase* (CK) levels were measured using ELISA to assess stress and tissue damage. Brain tissues were evaluated histopathologically using hematoxylin and eosin staining, and immunohistochemically for brain-derived neurotrophic factor (BDNF) and glial fibrillary acidic protein (GFAP) expression using specific monoclonal antibodies. CRD significantly increased serum cortisol and CK levels compared to the control group ($P<0.001$). Both DEX and BUT treatments reduced these elevations, with the combination therapy showing the most pronounced effect ($P<0.001$). Histopathological examination revealed reduced neuronal degeneration, hyperemia, and hemorrhage in the treatment groups compared to the CRD group. Immunohistochemical analysis showed significantly increased BDNF and GFAP expression in the BUT + DEX group ($P<0.001$). These findings suggest that DEX and BUT, particularly in combination, exert neuroprotective effects against CRD-induced brain injury by modulating oxidative stress, inflammation, and neurotrophic signaling pathways.

Keywords: Butafosfan, Circadian rhythm, Dexpanthenol, Neuroprotective, Vitamin B_{12}

Introduction

The circadian system governs the timing of physiological processes and behaviors across a 24-h cycle, ensuring adaptation to environmental changes and internal coordination within the body. Central to this system is the suprachiasmatic nucleus (SCN) in the hypothalamus, which acts as the master pacemaker, orchestrating the timing of various biological rhythms. However, the circadian rhythm extends beyond the SCN, encompassing peripheral clocks present in diverse tissues, each contributing to the regulation of specific metabolic functions. Understanding the complex interplay between central and peripheral clocks is critical for clarifying the circadian system's role in metabolic health and disease [1]. The central clock in the SCN serves as the primary regulator of circadian rhythms, entraining the body to the external light-dark cycle. Through neural and hormonal signaling pathways, the SCN coordinates physiological processes such as sleep-wake cycles, hormone secretion, and metabolism. Notably, disruptions in SCN function, induced by factors like shift work or jet lag, can lead to desynchronization of internal clocks, contributing to metabolic disturbances and increased susceptibility to chronic diseases [2]. In addition to the central clock, peripheral clocks distributed throughout peripheral tissues play a crucial role in maintaining metabolic homeostasis. These clocks exhibit intrinsic rhythmicity and respond to both systemic signals from the SCN and local cues such as nutrient availability and physical activity. Peripheral clocks regulate tissue-specific metabolic processes, including glucose and lipid metabolism, insulin sensitivity, and energy expenditure. Given the detrimental impact of circadian rhythm disorder (CRD) on neuronal integrity and inflammatory balance, identifying agents that can

counteract oxidative stress and promote cellular resilience is of paramount importance [3].

Dexpanthenol (DEX), also known as D-pantothenol, is an alcoholic analog of pantothenic acid (PA), a precursor of coenzyme A (CoA) and an essential component of cellular energy metabolism. DEX and its derivatives have been extensively studied for their ability to enhance intracellular ATP synthesis and increase concentrations of reduced glutathione (GSH), thereby exerting antioxidant and cytoprotective effects. Moreover, DEX has been shown to play a crucial role in cellular defense and repair mechanisms, making it a promising candidate for the treatment of various oxidative stress-related disorders. Animal studies have demonstrated the potent antioxidant properties of DEX in conditions such as lung fibrosis, necrotizing enterocolitis, ischemia-reperfusion injury, and testicular damage [4-7]. Additionally, DEX exhibits anti-inflammatory effects and contributes to tissue repair processes [8]. Furthermore, emerging evidence from human studies suggests that DEX plays a significant role in brain function and health, with brain levels reported to be significantly higher than plasma levels [9,10]. DEX is implicated in the synthesis of multiple neurotransmitters, underscoring its importance in neuronal communication and synaptic transmission [11]. Studies have shown that DEX therapy can attenuate oxidative stress and enhance the levels of amino acid neurotransmitters associated with brain damage, potentially mitigating the neurological consequences of oxidative insults [12].

In addition to redox-based neuroprotection, metabolic regulators like butafosfan may offer complementary benefits by modulating cellular energy metabolism and stress-induced immune responses. Butafosfan, a phosphonic acid derivative, has attracted scientific interest due to its proposed immunomodulatory properties and potential roles in promoting systemic physiological resilience. Although primarily used in veterinary medicine, particularly in combination with vitamin B_{12}, recent studies have explored its broader therapeutic potential beyond its role as an energy enhancer. Understanding the immunomodulatory mechanisms of butafosfan is crucial for elucidating its therapeutic effects and exploring its potential applications in human health [13,14]. The immunomodulatory effects of butafosfan have not been extensively characterized; however, available studies indicate its potential to enhance immune function. In an experimental study on pregnant ewes, subcutaneous administration of butafosfan and cyanocobalamin significantly improved metabolic markers, reduced oxidative stress indices, and effectively reduced the incidence of subclinical pregnancy toxemia [15]. However, the precise cellular mechanisms underlying its immunomodulatory effects remain unclear and warrant further investigation [16]. Moreover, research in various animal species, including cattle, horses, pigs, chickens, and mice, has demonstrated the beneficial effects of butafosfan and vitamin B_{12} combination (BUT) supplementation on overall health. These effects include increased feed intake, improved immune function, enhanced liver and muscle function, and improved hemostasis. Furthermore, BUT supplementation has been shown to support erythropoiesis and alleviate stress responses in animals, further highlighting its potential health benefits [17].

The circadian system regulates various physiological processes, including sleep-wake cycles, hormone secretion, and metabolism. Disruptions in circadian rhythms can have profound effects on health, including cognitive impairment and metabolic disorders. Dexpanthenol and BUT have been implicated in cellular metabolism and immune function, but their effects on the circadian system and brain remain poorly understood.

This study investigates the neuroprotective potential of the DEX and BUT combination against CRD induced brain injury by modulating stress-related biochemical changes and neurotrophic responses.

Material and Methods

Ethical Statement

This study was approved by the Animal Experiments Local Ethics Committee of Burdur Mehmet Akif Ersoy University (Approval No. 14.02.2025/1273).

Animals

A total of 35 male C57BL/6 mice, aged 10-12 weeks and weighing approximately 20-30 g, were obtained from the Burdur Mehmet Akif Ersoy University Laboratory Animal Production and Experimental Research Center. The mice were housed in a facility with maintained at room temperature 21-23°C, and humidity levels ranging from 55% to 65%. *Ad libitum* access to water and food was provided to the animals.

Circadian Rhythm Disorder Model

Baseline data collection started 1 week after the mice were transferred to plastic cages. The experimental protocol began with a 1-week baseline period with lights on at 09.00-21.00, 13 weeks of 12-h phase shifts in the light/dark cycle two times per week, and 10 days of recovery period with lights on at 09.00-2100. Light/dark cycle shifts always involved a 24-h period of lights on *(Fig. 1)*. The control mice were maintained on the same lighting schedule with lights on at 09.00-21.00 throughout the experimental period except that lights-on periods were extended to 24 h every Monday during the light/dark cycle shift period to maintain an equal amount of exposure to light

for both the control mice and the experimental mice.

Chemicals

DEX was obtained from dexpanthenol (Bepanthen®; 500 mg/2 mL, Bayer Corp., Istanbul, Türkiye), and the BUT was obtained from butafosfan and vitamin B_{12} (Catosal® 10%; 100 mg butafosfan/0.05 mg vitamin B_{12}, Bayer Animal Health GmbH, Leverkusen, Germany).

Experimental Design

The study was conducted using a total of 35 C57BL/6 mice, which were divided into five groups (n = 7). Prior to the experiment, all animals were housed under standard laboratory conditions with a 12:12-h light-dark cycle for a 10-day adaptation period [18]. To induce CRD, animals in the CRD, DEX, BUT, and BUT + DEX groups were housed for 19 consecutive days in specialized chambers with programmable light-dark cycles [19]. All animals in these groups were provided standard diet and water *ad libitum*. During this period, the DEX group received intraperitoneal dexpanthenol (1000 mg/kg/day), the BUT group received subcutaneous butafosfan + vitamin B_{12} (200 mg/kg/day), and the BUT + DEX group received both treatments simultaneously at the same dosages [16,19,20]. The control group remained under a normal 12:12-h light-dark cycle throughout the study and did not receive any pharmacological treatment. The CRD induction protocol is illustrated in *Fig. 1*, and the overall experimental design is summarized in *Fig. 2*.

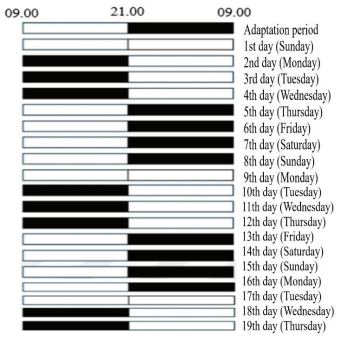

Fig 1. The program applied to create circadian rhythm disorder

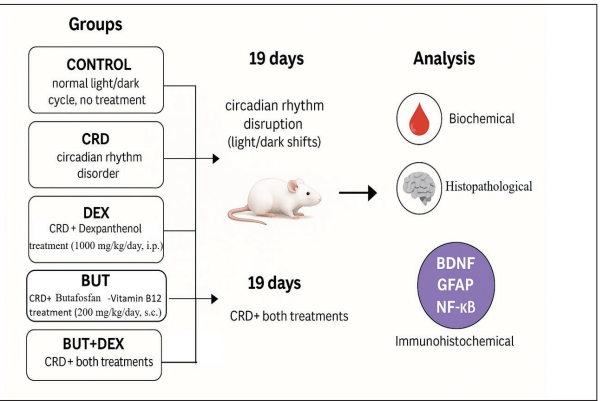

Fig 2. Schematic illustration of the experimental design

On day 20, all animals were anesthetized with ketamine HCl (90 mg/kg, i.p.) and xylazine HCl (10 mg/kg, i.p.), and cardiac blood samples were collected. Euthanasia was performed via cervical dislocation under deep anesthesia. Brain tissues were harvested and divided into two portions: one was homogenized for the biochemical analysis of cortisol and creatine kinase (CK) levels, while the other was fixed in 10% buffered formalin for histopathological and immunohistochemical examinations.

Biochemical Analyses

The blood samples were taken directly into tubes without anticoagulant and centrifuged at 4000 rpm for 10 min to separate the serum. The tissues were initially washed with 0.9% saline solution. Subsequently, the tissues were homogenized with 0.01 M pH 7.4 phosphate buffer solution (1 g tissue/9 mL PBS) as specified in the kits. The homogenate was then centrifuged at 15.000 rpm for 45 min at 4°C to obtain supernatants.

Determination of Creatine Kinase Levels

CK levels of serum and tissue homogenate samples were determined according to the procedure described in the commercial ELISA kit (BT-LAB E0609Mo) and measured at 450 nm with ELx800-Biotek. Total protein concentrations of brain homogenates were measured according to the Biuret method [21]. The results were expressed in mIU/mL tissue based on the prepared standard curve.

Determination of Cortizol Levels

Serum cortisol levels were determined using a commercial ELISA kit (BT-LAB E0609Mo) and measured at 450 nm with an ELx800-Biotek instrument. Cortisol levels are expressed as ng/ml.

Histopathological Examination

At the conclusion of the experimental phase, all groups of mice were humanely sacrificed. The brains were carefully extracted from the skull to prevent any damage to the delicate brain tissue during necropsy. Following removal, all brain samples underwent a meticulous gross examination. Brain tissues were fixed in 10% buffered formalin for 48 h, followed by routine paraffin embedding using an automated tissue processor. Serial sections, each with a thickness of 5 µm, were obtained using an automated rotary microtome. These sections were then stained with routine hematoxylin and eosin (HE) staining to facilitate microscopic examination. Histopathological evaluation of the brain tissue focused on identifying key lesions, including hyperemia, hemorrhage, and degenerative changes. A semi-quantitative ordinal grading system was

employed to assess these alterations. The severity of lesions was categorized as follows: normal (score = 0), mild (score = 1), moderate (score = 2), and severe (score = 3). The stained sections were examined under a light microscope, and the histological findings were systematically recorded.

Immunohistochemical Examination

Sections mounted on poly-L-lysine-coated slides were subjected to streptavidin-biotin peroxidase immunohistochemical staining. Immunohistochemical analysis was performed on brain sections using primary antibodies targeting brain-derived neurotrophic factor (BDNF) (Anti-BDNF antibody [EPR1292], ab108319), glial fibrillary acidic protein (GFAP) (Anti-GFAP antibody, ab7260), and nuclear factor kappa B (NF-κB) (Anti-NFκB p100/p52 antibody, ab227078). All primary antibodies were obtained from Abcam and diluted at a ratio of 1:100 using antibody dilution solutions. The immunohistochemical procedure was carried out following the manufacturer's instructions. For secondary detection, the Mouse and Rabbit Specific HRP/DAB Detection Kit - Micropolymer (ab236466) from Abcam (Cambridge, UK) was used. All sections underwent identical staining procedures. Negative controls were processed by replacing the primary antibodies with antibody dilution buffer to verify staining specificity. Immunohistochemical evaluation was performed by quantifying the percentage of positively stained cells in regions associated with circadian rhythm regulation. For each animal, 100 cells were evaluated per brain region, by analyzing 20 randomly selected cells from each of five non-overlapping fields under a 40X objective lens. ImageJ 1.46r software (National Institutes of Health, Bethesda, MD) was employed to determine the number of immunopositive cells. Microscopic imaging was conducted using an Olympus CX41 microscope, and the Database Manual Cell Sens Life Science Imaging Software System (Olympus Corporation, Tokyo, Japan) was utilized for microphotography.

