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A SYSTEMATIC REVIEW AND META-ANALYSIS OF CATTLE TICK INFESTATIONS AND ASSOCIATED RISK FACTORS IN SOUTH AFRICA

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Abstract

Cattle farming is a vital component of the South African agricultural landscape through its contribution to sustainable livelihoods and stability of the national economy. The South African cattle production system has various genotypes that succumb differently to environmental and management-related stressors. Tick-borne diseases are among the challenges faced by cattle farmers in South Africa. Ticks tend to evolve with time and their characterization is key for effective control measures and treatment of tick-related diseases. A systematic review of the tick species was carried out and risk factors associated with tick infestations in different cattle genotypes in South African communities were identified. Twenty-four studies covering the period from 2002 to 2022 were reviewed. The studies reported a diversity in cattle sample sizes that ranged from 52 to 650. The genotypes comprised Nguni, Bonsmara, Hereford, and non-descript cattle breeds. In summary, results showed that tick infestations were widespread in cattle. The most frequently reported tick species are *Rhipicephalus decoloratus*, *Rhipicephalus microplus*, *Amblyomma variegatum*, and *Hyalomma truncatum*. The review further indicates tick infestation as a significant challenge to communal cattle farmers in South Africa, affecting animal health, productivity, and economic well-being. The evaluation of risk factors has revealed an intricate interaction among animals, environmental, and management factors influencing the prevalence and intensity of tick infestations. Understanding the significance of these factors is essential for implementation of successful tick control measures, which should consider environmental variations, address the challenge of acaricide resistance through measures

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such as rotational grazing, and emphasize selection of tick-resistant genes.

Key words: Acaricide resistance, *Amblyomma variegatum*, cattle genotypes, *Rhipicephalus decoloratus*, tick-borne, tick-resistance

SISTEMATSKI PREGLED I META ANALIZA INFESTACIJA GOVEDA KRPELJIMA I FAKTORI RIZIKA U JUŽNOJ AFRICI

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Kratak sadržaj

Stočarstvo je ključna komponenta poljoprivrenog sektora Južne Afrike zbog doprinosa održivom razvoju i stabilnosti nacionalne ekonomije. Proizvodnja stoke u Južnoj Africi sastoji se od raznih genotipa koji različito reaguju na izvore stresa iz životne sredine kao i one vezane za menadžment. Jedan od izazova stočarske proizvodnje u Južnoj Africi su i bolesti koje prenose krpelji. Krpelji se tokom vremena razvijaju i njihova karakterizacija je ključa za efektivno sprovođenje mera kontrole kao lečenje bolesti koje oni izazivaju. Izvršen je sistematski pregled vrsta krpelja, a identifikovani su faktori rizika povezani sa infestacijama krpeljima kod različitih genotipova goveda u zajednicama Južne Afrike. Izvršena je analiza 24 studije iz perioda od 2002. do 2022. godine. Studije su otkrile da postoji raznolikost u veličini uzoraka, u rasponu od 52 to 650. Genotipi su se sastojali od rasa Nguni, Bonsmara, Hereford kao i nekih nedefiniranih rasa. Rezultati su pokazali da su infestacije krpeljima kod stoke izuzetno rasprostranjene. Najčešće vrste koje su pronađene u studijama bile su *Rhipicephalus decoloratus*, *Rhipicephalus microplus*, *Amblyomma variegatum*, i *Hyalomma truncatum*. Pregled dalje navodi da infestacija krpeljima predstavlja veliki izazov za farmere u Južnoj Africi kao i da utiče na zdravlje životinja, proizvodnju i ekonomiju. Procena faktora rizika pokazala je da na infestaciju krpeljima utiču faktori kao što su životna sredina, interakcija između životinja kao i upravljanje farmama. Razumevanje značaja ovih faktora je ključno za implementaciju uspešnih mera kontrole krpelja. Potrebno je uzeti u obzir

promene u životnoj sredini, rešiti problem otpornosti na akaricide kroz mere poput rotacionog sistema ispaše kao i naglasiti važnost selekcije gena otpornih na krpelje.