Statistical Analysis

The data obtained from the study were analyzed using SPSS version 22.0 for Windows (IBM Corp., Armonk, NY, USA). Results are presented as mean ± standard error (SE). Initially, the data were analyzed for normality using the Shapiro-Wilk test. It was determined that the histopathological scoring data exhibited a nonparametric distribution, while all other data exhibited a parametric distribution. Statistical analysis of biochemical parameters, antioxidant-oxidative stress markers, anti-inflammatory parameters, inflammatory parameters, transcription factor levels, and percentages of immunohistochemically positive cells was performed using One-Way ANOVA, and differences between groups were determined using the Tukey test. Values of $P<0.05$ were considered significant. Since histopathological scores did not show a normal distribution, the Kruskal-Wallis test and the Mann-Whitney U test with Bonferroni correction were used, with values of $P<0.05$ considered statistically significant.

Results

Effects of DEX and BUT on Blood and Brain Function

Both serum and brain tissue levels of CK and cortisol were significantly elevated in the CRD group compared to the control ($P<0.001$). Treatment with DEX, BUT, and their combination (BUT + DEX) led to a significant reduction in these parameters compared to the CRD group ($P<0.001$). Among the treatment groups, the BUT group showed the most pronounced decrease in serum cortisol and CK levels ($P<0.001$), while brain CK levels were lowest in the DEX group ($P<0.001$). These findings indicate tissue-specific effects of DEX and BUT on stress and damage markers *(Fig. 3)*.

Histopathological Findings

The control group exhibited normal brain histology without any pathological alterations. The CRD group exhibited a statistically significant increase in histopathological scores compared to the control group ($P<0.001$ for both) *(Fig. 4)*. On average, scores increased from approximately 0.4 in the control group to 3.1 in the CRD group, representing a **7.75**-fold increase, indicating marked neuronal degeneration, hyperemia, and mild perivascular hemorrhage. Treatment with BUT and DEX reduced histopathological scores by approximately 2.4-fold and 2.6-fold, respectively, compared to the CRD group. The combined BUT + DEX treatment provided the most prominent improvement, with an approximately 5.3-fold reduction in histopathological damage relative to the CRD group *(Fig. 5)*.

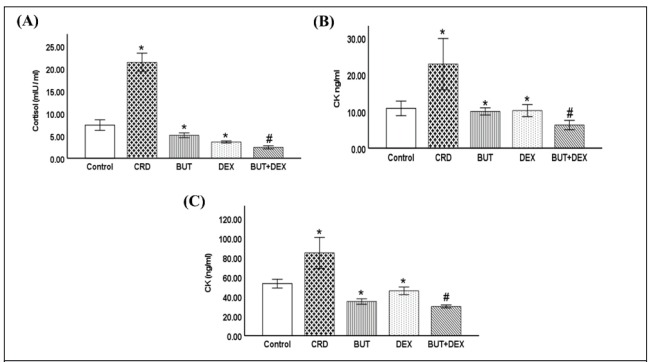

Fig 3. (A) Cortisol levels in serum, (B) creatine kinase levels in serum and (C) brain tissue control, CRD, BUT, DEX and BUT+DEX groups. All values are expressed as mean ± SEM (n = 7). *P<0.001 when compared to the control group. # P< 0.001 when compared to the CRD group

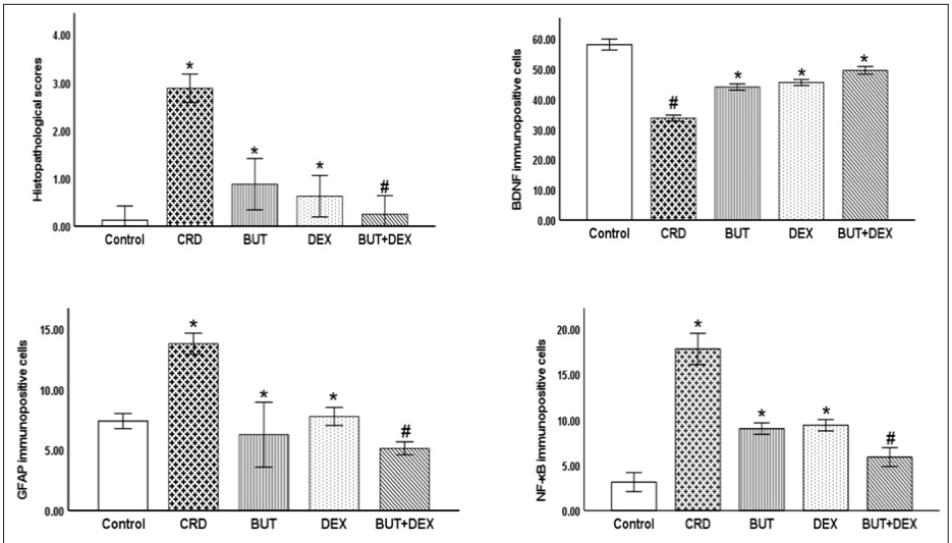

Fig 4. Histopathological scores and BDNF, GFAP, and NF-κB immunopositive cells between the control, CRD, BUT, DEX and BUT+DEX groups. All values are expressed as mean ± SEM (n = 7). *P<0.001 when compared to the control group. # P<0.001 when compared to the CRD group

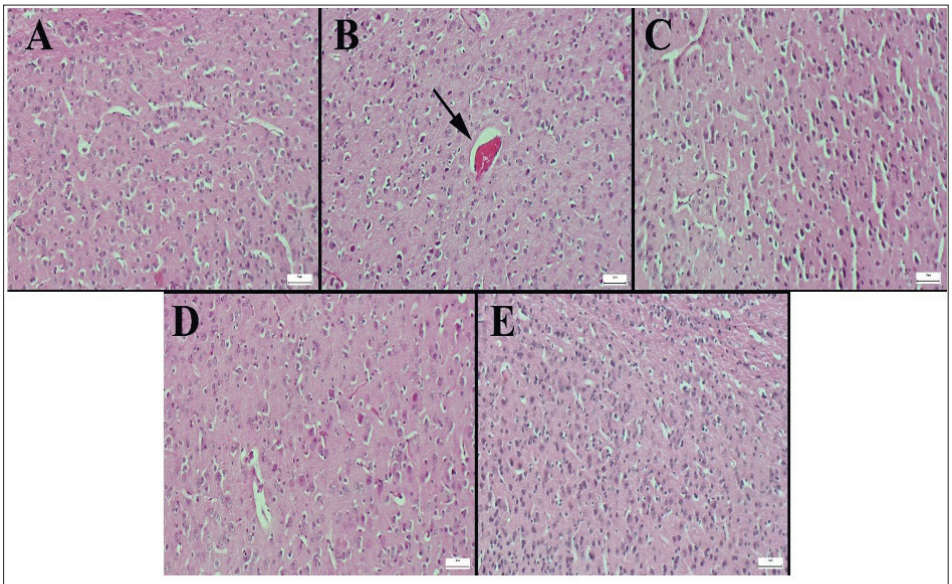

Fig 5. Histopathological comparison of brain tissues across experimental groups. (A) Normal brain tissue architecture in the control group. (B) The CRD group exhibits mild hyperemia and perivascular edema (arrow). (C) Decreased pathological alterations in the BUT-treated group. (D) Reduced pathological alterations in the DEX-treated group. (E) Markedly diminished pathological changes in the BUT+DEX-treated group. HE staining, scale bars = 50 µm

Immunohistochemical Findings

Immunohistochemical analysis revealed that the control group exhibited marked BDNF expression alongside slight to negative GFAP and NF-κB levels. In contrast, CRD group showed decreased BDNF and increased GFAP and NF-κB levels. Treatment with BUT and DEX led to a significant increase in BDNF expression while reducing GFAP and NF-κB levels in the treated groups. BDNF-positive cell counts increased by approximately 50% in the BUT + DEX group compared to CRD (P<0.001), while NF-κB and GFAP expression decreased by 65% and 55%, respectively *(Fig. 4)*. Notably, the combined therapy demonstrated greater effectiveness compared to single treatments, showing a more pronounced enhancement in BDNF and a stronger reduction in GFAP and NF-κB expression *(Fig. 6)*.

Discussion

This study investigates the impact of CRD on neuro-inflammation, glial activation, and neuroplasticity, highlighting the therapeutic potential of BUT and DEX treatments. Our results show that circadian misalignment

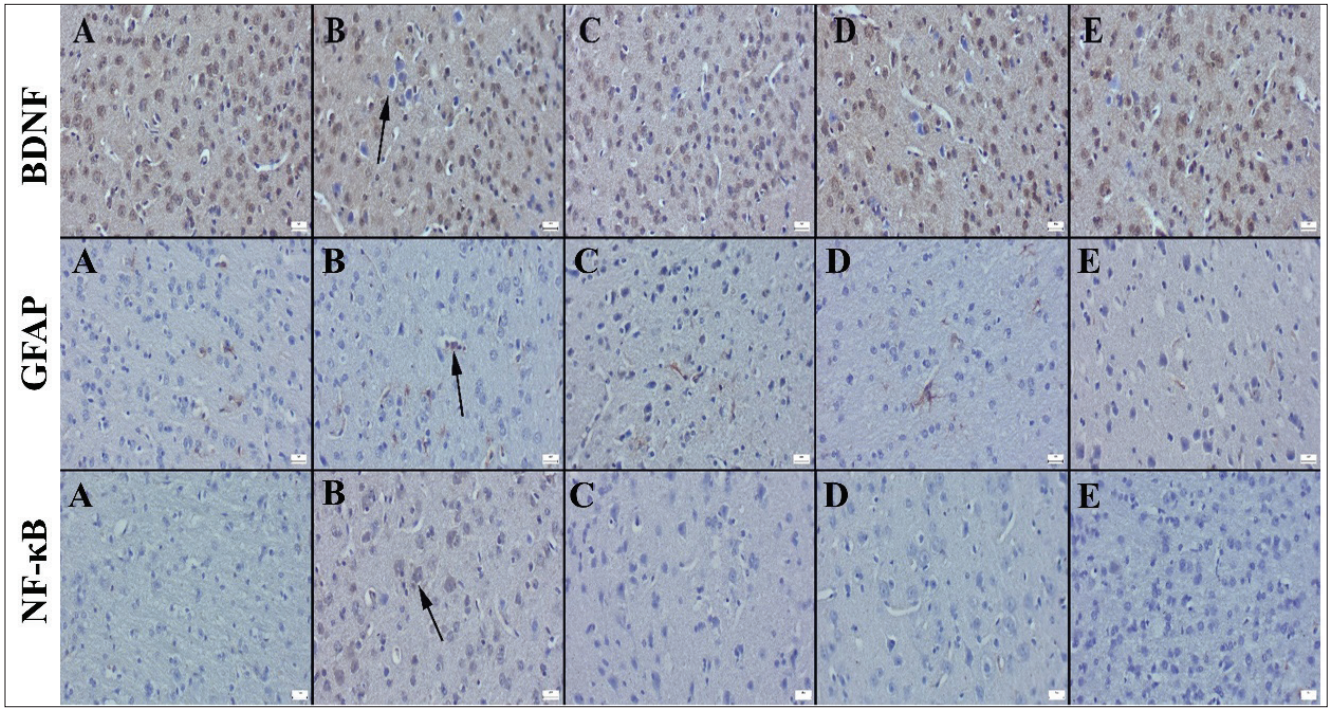

Fig 6. Immunohistochemical expression of BDNF (top row), GFAP (middle row), and NF-κB (bottom row) in brain tissues across experimental groups. (A) The control group shows strong BDNF expression with minimal to negative GFAP and NF-κB expression. (B) The CRD group exhibits decreased BDNF expression and significantly increased GFAP and NF-κB levels. (C) The BUT-treated group shows increased BDNF expression and reduced GFAP and NF-κB levels. (D) The DEX-treated group similarly demonstrates increased BDNF and decreased GFAP and NF-κB expression. (E) The BUT+DEX combination group exhibits the most pronounced increase in BDNF expression and the greatest

leads to elevated GFAP and NF-κB expression, alongside a significant reduction in BDNF levels ($P<0.001$ for all). These pathological changes were significantly attenuated by treatment, with the combination of BUT and DEX showing the most pronounced effect: BDNF levels increased by ~50%, NF-κB expression decreased by ~65%, and histopathological scores were reduced by ~81% compared to the CRD group.

Inflammatory processes play a crucial role in mediating brain injury under stress-related conditions. Agents with both antioxidant and anti-inflammatory properties offer a dual mechanism of neuroprotection. For instance, DEX and B-complex vitamins have been reported to exert beneficial effects in mitigating neuroinflammation, enhancing cellular repair mechanisms, and preserving blood-brain barrier integrity. The therapeutic efficacy of such compounds in experimental animal models further support their potential clinical applications in neurological disorders [22,23]. GFAP is a marker for astrocytic activation and gliosis, commonly associated with injury or inflammation [24]. The observed increase in GFAP expression in the CRD group is consistent with previous studies, such as those by Moriya et al.[25], which showed elevated GFAP levels following chronic CRD, indicating heightened astrocyte reactivity. Zimmermann et al.[26] further reported glial cell density reduction in the suprachiasmatic nucleus, further supporting the link between CRD and glial changes. NF-κB, a key regulator of neuroinflammation, was also upregulated in the CRD group. This finding aligns with Chen et al.[27], who demonstrated melatonin's role in modulating NF-κB activity to control neuroinflammation. Additionally, Başak et al.[28] observed oxidative stress-induced GFAP upregulation and increased NF-κB activity in pinealectomized rats, reinforcing the impact of CRD on neuroinflammatory processes. The reduction in NF-κB expression following DEX and BUT treatment underscores the potential of these therapies to counteract neuroinflammation. BDNF is crucial for neuroplasticity and cognitive function, and its significant reduction following CRD is consistent with findings by Liang et al.[29], Pang et al.[30], and Dingding et al.[31], who reported impaired BDNF signaling due to CRD or neurodegenerative conditions such as Parkinson's disease. Together, they may act to restore neurotrophic support and mitigate glial activation. This interpretation is supported by increased BDNF expression and reduced NF-κB and GFAP levels in the combination group, suggesting that targeting multiple pathological pathways enhances neuroprotection. Notably, DEX is known to enhance antioxidant enzyme activity, particularly via glutathione pathway modulation, while BUT influences energy metabolism and reduces proinflammatory cytokine release. Moreover, BUT, especially in combination with

vitamin B_{12}, may downregulate NF-κB and promote BDNF expression, providing a multifaceted approach to neuroprotection [32].

Additionally, we explored the effects of CRD on cortisol and CK levels. Disruptions in sleep patterns are known to alter cortisol rhythms [33,34], but the effects of BUT and DEX on cortisol and CK levels remain unclear and warrant further investigation. In conclusion, CRD significantly impacts neuroinflammation, glial activation, and neuroplasticity. The combined treatment of BUT and DEX effectively mitigated these effects, offering therapeutic potential. Future studies should investigate the long-term efficacy and mechanisms of these treatments, particularly their impact on cortisol and CK levels in human models, to inform potential therapies for CRD and stress-related conditions.

As highlighted by Nair and Jacob [35], dose extrapolation from animal models to humans is inherently complex and requires careful consideration of species-specific differences in metabolism and pharmacokinetics, typically using body surface area as a reference. Although the doses of DEX (1000 mg/kg/day) and BUT (200 mg/kg/day) used in this study are consistent with previous animal studies, translating these doses to human equivalents would require significant adjustment. Therefore, their translational relevance to human therapy remains to be determined. In light of the rising prevalence of CRD among shift workers, adolescents, and the elderly [36], the present findings may contribute to the development of adjunctive therapeutic strategies involving DEX and BUT to attenuate cognitive and neurological impairments. However, comprehensive clinical trials are warranted to assess the safety, optimal dosing, and therapeutic efficacy of these agents in human populations. The present study focused on acute effects following a 3-week CRD protocol. Chronic exposure studies are also needed to assess tolerance, toxicity, and behavioral outcomes.

This study demonstrates that CRD causes significant biochemical, histological, and immunohistochemical alterations in mouse brain tissue, indicative of cellular stress and damage. Treatment with DEX and BUT, particularly in combination, provided significant neuroprotection by reducing cortisol and CK levels, alleviating tissue damage, suppressing inflammatory NF-κB and astrocytic GFAP expression, and preserving BDNF expression. These findings suggest a potential therapeutic approach using antioxidant and metabolic support compounds in conditions related to CRD.

Declarations

Availability of Data and Materials: The data used in this article will be provided by correspondin author (S.G.) upon request.

Ethical Statement

This study was approved by the Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee dated 14.02.2025 and numbered 1273.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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Author Contributions: Forming the hypothesis and planning the study: S.G.; Carrying out the experimental phase: S.G., Ö. Ö. & Ş.T.; Obtaining data and writing the article: S.G. & Ö.Ö.

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Research Article

The Influences of *Spirulina platensis* as an Eco-friendly Anticoccidial Agent on Growth Performance, Blood Biochemistry, Immune Response, Gut Microbiota in *Eimeria* Challenged Broiler Chickens

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Abstract

The escalating threat of antibiotic resistance drives the search for alternatives, leading this study to investigate *Spirulina platensis* extract (SPE) as a substitute in broiler chicken feed. The research evaluated the impact of SPE on growth, antioxidant levels, blood profiles, and gut bacteria. SPE was found to contain antimicrobial organic active compounds, such as heptadecane and geosmin. A 420 broilers were divided into seven groups for a 35-day study: a control group, an *Eimeria*-infected group, and five SPE-supplemented groups. Of the supplemented groups, four received 0.5, 1, 2, or 3 mg/kg SPE, respectively, while one *Eimeria*-infected group also received 3 mg/kg SPE. Supplementation with 3 mg/kg SPE improved body structure, feed efficiency, weight gain, carcass quality, and gut pH. Liver enzymes and kidney markers were reduced. SPE lowered oxidative stress while maintaining antioxidant enzymes and improving gut microbiota and immunity. *Eimeria* infection in broiler chickens led to reduced growth performance, increased oxidative stress, and compromised immunity compared to the control group. However, the dietary inclusion of *Spirulina platensis* extract (SPE) at 3 mg/kg demonstrated a significant mitigating effect on these negative impacts in *Eimeria*-infected birds. Specifically, broilers infected with *Eimeria* and treated with SPE exhibited notable improvements in growth performance, reaching levels comparable to or surpassing those of non-infected chickens receiving SPE. Furthermore, SPE supplementation in infected birds effectively improved their antioxidant status, evidenced by reduced oxidative stress markers, and bolstered their immune response. These findings suggest that SPE possesses properties capable of counteracting the detrimental effects of *Eimeria* infection in broiler chickens, highlighting its potential as a supportive agent in managing coccidiosis and maintaining overall health and productivity in poultry.

Keywords: Anticoccidial, *Eimeria*, *Spirulina platensis,* Gut microbiota, Immune response

Introduction

The emergence of antibiotic-resistant microbes has become a significant global health concern, prompting the search for reliable and sustainable alternatives to antibiotics in livestock production. In poultry farming, antibiotics have traditionally been used to promote growth, prevent diseases, and improve feed efficiency [1]. However, the overuse of antibiotics has led to the development of resistant bacterial strains, posing risks to animal and human health [2]. Consequently, there is a growing interest in natural alternatives, such as plant extracts, probiotics, and algal-based supplements, which can enhance poultry health and productivity without contributing to antibiotic resistance.

One of the most promising natural alternatives is *Spirulina platensis*, a cyanobacterium known for its high nutritional value and bioactive compounds [3]. *Spirulina* is rich in proteins, vitamins, minerals, and anti-oxidants, making it a valuable dietary supplement for livestock [4]. Previous studies have demonstrated that *Spirulina* supplementation can improve growth performance, enhance immune function, and reduce oxidative stress in broiler chickens. For instance, research by Abdelfatah et al.[5] showed that *Spirulina* supplementation increased body weight gain and improved feed conversion ratios in broilers. Similarly, Spínola et al.[6] reported that *Spirulina* enhanced anti-oxidant enzyme activity and reduced lipid peroxidation in poultry. More recently, Alghamdi et al.[7] found that dietary supplementation with *Spirulina* improved gut microbiota

composition and immune responses in broilers, further supporting its potential as a natural growth promoter.

Despite these benefits, poultry farming faces significant challenges from infectious diseases, particularly coccidiosis caused by *Eimeria* species. Coccidiosis is one of the most prevalent and economically devastating diseases in the poultry industry, leading to reduced growth performance, impaired feed efficiency, and increased mortality [8]. *Eimeria* infection damages the intestinal epithelium, resulting in malabsorption of nutrients, diarrhea, and secondary infections [9]. Traditional control methods rely heavily on anticoccidial drugs and vaccines, but the emergence of drug-resistant *Eimeria* strains has necessitated exploring alternative strategies. Recent studies have investigated using natural products, such as plant extracts and probiotics, to mitigate the effects of coccidiosis. For example, Elbaz et al.[10] found that dietary supplementation with essential oils reduced oocyst shedding and improved growth performance in *Eimeria*-infected chickens. Similarly, El-Ghareeb et al.[11] reported that herbal extracts enhanced immune responses and reduced oxidative stress in broilers challenged with *Eimeria*.

The impact of *Eimeria* infection on poultry extends beyond growth performance, affecting the histological integrity of the intestinal tract. The parasite invades and replicates within the intestinal epithelial cells, causing severe tissue damage, inflammation, and necrosis. Studies have shown that *Eimeria* infection leads to villus atrophy, crypt hyperplasia, and increased cellular infiltration in the intestinal mucosa. For instance, Hussein et al.[12] demonstrated that *Eimeria acervulina* infection resulted in shortened villi and deepened crypts in the duodenum of broiler chickens, impairing nutrient absorption and leading to weight loss. Similarly, Attia et al.[13] reported that *Eimeria tenella* infection caused extensive damage to the cecal mucosa, characterized by epithelial sloughing, hemorrhage, and inflammatory cell infiltration. These histological changes compromise intestinal function and predispose birds to secondary infections, further exacerbating the economic losses associated with coccidiosis.

In addition to histological damage, *Eimeria* infection has profound effects on the growth performance of poultry. Infected birds exhibit reduced feed intake, poor weight gain, and decreased feed efficiency due to the malabsorption of nutrients and the metabolic demands of the immune response. Freitas et al.[14] found that broilers infected with *Eimeria maxima* had significantly lower body weight gains and higher feed conversion ratios than uninfected controls. More recently, Choi et al.[15] reported that *Eimeria*-infected chickens showed a 20-30% reduction in body weight gain and a 15-25% increase in feed conversion ratio, highlighting the detrimental impact of coccidiosis on poultry productivity. These findings underscore the need for effective strategies to mitigate the effects of *Eimeria* infection on growth performance and intestinal health.

Recent studies have further highlighted the potential of natural products in mitigating the effects of *Eimeria* infection. For example, Abd El-Ghany [16] demonstrated that dietary supplementation with garlic extract reduced oocyst shedding and improved intestinal morphology in *Eimeria*-infected broilers. Similarly, Memon et al.[17] found that supplementation with Bacillus subtilis probiotics enhanced gut health and immune responses in chickens challenged with *Eimeria*. In 2024, a study by Zhang et al.[18] revealed that *Spirulina*-based diets significantly improved intestinal integrity and reduced oxidative stress in *Eimeria*-infected broilers, further supporting its potential as a natural remedy for coccidiosis.

Given the potential of *Spirulina platensis* to improve poultry health and productivity, as well as the need for effective alternatives to control coccidiosis, this study aimed to evaluate the effects of *Spirulina platensis* extract (SPE) on broiler chickens, including those infected with *Eimeria*. The specific objectives of this study were to assess SPE's impact on broiler chickens' growth performance, including body weight gain, feed conversion ratio, and growth rate, compared to control groups. Evaluate the effects of SPE on anti-oxidant status by measuring oxidative stress markers (e.g., malondialdehyde) and anti-oxidant enzyme activity (e.g., superoxide dismutase and glutathione peroxidase). Analyze blood parameters such as liver enzymes, kidney markers, and lipid profiles to determine the systemic effects of SPE supplementation. To investigate the influence of SPE on cecal microbiota composition, focusing on the abundance of beneficial and pathogenic microbes. Examine the histological changes in the intestinal tract of *Eimeria*-infected broilers and determine whether SPE supplementation can mitigate tissue damage and inflammation. Compare the effects of SPE in healthy broilers versus *Eimeria*-infected broilers, assessing its potential as a therapeutic agent for coccidiosis.

Material and Methods

Ethical Approval

The animal study has been reviewed and approved by ZU-IACUC committee. was performed in accordance with the guidelines of the Egyptian Research Ethics Committee and the guidelines specified in the Guide for the Care and Use of Laboratory Animals (2024). Ethical code number ZU-IACUC/2/F/489/2024. Written informed consent was obtained from the owners for the participation of their animals in this study.

Table 1. Experimental layout design

Treatment	SPE (mg/kg)	*Eimeria tenella* (2.5×10^4)	Description
G1	–	–	Negative control (NC)
G2	–	+	Positive control (PC)
G3	0.5	–	SPE-treated
G4	1	–	SPE-treated
G5	2	–	SPE-treated
G6	3	–	SPE-treated
G7	3	+	Challenged and SPE-treated

Isolation, Cultivation, Extraction, and GC-MS Analysis of *Spirulina platensis*

Spirulina platensis was isolated and cultivated in Zarrouk's medium under controlled temperature and light conditions. A pure culture was established through streaking techniques and maintained on agar slants. Morphological identification was confirmed microscopically. The collected water sample was concentrated by the filtration method and examined under a compound microscope using low-power magnification. After repeated drop-by-drop microscopy, the cyanobacteria were fixed in the tube culture for multiplication. The isolated plankton community with cyanobacteria was cultured in the conical flasks with Zarrouk's media (Zarrouk C, 1966). Different pH levels were maintained in three culture containers, viz., 5.0, 7.0, and 9.0. Since the pH maintained was alkaline, there was a gradual eradication of other contaminant plankton as they could not thrive in higher pH. For extract preparation, *Spirulina platensis* powder underwent cold-water extraction involving freezing, thawing, and centrifugation. The supernatant was then freeze-dried to yield *Spirulina platensis* extract (SPE) [19]. Organic compounds within the SPE supernatant were identified using GC-MS spectroscopy [20]. 1 g of SPE was dissolved in 10 mL of Hexane (1:10, w/v), then sonicated for 10-30 min at room temperature. The obtained extract was filtered through centrifugation, and the supernatant was obtained. The solvent was removed under reduced pressure (rotary evaporator) to obtain the crude extract. An extract volume of 1 µL was injected into the GC-MS system (Agilent 6890, Foster City, CA), which had an HP-5 MS column and an Agilent mass spectrometer detector. The carrier gas was helium, with a flow rate of 1.0 mL/min. After adding 1 µL of volume to the sample, the solvent was left in place for 3 min. The rate of temperature increase was 8°C/min, starting at 40°C and reaching 260°C. The detector temperature was set to 280°C, while the injector temperature was maintained at 250°C. Wiley 9 datasets were used to determine peaks.