Ključne reči: Otpornost na akaricide, *Amblyomma variegatum*, genotipi stoke, *Rhipicephalus decoloratus*, bolesti koje prenose krpelji, otpornost na krpelje

INTRODUCTION

Ticks have severe implications on animal health due to their ability to transmit various diseases. Al-Hosary et al. (2021) stated that ticks are hematophagous arthropods that suck out blood from the host resulting in severe economic losses due to anemia, damage to animal hides, weight, and production losses as well as toxicoses. Furthermore, ticks act as vectors of pathogens such as *Theileria*, *Babesia*, *Ehrlichia*, and *Anaplasma* species which are the causative agents of tick-borne diseases in animals. Tick-borne diseases lead to losses through livestock mortality and decreased productivity, resulting in significant economic setbacks and adverse effects on food security. Global annual livestock losses due to tick-borne diseases and the costs associated with treatment are estimated to be between USD 22-30 billion, while annual losses due to heartwater disease in South Africa are estimated to be around USD 1.059 million (Makwarela et al., 2023). Nuttall (2022) observed that the presence and behavior of ticks in a geographical area are highly influenced by the local climate conditions. Changes in climate conditions can result in higher prevalences of ticks and tick-borne diseases. Makwarela et al. (2023) reported that tick species tend to be more active in warmer and humid climates as these conditions are suitable for metamorphosis. Yusufmia et al. (2010) noted that the generally warmer climate conditions in the southern African region promote the prevalence of various genera of ixodid ticks infesting cattle such as *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, and *Rhipicephalus* spp. Santos et al. (2022) reported genetic diversity in cattle populations as being one of the major factors that may influence cattle susceptibility to tick-borne diseases and the effectiveness of tick control measures. For example, the Nguni cattle breed is reported to possess a higher genetic resistance to ticks compared to other cattle breeds in South Africa. Muchenje et al. (2008) indicated that environmental factors, management practices, and climate conditions can further complicate the dynamics of tick infestations and their associated risks. Therefore, understanding the characteristics of tick populations

and risk factors influencing their prevalence is vital for developing targeted control strategies. This systematic review aims to highlight the existing knowledge on tick characteristics, infestations, and risk factors specific to various cattle genotypes in South Africa thereby contributing to improved cattle health care, sustainable agricultural practices, and in turn national economic stability.

MATERIAL AND METHODS

Article eligibility criteria

This review followed the principles and recommendations outlined in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement, as specified by (Page et al., 2021).

Search strategy

A comprehensive literature search was conducted to identify the studies on cattle tick infestations and associated risk factors in South Africa. The databases searched include PubMed, Google Scholar, Science Direct, and ResearchGate. The search was conducted using the key terms “tick species identification,” “prevalence,” “risk factors,” “mitigation strategies,” “cattle tick infestations,” and “South Africa”. Boolean operators (AND, OR) were used to refine the search. Reference lists of relevant articles were also screened for additional studies.

Article selection criteria

The criteria used to determine eligibility and inclusion in this search were studies focusing on tick characteristics and risk factors on different cattle genotypes within twenty-year period, from 2002 to 2022, and published in the English language. Figure 1 presents a PRISMA flowchart illustrating the filtering process.

Data extraction

The researchers extracted the study content and carefully reviewed it to ensure agreement on all key elements. To maintain the data accuracy and quality, each article underwent a thorough review, and the necessary information was carefully documented.