Anticoccidial Activity

Eimeria tenella pure strain (1×10^7 parasites/ml) was cultured in 96-well plates. Each well was supplemented with SPE concentrations (50, 100, 200, and 300 µg/mL) and then kept for 24 h at 28°C. The resazurin was added to each well for a colorimetric test [21].

Experimental Design

The same *E. tenella* employed for the anticoccidial activity was used for the experimental infection. *E. tenella* oocysts were obtained from the supernatant using the flotation technique, then cleaned with tap water and centrifuged at 1500 rpm for 10 min. To induce sporulation, the oocysts were immersed in a solution containing 2.5% potassium dichromate and kept for 72 h at room temperature. The sporulated oocysts were rinsed with PBS at 1500 rpm for 10 min. Sporulated oocysts were vigorously mixed with 0.5 mm sterilized glass beads [22]. The infective dose was adjusted to 2.5×10^4 *E. tenella* sporulated oocysts via the McMaster counting technique [13].

Seven groups were carefully assigned to 420 broiler chicks, ensuring a fair and balanced distribution for the study, with each group consisting of 3 replicates of 20 chicks. The standard basal diet was given to the control negative group (G1), and G2 was *Eimeria*-infected chickens. The other four groups (G3, G4, G5, and G6) received a basic diet supplemented with 0.5 mg SPE /kg, 1 mg SPE /kg, 2 mg SPE/kg, and 3 mg SPE/kg, respectively; meanwhile, G7 was *Eimeria*-infected chickens and treated with 3 mg SPE/kg *(Table 1)*. Using a randomized methodology, every chick in the study was grown on a litter model. In a shed with adequate ventilation, rice husk was employed as litter. All broiler chicks were provided standard management conditions and water availability throughout the experiment. We tracked the weekly weights of each bird and recorded the daily feed intake for all groups. At the end of the 35-day experiment, blood was collected from the wing veins of 5 brids from each group, and the samples were collected and stored in EDTA vials for further analysis.

Growth Performance

Broiler chickens were assessed for their live body weights (LBW) and feed consumption. By deducting the beginning live body weight (7 days old) from the ending live body weight (35 days old), the body weight increase (BWG) is calculated by the feed conversion ratio (FCR) and divided by feed consumption following Saad et al.[23]. The performance index (PI) and the growth Rate (GR) were estimated [24].

$$\text{Body weight gain (BWG)} = \text{FBW} - \text{IBW} \quad (1)$$

$$\text{GR} = (\text{LBW35} - \text{LBW7})/0.5 \times (\text{LBW7}+\text{LBW35}) \quad (2)$$

$$\text{PI} = \text{BWG}/\text{FC} \quad (3)$$

Carcass Traits

After the experiment, three birds were randomly selected from each replication, and their weights were measured. After collecting body measures, birds were butchered to evaluate carcass features, including carcass, dressing percent, and the weight of the visceral organs, involving giblets, heart, liver, and gizzard. Intestinal pH was also estimated.

Digestive Enzyme Activities

During the investigation, we carefully evaluated the concentrations of digestive enzymes in the intestine, namely amylase, protease, and lipase, with one measurement per duplicate. We dissected the chicken ileum. The contents of the intestine (ileum) were carefully gathered and put into sterile containers equipped with screw closures to avoid any contamination. The enzymes' activity in the ileum was evaluated using the methodology established by Najafi et al.[25].

Blood Parameter Determination

Plasma samples were obtained using gauge needles from the broiler chickens' wing veins (3 birds per replicate). The samples included 200 µL of EDTA, which was applied as an anticoagulant. To provide a comprehensive analysis of essential blood parameters, including red blood cells (RBCs), packed cell volume (PCV), haemoglobin, and white blood cells (WBCs), the plasma samples were obtained in labeled screw-top tubes.

Liver, Kidney Function

Blood samples were meticulously extracted from slaughtered chicks that were 35 days old and promptly preserved in an anticoagulant-containing tube for efficient plasma extraction using high-speed centrifugation at 4000 rpm for 10 min. After the plasma was collected, it was securely sealed in a sterile tube and stored at a temperature of -20°C till it was needed. Spectrophotometers (Apel 310 Spectrophotometer, Japan) were used to measure photometric biological processes. Calorimetric analysis was conducted using specific commercial kits to evaluate the biochemical characteristics of blood components. The biochemical profiles including alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, urea, total protein (TP), total globulin (TG), albumin/globulin (A/G) ratio.

Lipid Profile

The lipid profile, TC, TG, LDL, HDL, and VLDL, the assessment was conducted using a spectrophotometer and suitable kits following the instructions provided by the manufacturer.

Immunological Parameters

The colorimetric estimation of IgA and IgG immunoglobulin isotypes were tested using a spectrophotometer with respective kits [26].

Anti-oxidant Status

At slaughter, nine birds from each group were blood sampled and centrifuged for twenty minutes at 4500 rpm. Subsequently, the plasma was stored at -20°C to preserve its integrity. Key biomarkers, including SOD, CAT, glutathione, malondialdehyde, anti-oxidant enzymes, and total anti-oxidant capacity, were assessed using top-quality commercial kits from a leading biodiagnostic company.

Histopathological Investigation

Intestinal tissues were collected, fixed in 10% formalin for 48 h, and then processed using an automated tissue processor. Following fixation, tissues were washed in distilled water for 30 min and then dried using different immersions in alcohol with different concentrations (70% for 120 min, 90% for 90 min). The dehydration was cleared by applying numerous cycles of xylene. Briefly, tissues were submerged in xylene (50%) for 60 min and alcohol (50%), then pure xylene for an additional 90 min. The tissues were put with melted paraffin wax, sealed, then paraffin cut sections for 4-5 µm, and then stained with Hematoxylin & Eosin [27].

Estimation of Caecal Bacterial Load

Three birds from each replicate (a total of 9 birds/group) were sacrificed at 35 days of age to collect caecal content. The careful dissection of the caeca and collection of contents in sterile cups was performed using aseptic techniques to ensure the integrity of the samples. The inoculums were carefully diluted using a 1 mL to 9 mL ratio of the suspension to normal saline in sterile ependymal tubes. This approach ensured the accuracy and reliability of our research process. The caecal samples underwent a process of repeated dilution, resulting in a dilution level of 10^{-6}. Then, 0.1 mL of the dilution was pipetted out and incubated for total bacterial count, coliforms, *E. coli*,

Salmonella, enterococcus, total fungal count, in Nutrient, McConkey's agar, Eosin-Methylene blue agar, XLD, and *Enterococcus* agar, sabaroud dextrose agar respectively. The samples were applied to the agar surface using a sterilized glass spreader while rotating the Petri dish beneath. For the total bacterial count, the nutrient broth is employed. The Petri dish was incubated at a temperature of 37°C for 24 h, and individual colonies were calculated with a colony counter and quantified as: CFU/mL = (No. of colonies x dilution factor)/volume of the culture plate.

Statistical Analysis

The statistical analysis was conducted using one-way ANOVA in SPSS program (SPSS, 2021). The LSD test was used to compare all tested means (treatments) at a significance level of P<0.05. The sample size was calculated using the following equation: $n = (\frac{ZSD}{E})^2$.

Results

Table 2 highlighted the active components in SPE, where the GC-MS analysis table of *Spirulina platensis* extract highlights a diverse array of bioactive organic compounds, notably long-chain alkanes (such as octacosane, heneicosane, pentacosane, hexacosane), fatty acids (palmitic acid/n-hexadecanoic acid and cis-9-hexadecenoic acid), aromatic phenolic compounds, and organic esters. The most abundant constituents are octacosane and heneicosane, which together account for a substantial portion of the extract's composition. These compounds are well recognized for their membrane-disrupting, antioxidant, anti-inflammatory, and anti-parasitic properties, supporting the anticoccidial efficacy attributed to *Spirulina* extracts. The presence of both direct antiparasitic agents (alkanes, fatty acids) and supportive compounds (phenolics, esters) suggests a multimodal mechanism, contributing to gut protection and enhanced resistance against *Eimeria* infection. As mentioned in *Table 3*, the body weight demonstrated a substantial elevation in weeks three & five in all groups treated with SPE compared to control negative birds. From the first to third weeks of age, the body gain was considerably higher in all groups administered different dosages of SPE compared to the control group (T1), which fed a basic diet only. Compared to the birds in groups T1 and T5, there was a substantial elevation in body weight gain (BWG) for the birds in groups T2, T3, T4, and T5 throughout the third and fifth weeks of their lives. The cumulative body weight gain over the experimental time from week one to week five showed that all *Spirulina platensis* extract-supplied birds revealed a substantial elevation in BWG as opposed to T1, and the birds supplied with 3 mg SPE/kg diet revealed the best BWG (2405 g) at P-value <0.0001. The results in *Table 3* showed that feed intake declined substantially in all birds supplied with *Spirulina platensis* extract, contrasting with unsupplied birds (T1). Between the third and fifth weeks of age, it became apparent that all birds provided with SPE exhibited a substantial improvement in feed conversion ratio (FCR), indicating a reduction in FCR as opposed to the birds (T1). The cumulative FCR was the best (1.76 and 1.83) at (T4 and T5), respectively, (P-value (0.0235). *Eimeria*-infected chickens without SPE treatment showed reduced growth performance compared to the control group. However, *Eimeria*-infected chickens treated with SPE at 3 mg/kg demonstrate improved growth performance compared to untreated *Eimeria*-infected chickens, which is slightly lower than those treated with SPE at 3 mg/kg.

Table 4 shows no notable disparities in the liver's total weight and the percentage of the heart. The carcass and dressing exhibited a statistically significant increase in Group 5 (3 mg/kg diet) compared to Group 1 (birds), with values of 75.8 and 80.89, respectively, compared to 70 and 73 in the control birds. In addition, the examination of pH levels in the intestines revealed a substantial decrease

Table 2. *Organic active compounds in Spirulina platensis extract (SPE) detected by GC-MS*

Retention Time	Organic Active Compounds	% Area
9.60	Phenol, 2,5-bis(1,1-dimethyethyl)	1.01
15.16	Oxalic acid, isobutyl pentyl ester	0.52
27.02	Tricosane	2.63
29.13	Heneicosane	24.41
29.59	Pentacosane	10.75
30.86	Hexacosane	10.63
33.65	Octacosane	28.66
34.10	cis-9-Hexadecenoic acid	1.95
34.28	n-Hexadecanoic acid	2.12
36.49	Tetrapentacontane, 1,54-dibromo	1.86

a-f different lowercase letters at the same column indicate significant differences at P<0.05

Table 3. *The influence of dietary Spirulina platensis extract (SPE) at four concentrations at the growth performance parameters of broiler chickens*

SPE Treatments (mg/kg)	LBW (g)		BWG (g)	FI (g)	FCR	GR	PI
	1d	35d	1-35d	1-35d	1-35d	1-35d	1-35d
NC	44.9	2312e	2267e	3754d	1.59d	196b	131c
PC	44.8	2100f	2055f	3800e	1.85e	180d	120d
SPE 0.5	45.1	2339d	2294d	3768cd	1.65c	199a	134b
SPE 1	45.0	2370c	2325c	3778c	1.70bc	202a	136ab
SPE 2	45.7	2412b	2366b	3812b	1.76b	206a	139a
SPE 3	45.5	2450a	2405a	3878a	1.83a	210a	143a
PC+SPE 3 mg/kg	45.3	2380c	2335c	3820b	1.78b	204a	138a
P-value	0.89	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

a-e different lowercase letters at the same column indicate significant differences at P<0.05. SEM[1]: Pooled standard error, LBW: Live body weight, BWG: body weight gain, FCR: feed conversion ratio, PI: performance index, GR: growth rate, Control = basal diet + 0 mg/kg SPE; SPE 0.5 = basal diet + 0.5 mg/kg SPE, SPE 1 = basal diet + 1 mg/kg SPE, SPE 2 = basal diet + 2 mg/kg SPE; SPE 3 = basal diet + 3 mg/kg SPE

Table 4. *The impact of dietary Spirulina platensis on Carcass traits and intestinal pH of broiler chickens*

Traits (%)	SPE Treatments (mg/kg)							*P*-value
	NC	PC	0.5	1	2	3	PC+SPE	
Carcass	70.0c	68.5d	73.11b	74.36a	74.9a	75.8a	72.5b	0.001
Liver	2.20	2.18	2.23	2.22	2.17	2.20	2.19	0.9
Gizzard	1.9b	1.8c	2.2a	2.25a	2.26a	2.25a	2.1b	0.05
Heart	0.84b	0.80c	0.93ab	0.95a	0.96a	0.95a	0.90b	0.04
Dressing	73.23d	71.50e	75.11c	77.24b	78.88b	80.89a	76.50c	0.001
Intestinal pH	6.8b	7.0a	6.5b	6.7b	6.8b	7.2a	6.9a	0.05

a-e Means within the same raw with different superscripts differ significantly (P≤0.05). Control = basal diet + 0 mg/kg SPE; SPE 0.5 = basal diet + 0.5 mg/kg SPE, SPE 1 = basal diet + 1 mg/kg SPE, SPE 2 = basal diet + 2 mg/kg SPE; SPE 3 = basal diet + 3 mg/kg SPE

Table 5. *The influence of dietary Spirulina platensis extract (SPE) at four concentrations on the serum digestive enzymes of broiler chickens*

SPE Treatments (mg/kg)	Amylase	Lipase	Trypsin	*P*-value
0 (Control)	310d	12c	25d	<0.0001
0 (*Eimeria*-infected)	280e	10d	20e	<0.0001
0.5	420c	17d	30c	<0.0001
1	480b	22c	36b	<0.0001
2	500ab	26b	42ab	<0.0001
3	510a	29a	45a	<0.0001
3 (*Eimeria*-infected)	490b	24b	40b	<0.0001

a-e Means within the same column with different superscripts differ significantly (P≤0.05). Control = basal diet + 0 mg/kg SPE; SPE 0.5 = basal diet + 0.5 mg/kg SPE, SPE 1 = basal diet + 1 mg/kg SPE, SPE 2 = basal diet + 2 mg/kg SPE; SPE 3 = basal diet +3 mg/kg SPE

in G2 (6.5) and G5 (7.2) compared to the birds in G1 (6.8) (P-value of 0.05). The *Eimeria*-infected chickens without SPE treatment showed reduced carcass traits and altered intestinal pH compared to the control group. The treatment with SPE at 3 mg/kg demonstrated improved carcass traits and intestinal pH compared to untreated *Eimeria*-infected chickens.

Table 5 demonstrated that the digestive enzyme levels as the lipase enzyme level showed that lipase level exhibited a substantial elevation in T5 as opposed to T1 (P-value <0.0001) while the level of protease exhibited a significant elevation of all groups supplied with *Spirulina platensis* in contrast with unsupplied birds (G1). The amylase level substantially elevated T4 & T5 compared to T1, T2, and T3 birds. The *Eimeria*-infected chickens without SPE treatment reduced the activities of digestive enzymes compared to the control group. Meanwhile, the SPE (3 mg/kg) treatment improved digestive enzyme levels compared to untreated *Eimeria*-infected chickens.

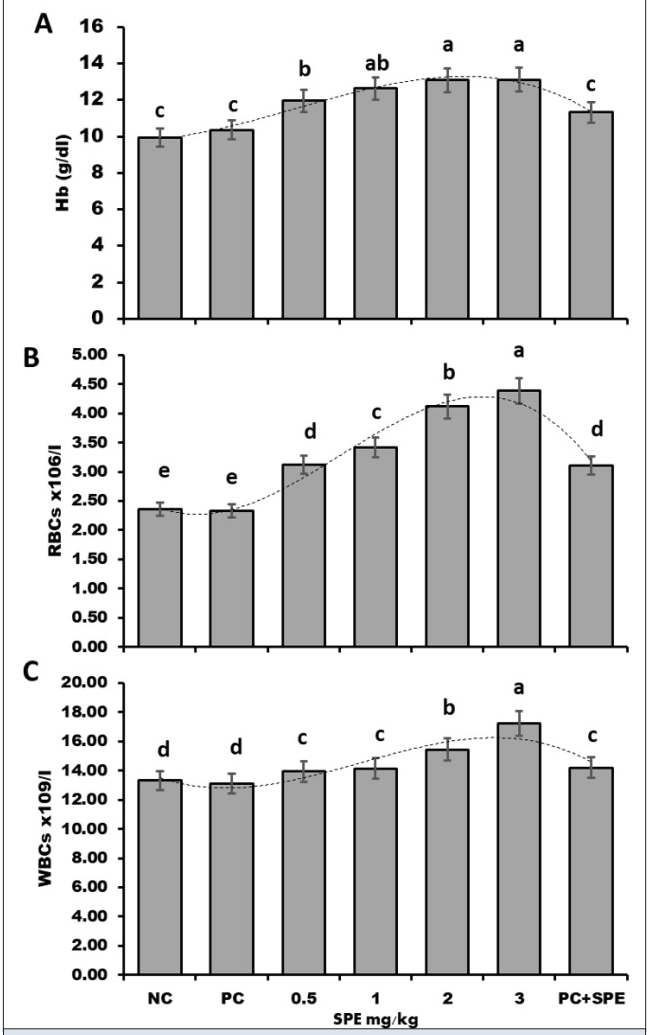

Fig 1. The effect of dietary *Spirulina platensis* extract (SPE) at four concentrations on the hematology of *Eimeria-infected* broiler chickens

Fig. 1 revealed that the blood parameters of birds supplied with dietary *Spirulina platensis* were significantly improved compared to those of birds in the control group (T1). The haemoglobin (Hb) level demonstrated a substantial rise in groups (T2, T3, T4, and T5), contrary to group (T1). When comparing G5 (4.5) to G1 (2.4) birds fed a 3 mg SPE/kg diet, the RBCs count revealed a considerable rise. The WBC count significantly increased T5 (17.33) compared to G1 (13.41) birds.

The *Eimeria* challenge (PC group) resulted in slightly increased liver enzyme levels (AST and ALT) compared to the healthy control (NC), although this increase was not always statistically significant. However, SPE treatment consistently and significantly reduced both AST and ALT compared to the challenged group, with the 3 mg/kg dose of SPE showing the most pronounced decrease (approximately a 34.4% reduction in AST and a 49.2% reduction in ALT relative to PC), bringing levels closest to those of the healthy control. Even in the presence of the parasite (PC+SPE group), SPE significantly lowered these enzymes (approximately 29.6% reduction in AST and 35.5% reduction in ALT relative to PC), demonstrating its ability to mitigate liver damage during infection. Notably, the AST and ALT values in the PC+SPE group approached those observed in the SPE 3 mg/kg group, highlighting its therapeutic potential *(Table 6)*.

Eimeria challenges slightly elevated uric acid, but didn't significantly impact creatinine. SPE treatment reduced uric acid considerably across all doses. It slightly decreased creatinine at higher doses, with the 3 mg/kg SPE group exhibiting the lowest uric acid levels (approximately 50% reduction relative to PC). The PC+SPE group also showed a considerable decrease in uric acid (approximately 35% relative to PC), suggesting that SPE benefits kidney function even during infection. The *Eimeria* challenge resulted in a reduction of total protein and albumin (approximately 10.7% and 5.9% reductions, respectively, relative to the NC). SPE treatment dose-dependently increased both, with the 3 mg/kg SPE group surpassing control levels (approximately 64.3% increase in total protein and 50% increase in albumin relative to PC). The PC+SPE group also showed a significant increase in total protein (approximately 60% increase relative to PC) and albumin (approximately 35.3% increase relative to PC), indicating SPE's role in recovery. Globulin levels also decreased with *Eimeria* (approximately an 8.3% reduction relative to NC), and SPE treatment increased them, with the 3 mg/kg dose showing the highest values (approximately a 90.9% increase relative to PC), similar to the PC+SPE group (approximately a 63.6% increase relative to PC). The albumin/globulin ratio was slightly higher in the challenged group (approximately a 3.3% increase relative to the NC group), and SPE treatment decreased it, with the 3 mg/kg SPE and PC+SPE groups showing comparable lower ratios (approximately a 17.7% and 17.4% reduction, respectively, relative to PC).

Eimeria challenges result in a slight increase in total cholesterol, triglycerides, and LDL (approximately 6.4%, 4.2%, and 4.2% increases relative to NC), while decreasing HDL (approximately 5.9% reduction relative to NC). SPE treatment significantly and dose-dependently reduced total cholesterol, triglycerides, LDL, and VLDL and increased beneficial HDL levels. The 3 mg/kg SPE group consistently demonstrated the most substantial improvements in all lipid parameters (approximately 55.6% reduction in total cholesterol, 62.5% reduction in triglycerides, 70% reduction in LDL, and 62.5% reduction in VLDL relative to PC, and a 37.5% increase in HDL relative to PC), reaching the lowest levels of harmful lipids and the highest HDL. The PC+SPE group also showed marked reductions in cholesterol (approximately 48% reduction), triglycerides (approximately 52.5% reduction), LDL (approximately 60% reduction), and

Table 6. *The influence of dietary Spirulina platensis extract (SPE) at four concentrations at the serum biochemical parameters of broiler chickens*

Serum Biochemistry		SPE Treatments (mg/kg)							P value
		NC	PC	0.5	1	2	3	PC+SPE	
Liver and kidney functions	AST (U/L)	255a	270a	220b	208c	189d	177e	190d	<0.0001
	ALT (U/L)	5.9a	6.2a	5.2b	4.7c	3.9d	3.1e	4.0d	<0.0001
	Uric acid (mg/dL)	5.5a	6.0a	4.8b	4.5c	3.8d	3.0e	3.9d	<0.0001
	Creatinine (mg/dL)	0.36a	0.38a	0.35a	0.32ab	0.28b	0.27b	0.29b	0.05
	Total protein (g/dL)	2.8c	2.5d	3.6bc	3.9b	4.2ab	4.6a	4.0b	0.00123
	Albumin (g/dL)	1.8d	1.7d	1.92c	2.1b	2.4ab	2.7a	2.3b	<0.0001
	Globulin (g/dL)	1.2c	1.1c	1.4bc	1.7b	1.9ab	2.1a	1.8b	0.0011
	Albumin/Globulin (%)	1.5a	1.55a	1.35b	1.23d	1.26c	1.28c	1.27c	0.0023
Lipid profile	Total cholesterol (mg/dL)	235a	250a	196b	141c	125d	111e	130d	<0.0001
	Triglycerides (mg/dL)	192a	200a	187b	168c	100d	74e	95d	<0.0001
	HDL (mg/dL)	85d	80d	92c	96c	100b	110a	98b	<0.0001
	LDL (mg/dL)	48a	50a	33b	25c	18d	15e	20d	<0.0001
	VLDL (mg/dL)	46a	48a	35b	28c	20d	18e	22d	<0.0001
	Abdominal fat	1.33a	1.48a	1.21b	0.91c	0.85d	0.69e	0.8d	<0.0001
Oxidative stress	GSH (ng/mL)	0.35c	30d	0.52bc	0.59b	0.67ab	0.69a	0.65b	<0.0001
	SOD (U/mL)	0.51e	0.45e	0.68d	0.72c	0.83b	1.01a	0.80b	<0.0001
	CAT (ng/mL)	0.33d	0.28d	0.51c	0.62bc	0.68b	0.77a	0.70b	<0.0001
	MDA (nmol/mL)	0.55a	0.60a	0.41b	0.35c	0.30c	0.21d	0.32c	<0.0001
	TAC (ng/mL)	0.35c	0.30c	0.42c	0.55c	0.68b	0.85a	0.70b	<0.0001
Immunity	IgG (mg/dL)	960e	900e	1050d	1071c	1099b	1120a	1100b	<0.0001
	IgA (mg/dL)	177.8e	170e	188.2d	191.3c	205.6b	211a	200b	<0.0001

a-e different lowercase letters at the same raw indicate significant differences at P<0.05. ALT Alanine aminotransferase: AST Aspartate aminotransferase, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, T_3: Triiodothyronine T_4: Thyroxine IgG, IgA Immunoglobulins Isotypes G, and A. Control = basal diet + 0 mg/kg SPE; SPE 0.5 = basal diet + 0.5 mg/kg SPE, SPE 1 = basal diet + 1 mg/kg SPE, SPE 2 = basal diet + 2 mg/kg SPE; SPE 3 = basal diet + 3 mg/kg SPE

VLDL (approximately 54.2% reduction) relative to PC, and an increase in HDL (approximately 22.5% increase relative to PC), though generally not as extreme as the highest SPE dose alone. Abdominal fat was also slightly higher in the challenged group (approximately 11.3% increase relative to NC). SPE treatment significantly reduced it, with the 3 mg/kg SPE group showing the greatest reduction (approximately 53.4% relative to PC). The PC+SPE group also exhibiting a significant decrease (approximately 45.9% reduction relative to PC).

Eimeria challenge significantly increased markers of oxidative stress (MDA) and decreased anti-oxidant defenses (GSH, SOD, CAT, TAC) (approximately 9.1% increase in MDA, 14.3% decrease in GSH, 11.8% decrease in SOD, 15.2% decrease in CAT, and 14.3% decrease in TAC relative to NC). SPE treatment significantly and dose-dependently reversed these effects, with the 3 mg/kg SPE group showing the most substantial increase in anti-oxidant enzyme activity and non-enzymatic anti-oxidant levels (approximately 130% increase in GSH, 124.4% increase in SOD, 175% increase in CAT, and 183.3% increase in TAC relative to PC), and the greatest reduction in MDA (approximately 65% reduction relative to PC). The PC+SPE group also demonstrated a significant recovery in all anti-oxidant markers (approximately 116.7% increase in GSH, 77.8% increase in SOD, 150% increase in CAT, and 133.3% increase in TAC relative to PC) and a reduction in MDA (approximately 46.7% reduction relative to PC), indicating SPE's protective effect against oxidative damage even during infection, with values often approaching those of the 3 mg/kg SPE group.

Eimeria challenge slightly decreased IgG and IgA levels (approximately 6.2% and 4.4% reduction, respectively, relative to NC). SPE treatment significantly and dose-dependently increased both immunoglobulins, with the 3 mg/kg SPE group exhibiting the highest levels (approximately 24.4% increase in IgG and 24.1% increase in IgA relative to PC). The PC+SPE group also showed a considerable increase in both IgG (approximately 22.2% increase relative to PC) and IgA (approximately 17.6% increase relative to PC), suggesting an enhanced immune response even in the presence of the parasite, with levels comparable to the higher SPE doses (*Table 6*).