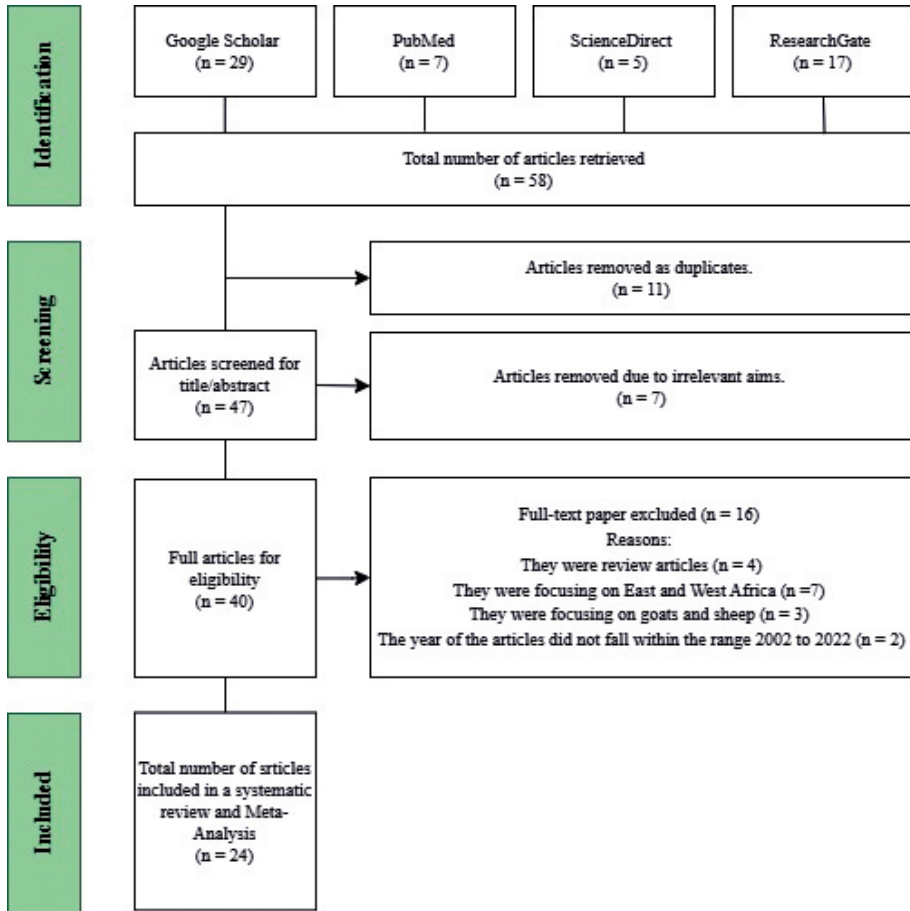


Figure 1. PRISMA flow diagram according to Page et al. (2021)

RESULTS

The outcome of the literature search

A total of 58 papers were gathered from four databases: Google Scholar (29), PubMed (7), ScienceDirect (5), and ResearchGate (17). After the initial screening, 11 duplicate articles were removed, leaving 47 articles for further consideration. During the title and abstract screening process, 7 papers were excluded because they had different aims. Subsequently, 40 articles were

deemed ineligible after screening. Upon full-text analysis, 16 papers were excluded; 4 were reviews, 7 originated from the countries outside of South Africa, 3 focused on sheep, goats, and non-domesticated animals and 2 did not cover the years 2002 to 2022. Twenty-four articles were considered appropriate and are discussed in this review.

Characteristics of studies that were included in the systematic review

Table 1 summarizes the key characteristics of the studies that formed part of this review. These studies primarily relied on morphological methods for tick identification in multiple communities across South Africa. Based on the studies, *Rhipicephalus* (*Boophilus*) species were the predominant tick species. This observation was consistent across numerous studies such as Nyangiwe and Horak (2013), Mapholi et al. (2017), and Dzemo et al. (2023). The studies varied in terms of sample sizes, with the number of cattle genotypes sampled ranging from 52 to 650. The cattle genotypes included Nguni, Bonsmara, Hereford, and non-descript cattle breeds. Most (88%) of the eligible studies focused on tick sampling from non-descript and Nguni cattle breeds.

Table 1. Characteristics of the reviewed studies

Authors and Year	Location	Province	Cattle breed	Sample size (cattle)	Tick species identified
Bryson et al., 2002	Rietgat, Madinyane, Bethany, and Geluk	Northwest	Non-descript cattle breed	110	<i>Amblyomma hebraeum</i> , <i>Boophilus decoloratus</i> , <i>Boophilus microplus</i> , <i>Hyalomma marginatum rufipes</i> , <i>Hyalomma truncatum</i> , and <i>Rhipicephalus tricuspis</i> .
Mapholi et al., 2022	Ulundini	KwaZulu-Natal	Bonsmara	125	<i>Amblyomma hebraeum</i> , and <i>Rhipicephalus appendiculatus</i>

Authors and Year	Location	Province	Cattle breed	Sample size (cattle)	Tick species identified
Choopa, 2015	Mnisi	Mpumalanga	Non-descript cattle breed	145	<i>Rhipicephalus (Boophilus) microplus</i> , <i>Rhipicephalus decoloratus</i> , <i>Amblyomma hebraeum</i> , <i>Rhipicephalus appendiculatus</i> and <i>Rhipicephalus evertsi evertsi</i> .
Dzemo et al., 2023	Bozisa, Mapuzi, Zanci, Mpafane and Ndakana	Eastern Cape	Nguni and Bonsmara	75	<i>Rhipicephalus (Boophilus) microplus</i>
Guo et al., 2019	Amathole, Hooningkloof, and Umsinga	Eastern Cape	Non-descript cattle breed	102	<i>Amblyomma hebraeum</i>
Hlatshwayo and Mbati, 2005	Bochabela, Namahadi, Tseki, and Lejwaneng	Free State	Non-descript cattle breeds	98	<i>Rhipicephalus (Boophilus) microplus</i> , <i>Rhipicephalus decoloratus</i> , <i>Amblyomma hebraeum</i> , <i>Rhipicephalus appendiculatus</i> and <i>Rhipicephalus evertsi evertsi</i> .
Katswara and Mukaratirwa, 2021	Lucingweni, Mthata, and Caquba	Eastern Cape	Non-descript cattle breed	76	<i>Amblyomma hebraeum</i> , <i>Rhipicephalus appendiculatus</i> and <i>Rhipicephalus (Boophilus) microplus</i>
Makgabo et al., 2023	Mnisi	Mpumalanga	Non-descript cattle breed	105	<i>Rhipicephalus decoloratus</i>