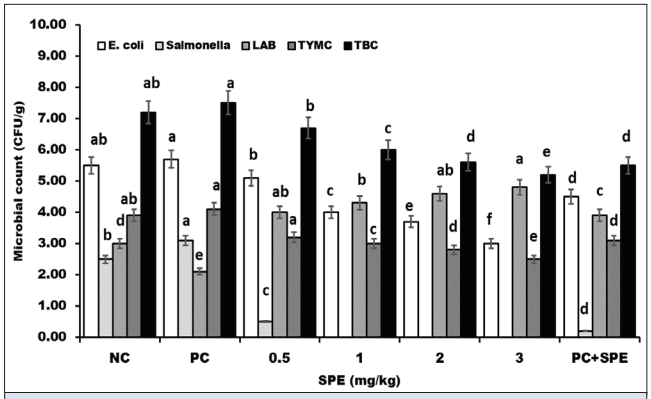

Fig 2. The influence of dietary *Spirulina platensis* extract (SPE) at four concentrations on the intestinal microbiota of *Eimeria-infected* broiler chickens

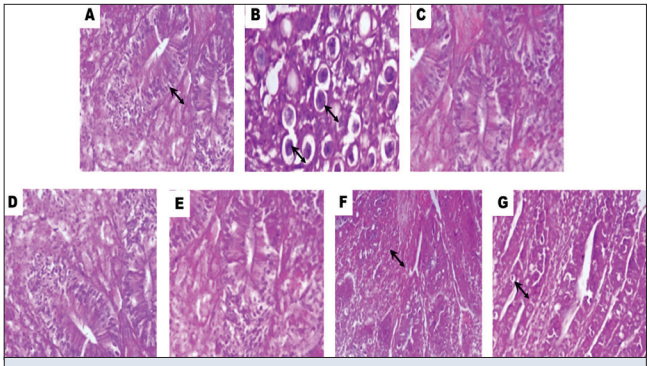

Fig 3. Photomicrographs, stained with Hematoxylin and Eosin (H&E), offer a histological evaluation of the impact of different concentrations of *Spirulina* extract on coccidiosis in broiler intestine. A, representing the negative control group, B, *Eimeria*-infected broiler chickens, C-F were SPE-treated broilers at different concentrations (0.5, 1, 2, 3 mg/kg), and G was *Eimeria-infected* broiler chickens and treated with SPE (3 mg/kg)

Spirulina extract, particularly at 3 mg/kg, generally mitigated the negative biochemical changes induced by *Eimeria* challenge to the greatest extent, often reversing the effects by significant percentages. SPE treatment consistently showed protective and restorative effects, and the PC+SPE group demonstrated that SPE could effectively counteract many of the detrimental effects of the parasite, often with results showing substantial percentage improvements compared to the challenged group alone and sometimes approaching the efficacy of the highest SPE dose.

Additionally, the total yeast and fungal count showed a substantial decrease in T2, T3, T4, & T5, contrasting with T1. Regarding the *E. coli* count, the birds fed a diet with the elevated SPE exhibited a substantial drop in contrast with their counts in control birds (G1). Furthermore, compared to control birds (T1), The SPE treatments showed a significant decline in TBC counts with a considerable rise in LAB count *(Fig. 2)*.

Fig. 3 shows that the photomicrographs, stained with Hematoxylin and Eosin (H&E), illustrate the effect of different *Spirulina* extract concentrations on broilers' intestinal histology. *Fig. 3-A*, representing the control group, exhibits normal intestinal villi architecture with intact villi, as indicated by the arrow. *Fig. 3-B* depicts a group under an unspecified condition, revealing significant alteration and parasitic presence within the intestinal tissue, marked by the arrow, as a reference for the treated groups. *Fig. 3-C* shows the intestinal tissue of broilers treated with 0.5 mg/kg *Spirulina* extract, displaying some preservation of villi structure with minimal pathological changes compared to *Fig. 3-B*. The group treated with 1 mg/kg *Spirulina* extract *(Fig. 3-D)* demonstrates improved intestinal villi structure and reduced pathological changes compared to the lower dose. Further improvement in villi integrity and minimal signs of damage are observed in Image E, representing the 2 mg/kg *Spirulina* extract treatment group. Image F showcases the intestinal tissue of broilers treated with the highest concentration of *Spirulina* extract, 3 mg/kg, exhibiting well-preserved intestinal villi architecture, closely resembling the control group (A). Finally, Image G illustrates the intestinal histology of an *Eimeria*-infected broiler treated with 3 mg/kg *Spirulina* extract, showing some preservation of villi structure and a reduction in the severity of damage typically associated with *Eimeria* infection, as indicated by the arrow, suggesting a protective effect of *Spirulina* extract in this context compared to a potentially untreated infected group.

From the microscopic images, control Group exhibits a healthy intestinal architecture, with a high VH:CD ratio (10.00), indicating optimal absorptive function and low crypt cell proliferation. Meanwhile *Spirulina*-treated group shows an increased villus height and a shallower crypt compared to control, resulting in a markedly higher VH:CD ratio (14.44). This suggests an enhancement in gut absorptive capacity and mucosal health-typically interpreted as a protective or promotive effect. However, *Eimeria*-challenged group demonstrates significant villus atrophy and deepened crypts, reflected in a much lower VH:CD ratio (4.67), indicative of intestinal damage and reduced absorption, commonly associated with coccidial infection.

Discussion

Coccidiosis is a widespread and economically significant parasitic disease in poultry caused by protozoan parasites of the genus *Eimeria*. These parasites infect the intestinal tract of chickens, leading to malabsorption of nutrients, diarrhea (often bloody), reduced growth rates, increased susceptibility to other diseases, and potentially high mortality rates, particularly in young birds. Control strategies have traditionally relied on anticoccidial drugs, but the emergence of drug-resistant *Eimeria* strains necessitates the exploration of alternative preventative and therapeutic approaches.

Discover the power of *Spirulina platensis*, a naturally occurring blue-green algae that grows in freshwater lakes [28]. Since ancient times, humans have consumed *Spirulina platensis*, and recent scientific findings confirm that *Spirulina platensis* is packed with essential fatty acids, protein, minerals, vitamins, amino acids, and volatile compounds, i.e., heptadecance that possess considerable activity [29]. These compounds showed anticoccidial properties through different mechanisms, where active compounds pass through membrane interference and disruption of parasite cellular processes, multiple alkanes and fatty acids present in the extract can reduce *Eimeria* oocyst viability and hinder parasite development. Also, phenolics and fatty acids found in *Spirulina* can modulate the inflammatory response, attenuate tissue damage, and foster mucosal recovery during infection episodes. Several identified compounds-particularly phenolics and organic esters-bolster the antioxidant capacity of the gut, mitigating the oxidative tissue injury caused by coccidial infection [29]. The array of bioactive organic compounds in *Spirulina platensis* extract-especially various alkanes, fatty acids, and phenolic agents-provides a multifaceted anticoccidial effect by disrupting parasite integrity, supporting host immune and antioxidative responses, and aiding recovery from intestinal pathology.

The main aim of this research was to evaluate the practicality of including SPE (selenium) in the diet of broiler chicks and its effects on their performance, lipid profiles, anti-oxidant activity, immune response, blood measurements, gut enzymes, and microbiota. *Table 2* and *Table 3* illustrates the impact of a food supplement containing SPE on the growth of Japanese broiler chicks from day 1 to day 35. The data indicates that broiler chicks were provided with *Spirulina platensis* supplementation for 35 days. Upon the completion of the experiment, the birds that were given a diet containing 3 mg of *Spirulina platensis* per kilogram of food had noticeably elevated body weight (BW), body weight gain (BWG), and lower feed conversion ratio (FCR) as opposed to the birds in the control group (G1) who fed a standard diet. This may be attributed to the fact that *Spirulina platensis* aids in preserving birds' natural microflora health, facilitating their ability to digest food and carry out efficient metabolic processes by absorbing essential vitamins and minerals. The findings align with Hanafy [30] study, which also saw comparable outcomes in broiler chickens given *Spirulina platensis* supplements. Hanafy noted that the enhanced absorption of minerals and vitamins caused the growth of the chicks' live body weight to rise. Also, *Spirulina platensis* might be applied as a substitute for antibiotics to enhance growth [31].

The study's findings, which evaluated the impact of several dietary doses of SPE (0.5, 1, 2, and 3 mg/kg) on several slaughter parameters, including the internal organs weight (Liver, heart, gizzard, and percentage of carcass body weight at 35 days), are shown in *Table 4*. When the subjects were 35 days old, the results indicated that adding *Spirulina platensis* to their diet significantly improved several slaughter criteria. The outcomes of this research are consistent with Hanafy [30] observations, which demonstrated enhanced carcass features and decreased abdominal fat in chickens that were given *Spirulina platensis*.

In contrast to the control group, the whole giblet data, which included the liver, heart, and gizzard, showed a substantial rise. The findings reported by Zahir et al.[31] are consistent with this observation. Moreover, the prior data align with the findings of Abou-Zeid et al.[32], which indicate that the collected data demonstrate that birds fed 2 mg SE/kg diet achieved higher average body weight. There were significant differences in the percentage of carcass and abdominal fat among the groups, but no significant differences were observed in the weight of the liver, heart, and gizzard.

Our research suggests that using the SPE raised the efficiency of digestive enzymes, potentially explaining the reason for the bird's increased output. Furthermore, these enzymes improved the bird's ability to digest feed and absorb nutrients. In addition, the groups that were provided with SE had a decreased microbial burden in the caecum as opposed to the control birds (G1). *Spirulina platensis* powder positively impacts the structure of the intestines, leading to longer villi and an increased number of goblet cells [33]. Additionally, it promotes a healthier population of microorganisms in the intestines, characterized by a higher abundance of *Lactobacillus* sp. and a decrease in *E. coli*. Phyto-additives positively impact nutritional digestibility primarily by increasing the production and enhancing the activity of digestive enzymes and improving gut morphology [34]. Enhanced absorption may cause improved protein digestibility, as seen in feed supplemented with *Spirulina platensis,* promoting broiler chickens' development [35].

In this study, groups supplied with SE significantly improved blood parameters, including WBCs, RBCs, and Hb levels. The present investigation assessed the clinical blood biochemicals, the state of the birds' health, the levels of circulating total protein, globulins, A/G ratio, and liver and kidney functions. These parameters showed improvement in the groups that were provided with SE. *Spirulina platensis* supplementation directly impacted blood hemoglobin levels because of its tendency and rich mineral content to alter the permeability and health of the intestines [36]. Furthermore, it was shown that increased hemoglobin levels were seen when 5 or 10 g of *Spirulina platensis* powder were added to the broiler diet [33].

The current study found that birds that got SE substantially improved their lipid profile compared to the control group (G1). Similar results were found by Abd El-HadyEl-Ghalid [37], who stated that compared to the control group, adding *Spirulina platensis* (3-6%) to broiler diets substantially decreased TC, TG, and LDL. The *Lactobacillus* population is increased by the dietary supplementation of *Spirulina platensis*, which results in the hypogastric-intestinal tract, which could be responsible for the cause of the broiler chickens' decreased blood lipid profile after consuming *Spirulina platensis* [38]. Serum high-density lipoprotein (HDL) concentration of broilers of 1, 2 gm *Spirulina platensis* significantly ($P \leq 0.05$) increased. The reduction in serum cholesterol was attributed to the impact of *Spirulina platensis* on lipoprotein metabolism and the elevation of lipoprotein enzyme activity levels. *Spirulina platensis* enhances the functions of hepatic triglyceride lipase and lipoprotein lipase, thereby reducing cholesterol levels in the liver and circulation. The lipases break down triglycerides and cholesterol. *Spirulina platensis* may increase the activity of these enzymes, which can reduce cholesterol levels while promoting overall heart health [39]. SA Majid et al.[40] noticed that oxidative stress increases the formation of ROS, which causes biomolecules, including nucleic acids, proteins, and enzymes, to become denaturized. Reduced protein consumption and a deficiency in vital amino acids may exacerbate oxidative damage brought on by heat stress, which may also be associated with the decline in blood protein levels [41].

Our results showed that the anti-oxidant and immunological status were improved in broiler chicks supplied with SPE instead of control birds (G1). Polyphenols are natural anti-oxidants with anti-oxidant and antimicrobial activity [42]. Anti-oxidants can inhibit lipid oxidation by performing the following actions: By scavenging free radicals, anti-oxidants can neutralize these harmful molecules before they can start the process of lipid oxidation. Interrupting sequential processes: Anti-oxidants can interrupt the consecutive events that transpire when lipids oxidize. Anti-oxidants may decrease the level of oxygen in a particular region by breaking down peroxides, which are substances formed during the oxidation of lipids. Anti-oxidants may reduce the oxygen supply for lipid oxidation.

Moreover, anti-oxidants such as metal ions and other catalysts can bind to chain-initiating catalysts, possibly initiating lipid oxidation [42]. The anti-oxidant qualities of these materials could potentially explain this. In their study, Park et al.[35] found that incorporating additives loaded with omega-3 fatty acids into the diet of broilers can improve the nutritional value and healthiness of their thigh meat. This is because essential fats like Omega-3 fatty acids can alleviate inflammation, strengthen the heart, and improve cognitive function, among other health advantages.

Chicken meat quality refers to physical, chemical, and biological attributes, including both the essential nature of the meat and its attributes. While the crucial quality of chicken meat relates to taste, color, muscle tissue texture, and meat nutritional quality, the specific characteristics of chicken meat include nutrient content, water-holding capacity, and pH value. Fatty acid content and flavor are also essential features. In addition, with the vast consumption of meat products, people are increasingly pursuing lower-fat, low-calorie, and high-nutrient content. Proper modification of the nutritional content of chicken meat can improve the quality of chicken meat and its market competitiveness, and it will be beneficial to the upgrade of the chicken industry [43]. Correlating with this study, microalgae, especially SPE, can be employed to improve the quality of meat and the performance of broiler poultry [44]. *Spirulina platensis* led to a varied meat color in their muscles. This effect was statistically significant ($P<0.01$) when *Spirulina platensis* was included in chicks' diets [45].

The cecum has a crucial role in preventing the establishment of infections, neutralizing harmful substances, reusing nitrogen, producing vitamins via microbial activity, breaking down certain carbohydrates, and absorbing additional minerals. The findings of our study demonstrated a substantial reduction in the overall levels of bacteria, coliforms, *E. coli*, *Salmonella* spp., *Enterococcus* spp., and fungi in the groups provided with SPE. The results of our investigation align with the findings of Ansari et al.[46], who stated the effects of feeding broiler chicks with *Spirulina platensis* on the microbes in their caecum; they found that supplementing the broiler meal with SE led to an elevated concentration of *Lactobacillus* in the caecum. Nevertheless, there was no fluctuation in the abundance of coliform bacteria. Numerous researches indicate that microalgae have a variety of antibacterial properties. *Candida albicans* growth is suppressed by 17.6% of different extracts of *Spirulina platensis*.

In contrast, the extract increased the proliferation of *Lactococcus* by 50% [47]. According to Kaushik and Chauhan [48] research, SE can eradicate or halt the growth of dangerous bacteria. The growth of *Lactobacillus acidophilus* was significantly enhanced by applying 10 mg/mL of dried *Spirulina platensis* to the LAB growth medium (de Man, Rogosa, and Sharpe medium), resulting in an 186% increase. This suggests that the microalgae may have prebiotic properties [49].

In addition, Abedin and Taha [50] research observed that the antibacterial activities of SE's lipopolysaccharides and alkaloids were practical against *E. coli*. Administering

chlorella microalgae to laying hens in a live experiment increased the cecum's count of lactic acid bacteria (LAB). Few studies have investigated the antibacterial characteristics of microalgae, mainly SE, in avian species. This research shows that adding a *Spirulina platensis* supplement would help sustain the LAB community and improve growth and digestibility.

In conclusion, including *Spirulina* extract (SPE) in the broiler diet at a 3 mg/kg ratio is advisable for improving overall production and health. This dietary supplementation has been shown to positively influence various aspects of broiler performance, including enhancements in body weight and weight gain, improved carcass characteristics, and a better feed conversion ratio. Furthermore, incorporating SPE at this level contributes to general health parameters by positively modulating blood and lipid profiles, enhancing digestive enzyme activity, and supporting healthy hepatic and renal functions. SPE at 3 mg/kg also boosts the immune system and elevates antioxidant levels, improving disease resistance. These beneficial effects ultimately reflect on the quality of the meat produced.

Spirulina platensis demonstrates promise as an eco-friendly anticoccidial agent in broiler chickens challenged with *Eimeria,* offering benefits for growth, immunity, and gut health. However, there are notable limitations and disadvantages. The active compounds in *Spirulina* can vary due to differences in cultivation and processing, leading to inconsistent efficacy. Determining optimal dosing is still challenging, and high inclusion rates may negatively affect feed palatability and intake. Most research is short-term and under controlled conditions, so results may not fully translate to commercial poultry settings. There are practical concerns related to production costs, supply scalability, and the risk of contamination with heavy metals or toxins if quality control is inadequate. *Spirulina* may also produce unintended changes in gut microbiota or, in rare cases, trigger hypersensitivity. Additionally, its anticoccidial effects may not match those of conventional drugs in severe infections and should not replace standard biosecurity and vaccination measures. Overall, while *Spirulina* has multiple advantages as a natural alternative, its application in poultry production should take these limitations into careful consideration.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests: The author declared that there is no conflict of interest.

Ethical Approval: The animal study has been reviewed and approved by ZU-IACUC committee. was performed in accordance with the guidelines of the Egyptian Research Ethics Committee and the guidelines specified in the Guide for the Care and Use of Laboratory Animals (2024). Ethical code number ZU-IACUC/2/F/489/2024. Written informed consent was obtained from the owners for the participation of their animals in this study.

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Letter to Editor

A Critical Choice in Data Presentation: "Should Standard Deviation (SD) or Standard Error of the Mean (SEM) be Given?"

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Dear Editor

In scientific studies conducted in the fields of veterinary, agricultural, and health sciences, the manner of data presentation is of paramount importance for the accurate interpretation of findings and the transparent communication of information. In this context, the question of whether to report the standard deviation (SD) or the standard error of the mean (SEM) as a measure of variability alongside the mean has led to persistent confusion and erroneous practices in the literature. This letter aims to clarify the functional differences between these two fundamental statistical measures and to emphasize correct usage practices in the interest of upholding scientific integrity.

The fundamental distinction lies in what these two measures represent. The standard deviation (SD) is a descriptive statistic that indicates how much individual data points in a sample deviate from that sample's mean. Its purpose is to reveal the internal spread or biological variability of the data. In contrast, the standard error of the mean (SEM) is an inferential statistic that indicates how precisely the sample mean estimates the true population mean (SEM = SD/$\sqrt{n}$). Consequently, these two concepts answer different scientific questions and cannot be used interchangeably [1].

However, it is frequently observed in the scientific literature that SEM is incorrectly used to mask the true variability of the data. Because SEM decreases as the sample size (n) increases, it makes error bars appear narrower, creating a misleading impression that the data are less variable and more precise than they actually are [2]. This situation can cause the reader to misjudge the overlap between groups and to exaggerate the significance of the findings. One study highlighted how this deceptive perception of precision adversely affects scientific communication and urged researchers to make a conscious choice in this regard [3]. Similarly, an analysis of animal experiments emphasized that the use of SEM does not reflect true biological variation and that SD should be preferred, as required by the principle of scientific transparency [4].

The scientific consensus on this issue is also reflected in the publication guidelines of prestigious journals. For instance, a study in the field of veterinary medicine has stated that adopting the mean ± SD format for presenting

experimental results should be a standard for data transparency and reproducibility [5]. Likewise, articles published in prominent journals like the BMJ have repeatedly warned against the potential of SEM to conceal variability [1,6].

In conclusion, when presenting their data, researchers should ask themselves the following fundamental question: Is my goal to demonstrate the spread and heterogeneity of the data within my sample, or is it to report the precision with which my sample mean estimates the population mean? If the purpose is descriptive -that is, to show the distribution of the data itself- then the correct and transparent choice is unequivocally the standard deviation (SD). The standard error of the mean (SEM) is a technical component used in inferential statistics for calculating confidence intervals and should only be reported in cases where its purpose is explicitly defined in the text.

In accordance with the principles of transparency and integrity that form the foundation of scientific publishing, authors, reviewers, and editors must be more meticulous regarding this critical distinction. This is an indispensable step for the credibility of science and the correct interpretation of findings.

Sincerely.

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ETHICAL PRINCIPLES AND PUBLICATION POLICY

Kafkas Universitesi Veteriner Fakultesi Dergisi follows and implements internationally accepted ethical standards to provide the necessary support to original scientific ideas and to publish high quality, reliable scientific articles in this direction. The journal's publication policy and ethical principles include the ethical standards of conduct that should be followed by author(s), journal editor(s), associate editors, subject editors, reviewers, and publishers who are the participants of this action.

The ethical statement of Kafkas Universitesi Veteriner Fakultesi Dergisi is based on the principles indicated in the "COPE Code of Conduct and Best Practice Guidelines for Journal Editors" (http://publicationethics.org/files/Code_of_conduct_for_journal_editors_Mar11.pdf) and "COPE Best Practice Guidelines for Journal Editors" (http://publicationethics.org/files/u2/Best_Practice.pdf).

GENERAL ETHICAL PRINCIPLES

• Objectivity and Independence

Editor-in-chief, editors, associate editors, and referees conduct the evaluation process of the manuscript sent to the journal objectively and in coordination within the framework of ethical principles. Editorial decisions are independent, and internal or external factors cannot influence these decisions. In accordance with the principle of impartiality, academics working in our institution are not deemed eligible to work as a section editor in Kafkas Universitesi Veteriner Fakultesi Dergisi, in order not to be effective in the evaluation of articles due to conflict of interest.

• Privacy

The content of the articles and the personal information of the authors such as name, e-mail address, and telephone numbers that are sent to Kafkas Universitesi Veteriner Fakultesi Dergisi are used only for the scientific purposes of the journal and not for other purposes, and cannot be shared with third parties. Article evaluation processes are also carried out confidentially.

• Authorship and Authors Rights

The authors of the manuscripts sent to Kafkas Universitesi Veteriner Fakultesi Dergisi must have contributed significantly to the design, execution or interpretation of the study. For example, in view of the research and publication ethics as well as authors rights, it is not acceptable to include those as authors who do not actively contribute to the research but just only help in writing or data collection processes, which may not require any scientific knowledge. All the authors in a publication should be in agreement of the names and the orders of the authors in the manuscript.

The competence of the authors to the subject of the study is evaluated by the editor within the framework of deontological rules and the professional fields of each author.

The corresponding author of the article should declare the contributions of the authors to the work under the title of "Author contributions". The corresponding author is primarily responsible for the problems that may arise in this regard.

In multidisciplinary studies, 2 authors who are from different disciplines can be "equivalent first name authors" and up to most 3 authors who are also from different disciplines can be "equivalent second name authors".

• Generative Artificial Intelligence (AI)

This declaration outlines the acceptable uses of generative AI technology in writing or editing manuscripts submitted to Kafkas Universitesi Veteriner Fakultesi Dergisi. During the writing process, AI and AI-assisted technologies are prohibited from writing or creating the article, tables, or figures. Authors should use AI and AI-assisted technologies solely to enhance the readability and language of the article. Authors should carefully review and edit the result of assisted parts of the manuscript by AI in term of reliability of the applying technologies.

When using generative AI and AI-assisted technologies in scientific writing, authors must declare this by including a statement in the cover letter when the article is first submitted. Once the article is accepted for publication, this statement should be included in the declarations section of the manuscript's final part.

If anything other than the declared conditions is detected, the articles in the evaluation stage will be rejected, and if it is detected in the published articles, the article will be retracted.

• Originality of Research Findings

The authors should declare that the article they presented contained the original research results, that the study data were analyzed correctly, and that they were prepared for publication using adequate and appropriate references, in the "cover letter" section of the on-line system at the submission stage. Using expressions such as "it is the first study done", "there has been no previous study on this subject" and "there is a limited number of studies" to add originality and importance to the article is not acceptable and may cause prevention of the scientific evaluation of the article by the editor.

• Similarity

Articles submitted to the journal are subjected to similarity analysis using appropriate software (iThenticate by CrossCheck) at the beginning and at every required stage. If unethical similarities are detected regardless of the rate of similarity, this situation is reported to the authors and corrections are requested or articles containing excessive similarities are rejected at the first evaluation stage without being evaluated.

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Kafkas Universitesi Veteriner Fakültesi Dergisi applies publication ethics and verifies the originality of content submitted before publication and checks all submitted manuscripts for plagiarism/self-plagiarism, similarity and duplication. All submitted manuscripts are meticilously screened by a similarity detection software (iThenticate by CrossCheck). Papers previously presented at scientific meetings and published only as an "abstract" should be indicated in the Title Page file as stated in the "Guidance for Authors". Authors do not have the right to use entire paragraphs from their previous publications into a new submission. These actions are also considered as a plagiarism. In any case, the manuscript should be original in terms of scientific contents and writing. In the event of alleged or suspected research misconduct, the Editorial Board will follow and act in accordance with "COPE Guidelines".

• Multi-part Publication (Piecemeal Publication)

Some authors may tend to divide study data into two or more articles and publish the results in different journals also having different authors names and orders. In principal, Kafkas Universitesi Veteriner Fakultesi Dergisi is against multi-part publication. When necessary, the ethical committee approval information of the study, project information, congress presentations, etc. are checked and such situations that will create an ethical problem are identified and reported to the authors.

Authors may think that their work should be published in multi parts that complement each other. For this, each part of the article should be titled "Part-I", "Part-II" and submitted to the journal "simultaneously". This issue can be evaluated by the editor-in-chief/subject editors/referees who may suggest that the article can be published in parts or as a whole. In addition, rejection of a submission presented in parts means that all parts will be rejected.

• Animal Rights and Ethics

The authors are responsible for conducting experimental and clinical studies on animal experiments within the framework of existing international legislation on animal rights. Authors must also obtain permission from the Animal Experiment Ethics Committees and provide relevant information in the Material and Method section to experiment with animals. In clinical studies, as well as the approval of the ethics committee, an "informed consent form" should be obtained from the animal owners and the information related to it should be declared in the Material and Method section. Declaration of "informed consent form" is sufficient for the articles in the "Case report" and "Letter to the Editor" category.

Ethics committee permission taken for a study can only be used in one article. It is unacceptable to use the same ethics committee approval number in articles with different names and contents. The editor/subject editors can request from the corresponding author, if necessary, to send a copy of the ethics committee approval form to the journal (electronically or by post).

In cases of violation ethical rules, the article is not taken into consideration or if it is in the evaluation stage, the procedure is terminated and the article is rejected.

• Conflicts of Interest/Competing Interests

The editor-in-chief pays attention to whether there is a conflict of interest or union of interest between editors, reviewers and author (s) for an objective and unbiased evaluation of the article. In addition, the authors should disclose any financial interests or links or any conditions that may raise the bias issue in research and article under the above heading.

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• Withdrawal of a Submission

In case of if the authors detect a significant error or deficiency in their article under review or if this error is reported to them by the editor/subject editor/referees they can contact immediately to the editor-in-chief and ask the request to withdraw the article by stating the reason. The decision on this issue is up to the editorial board.

• Erratum

After an article has been published, the corresponding author may request the editor to publish "erratum" for any errors or inaccuracies noticed by the authors, editors or readers. In collaboration with the authors, the editor prepares and publishes the Erratum article in the first upcoming issue. These articles, like other publications, should contain the publication tag and DOI number.

• Retraction

If any ethical problem is detected about the article that cannot be compensated and cannot be eliminated with erratum after the article is published, the editor-in-chief and associate editors prepare a justification about the article and apply the retraction procedure to the article. The text file on the web page of a retracted article is blocked and the reason for retraction is added to the system as a file, ensuring that it is constantly in the archive.

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ARTICLE EVALUATION AND PUBLICATION PROCESS

• Initial Evaluation Process

Articles submitted to Kafkas Universitesi Veteriner Fakultesi Dergisi are primarily evaluated by the editors and associate editors. At this stage, articles not having suitable scope and aims, with low original research value, containing scientific and ethically important errors, having low potential to contribute to science and the journal, and having poor language and narration are rejected by the editor without peer-review process. Initial evaluation process takes up to most 2 weeks.

• Preliminary Evaluation Process

Articles that are deemed appropriate for editorial evaluation are sent to the subject editor related to the category of articles to be examined in terms of scientific competence and to the statistics editor for evaluation in terms of statistical methods. The suject editors examine the article in all aspects and report their decisions (rejection, revision or peer-review) to the chief editor. This stage takes about 1 month.

• Peer-review Process

Double-blind peer-review is applied to the articles that have completed preliminary evaluation process. Suggestions of subject editors are primarily considered in referee assignment. In addition, reviews can be requested from the referees registered in the journal's referee pool. At least 2 referees are assigned for peer-review. Opinion of more referees can be required depending on the evaluation process. At this stage, referees send their decision (reject, revision or accept) about the article to the editor-in-chief. If the rejection decision given by a referee reflects sufficient examination and evidence-based negativities or ethical problems about the scientific content and accuracy of the article, this decision is checked by the editor-in-chief and associate editors and submitted to the authors regardless of the other referees' decisions. The time given to referees to evaluate an article is ~4 weeks.

• Publication Process of an Article

Total evaluation period of an article, which is completed in the peer-review phase after completing the initial and preliminary evaluation process, takes 4-6 months. The articles that have completed the subject editorial and peer-review evaluation stages and accepted by the editorial are sent to the corresponding author for final checks and necessary final additions. After the acceptance, the article designed in the publication format of the journal is given an DOI number and published immediately on the Article in Press page. When it is time to publish the periodic edition of the journal, a selection is made from the articles kept on the Article in Press page, taking into account the submission date. The time it takes for the article to be published by taking the page number is 6-12 months.

NO PUBLICATION FEE

Processing and publication are free of charge with the journal. There is no article processing charges, submission fees or any other fees for any submitted or accepted articles.

RESPONSIBILITIES OF THE PUBLISHER, EDITORS AND ASSOCIATE EDITORS

The publisher (Dean of the Faculty of Veterinary Medicine of Kafkas University) contributes to the execution of the journal's routine processes such as printing, archiving, and mailing, in line with requests from the editor.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and assistants in the article evaluation process and decisions.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and associate editors in the article evaluation process and decisions.

Editor-in-chief/editors/associate editors of Kafkas Universitesi Veteriner Fakultesi Dergisi evaluate the articles submitted to the journal regardless of their race, gender, religious belief, ethnicity, citizenship or political views. In addition, it undertakes not to give any information about the article except for the authors, subject editors and referees.

Kafkas Universitesi Veteriner Fakultesi Dergisi follows internationally accepted principles and criteria and takes the necessary decisions to apply in the journal.

Editor-in-chief/editors/associate editors conduct the evaluation and decision process in the journal in coordination within the principles of confidentiality and have independent decision-making authority and responsibility without being affected by any internal or external factors.

Editor-in-chief/editors/associate editors make and implement all kinds of planning for the development of the journal and its international recognition. They also follow national and international meetings or events on the development of journals and article evaluation, and ensures that the journal is represented on these platforms.

The editor-in-chief/editors/associate editors make every effort to ensure that the journal's subject editors and referee pool have international qualifications. Likewise, it makes the necessary attempts to strengthen the author's profile.

Editor-in-chief/editors/associate editors make plans to improve the quality of the articles published in the journal and carry out the necessary process.

Editor-in-chief/editors/associate editors regularly conduct and control the initial evaluation, preliminary evaluation, peer review and acceptance-rejection decisions of articles submitted to the journal. While carrying out these procedures, features such as the suitability of the study for the aims and scope of the journal, its originality, the up-to-date and reliability of the scientific methods used, and the potential it will contribute to the development of the journal as well as its benefit to science/practice are taken into consideration.

Editor-in-chief/editors/associate editors systematically review, inspect and make decisions about the articles submitted to the journal in terms of features such as author rights, conflict of interest, observance and protection of animal rights, and compliance with research and publication ethics.

The editor-in-chief conducts the evaluation/revision process between the authors and subject editors and referees, and ensures that it is completed within the prescribed time.

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ARCHIVE POLICY

The editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi and the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) keep all the articles (electronic and printed) published in the journal in their archives. All articles and their attachment files sent to the journal are kept securely in the archive. In light of the technological developments, the editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi regularly performs electronic processes for the development and updating of materials in digital environment and presents them to its readers on condition of keeping in safe the original documents and information regarding the articles.

Even if the journal ceases to be published for any reason, the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) will continue to protect the journal content in the long term and provide convenient access to users. Electronic services of Kafkas University Information Technologies Department will be used for the journal to maintain this responsibility.

RESPONSIBILITIES OF SUBJECT EDITORS

Subject editors do reviews and evaluations in accordance with the main publication goals and policies of the journal and in line with the criteria that will contribute to the development of the journal.

Author information is kept confidential in articles sent to the subject editor for preliminary evaluation by the editor.

Subject editors thoroughly examine the sections of the introduction, materials and methods, results, discussion and conclusion, in terms of journal publication policies, scope, originality and research ethics. Subject editor submits its decision (rejection, revision or peer-review) after evaluation to the chief editor in a reasoned report.

Subject editor may request additional information and documents related to the study from the authors, when necessary.

In multidisciplinary studies, the article can be submitted for the evaluation of multiple subject editors.

RESPONSIBILITIES OF REFEREES

Double-blinded peer-review procedure is applied in Kafkas Universitesi Veteriner Fakultesi Dergisi in order to evaluate the articles submitted to the journal in accordance with the principle of impartiality and in objective criteria; that is, referees and writers do not know about each other.

The referees submit their opinions and reports to the editor-in-chief to ensure the control and suitability of a submitted article, its scientific content, scientific consistency and compliance with the principles of the journal. When a referee makes a decision "reject" about an article, he/she prepares the reasons for the decision in accordance with the scientific norms and presents it to the editor.

The referee(s) also gives the authors the opportunity to improve the content of the article. Accordingly, the revisions requested from the authors should be of a quality that explains/questions specific issues rather than general statements.

Referees appointed for the evaluation of the articles agree that the articles are confidential documents and will not share any information about these documents with third parties, except for the editors participating in the evaluation.

Referees should place their criticism on scientific infrastructure and write their explanations based on scientific evidence. All comments made by the referees to improve the articles should be clear and direct, and should be written away from disturbing the feelings of the author. Insulting and derogatory statements should be avoided.

If a referee has an interest relationship with the author(s) on one or more issues, he/she must report the situation to the editor and ask his/her to withdraw from the referee position. The same is also applicable when the authors illegally obtain information about the referees of the article and try to influence them.

The editor-in-chief can share the comments and reports from the referees with the editors/associate editors and the relevant subject editor, as necessary, to ensure that the decision on the article is optimal. If necessary, the editor may share the critical decision and its grounds that a referee has sent about the article with the other referee(s) and present them to their attention.

Referee(s) may request revision many times for the article they evaluated.

The content of the referee reports is checked and evaluated by editor-in-chief/editors/associate editors. The final decision belongs to the editorial.

RESPONSIBILITIES OF AUTHOR(S)

It is not tolerable for the author (s) to send an article, which has been already sent to another journal, to Kafkas Universitesi Veteriner Fakultesi Dergisi within the scope of "which accepts" or "which publishes first" approach. If this is detected, the article is rejected at any stage of the evaluation. As a possible result of these actions, in the process following the previous acceptance of the article sent to another journal, the withdrawal request with this excuse that the authors submit for this article, the evaluation process of which is going on in our journal, is evaluated by the editors and associate editors of the journal and disciplinary action on the grounds of ethical violations about those responsible is started. This unethical action is also informed to the journal editor (if known) who accepted the article.

It is essential that the articles to be sent to Kafkas Universitesi Veteriner Fakultesi Dergisi include studies that have up-to-date, original and important clinical/practical results and prepared in accordance with the journal's writing rules.

Authors should choose the references they use during the writing of the article in accordance with the ethical principles and cite them according to the rules.

The authors are obliged to revise the article in line with the issues conveyed to them during the initial evaluation, preliminary evaluation and peer-review phases of the article and to explain the changes they made/did not make sequentially in the "response to editor" and "response to reviewer comments" sections.

If information, documents or data regarding to the study are requested during the evaluation process, the corresponding author is obliged to submit them to the editorial.

Authors should know and take into account the issues listed in the "General Ethical Principles" section regarding scientific research and authors.

The authors do not have the right to simultaneously submit multiple articles to Kafkas Universitesi Veteriner Fakultesi Dergisi. It is more appropriate to submit them with acceptable time intervals for the journal's policy.

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INSTRUCTION FOR AUTHORS

1- Kafkas Universitesi Veteriner Fakultesi Dergisi (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly (E-ISSN: 1309-2251). We follow a double-blind peer-review process, and therefore the authors should remove their name and any acknowledgment from the manuscript before submission. Author names, affiliations, present/permanent address etc. should be given on the title page only.

The journal publishes full-length research papers, short communications, preliminary scientific reports, case reports, observations, letters to the editor, and reviews. The scope of the journal includes all aspects of veterinary medicine and animal science.

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2- The official language of our journal is English.

3- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing, and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table and their appropriate position should be indicated in the text. Refer to tables and figures in the main text by their numbers. Also figure legends explanations should be given at the end of the text.

The figures should be at least 300 dpi resolution.

The manuscript and other files (figure etc.) should be submitted by using online manuscript submission system at the address of http://vetdergi.kafkas.edu.tr/

During the submission process, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the Copyright Agreement Form signed by all the authors should be sent to the editorial office.

4- The authors should indicate the name of the institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, the editorial board may also request the official document of the ethical commission report. In case reports, a sentence stating that "informed consent" was received from the owner should be added to the main document. If an ethical problem is detected (not reporting project information, lack of ethical committee information, conflict of interest, etc.), the editorial board may reject the manuscript at any stage of the evaluation process.

5- Authors should know and take into account the "Generative Artificial Intelligence (AI)" and other matters listed in the **"Ethical Principles and Publication Policy"** section regarding scientific research and authors.

6- Types of Manuscripts

Original (full-length) manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, introduction, material and methods, results, discussion, and references and it should not exceed 12 pages including text. The number of references should not exceed 50. The page limit does not include tables and illustrations. Abstract should contain 200±20 words.

Short communication manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but the abstract should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages in total. The page limit does not include tables and illustrations. Additionally, they should not contain more than 4 figures or tables.

Preliminary scientific reports are a short description of partially completed original research findings at an interpretable level. These should be prepared in the format of full-length original articles. The length of the text should be no longer than 4 pages in total.

Case reports describe rare significant findings encountered in the application, clinic, and laboratory of related fields. The title and abstract of these articles should be written in the format of full-length original articles (but the abstract should not exceed 100 words) and the remaining sections should be followed by the Introduction, Case History, Discussion and References. The reference numbers should not exceed 15 and the length of the text should be no longer than 4 pages in total. The page limit does not include tables and illustrations.

Letters to the editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases. The length of the text should be no longer than 3 pages in total. The page limit includes tables and illustrations.

Reviews are original manuscripts that gather the literature on the current and significant subject along with the commentary and findings of the author on a particular subject (It is essential that the author/s have international scientific publications on this subject). The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should be followed by introduction, text (with appropriate titles), conclusion, and references.

"Invited review" articles requested from authors who have experience and recognition in international publishing in a particular field are primarily published in our journal.

Review articles submitted to our journal must be prepared in accordance with any of the three categories listed below.

Narrative reviews describe current published information on a scientific topic. However, it does not include a specific methodological process.

Systematic reviews include the search for original studies published in that field on a specific topic, the evaluation of validity, synthesis and interpretation within a systematic methodology.

Meta-analysis is a method of evaluating the results of many studies on a subject with the methods defined in this category and statistical analysis of the obtained findings.

7- The necessary descriptive information (thesis, projects, financial supports, etc.) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of the title.

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If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in a book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of the book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

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9- Latin expression such as species names of bacteria, virus, parasite, and fungus and anatomical terms should be written in italic character, keeping their original forms.

10- The editorial board has the right to perform necessary modifications and a reduction in the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by the editorial board, the article can only be published after the approval of the field editor and referee/s specialized in the particular field.

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12- The editorship may request the language editing of the manuscript submitted to the journal. If the article is accepted, it will not be published without language editing. Before publication, a declaration and/or certificate stating that proofreading is done by a registered company will be requested from the corresponding author.

13- No fee is charged at any stage in Kafkas Üniversitesi Veteriner Fakültesi Dergisi (No APC/APF)

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