Authors and Year	Location	Province	Cattle breed	Sample size (cattle)	Tick species identified
Manamela et al., 2001	Ixopo, Umviti, and Ladysmith	KwaZulu-Natal	Non-descript cattle breed	450	<i>Rhipicephalus appendiculatus</i> and <i>Rhipicephalus evertsi evertsi</i> .
Mapholi, 2015	Matatiele	Eastern Cape	Nguni and Bonsmara	650	<i>Rhipicephalus (Boophilus) microplus</i>
Mapholi et al., 2017	Mukhuthali Nguni community	KwaZulu-Natal	Nguni	87	<i>Rhipicephalus (Boophilus) microplus</i> , <i>Rhipicephalus decoloratus</i> , <i>Amblyomma hebraeum</i> , <i>Rhipicephalus appendiculatus</i> and <i>Rhipicephalus evertsi evertsi</i> .
Marufu et al., 2010	Magwiji and Cala	Eastern Cape	Nguni	119	<i>Rhipicephalus appendiculatus</i> , <i>Rhipicephalus evertsi evertsi</i> , <i>Rhipicephalus (Boophilus) microplus</i> , and <i>Hyalomma spp.</i>
Marufu, 2008	Magwiji, and Ukhahlamba.	Eastern Cape and Kwa-Zulu-Natal	Nguni	418	<i>Amblyomma hebraeum</i>

Authors and Year	Location	Province	Cattle breed	Sample size (cattle)	Tick species identified
Marufu et al., 2011	Sakhisizwe	Eastern Cape	Nguni	149	<i>Rhipicephalus appendiculatus</i> , <i>Rhipicephalus evertsi evertsi</i> , <i>Rhipicephalus (Boophilus) microplus</i> , and <i>Hyalomma spp.</i>
Moyo et al., 2009	Cala	Eastern Cape	Non-descript cattle breed	59	<i>Rhipicephalus (Boophilus) microplus</i>
Moyo and Masika, 2009	Qolora and Nontshinga	Eastern Cape	Nguni and Bonsmara	52	<i>Rhipicephalus (Boophilus) microplus</i> , <i>Rhipicephalus decoloratus</i> , <i>Amblyomma hebraeum</i> , <i>Rhipicephalus appendiculatus</i> and <i>Rhipicephalus evertsi evertsi</i> .
Msimang et al., 2021	Mokala	Northern cape	Non- de-script cattle breed	579	<i>Hyalomma truncatum</i> and <i>Hyalomma rufipes</i>
Ndlovu et al., 2009	Switsha, Nyandeni, Matobo, and Gwanda	Matabeleland South	Non-descript cattle breed	286	<i>Amblyomma hebraeum</i> , <i>Boophilus decoloratus</i> , <i>Rhipicephalus simus</i> , <i>Hyalomma truncatum</i> , and <i>Rhipicephalus tricuspis</i> .

Authors and Year	Location	Province	Cattle breed	Sample size (cattle)	Tick species identified
Ndlovu and Combrink, 2015	Skukuza	Mpumalanga	Non-descript cattle breed	348	<i>Rhipicephalus (Boophilus) microplus</i> , and <i>Rhipicephalus decoloratus</i>
Nyangiwe and Horak, 2013	Dohne	Eastern Cape	Non-descript cattle breed	146	<i>Rhipicephalus (Boophilus) decoloratus</i> and) <i>microplus</i>
Ramudzuli, 2014	Tshamutava, Maramanzhi, and Mbodi	Limpopo	Non- de- script cattle breeds	280	<i>Rhipicephalus decoloratus</i>
Randela, 2005	Louis Trichardt	Limpopo	Non-descript cattle breed	427	<i>Rhipicephalus appendiculus</i>
Spickett et al., 2011	Tswaing and Mafikeng	Northwest	Nguni, Bonsmara, and Hereford	302	<i>Rhipicephalus (Boophilus) microplus</i> , <i>Rhipicephalus decoloratus</i> , <i>Amblyomma hebraeum</i> , <i>Rhipicephalus appendiculatus</i> and <i>Rhipicephalus evertsi evertsi</i> .
Yawa et al., 2018	Ulundini, Senqu, and Walter Sezulu	Free State	Non-descript cattle breed	177	<i>Rhipicephalus decoloratus</i>

Publications by year

Figure 2 illustrates the publication timeline of the 24 studies that were reviewed in the study. These studies cover a period of over 20 years, from 2002 to 2022.

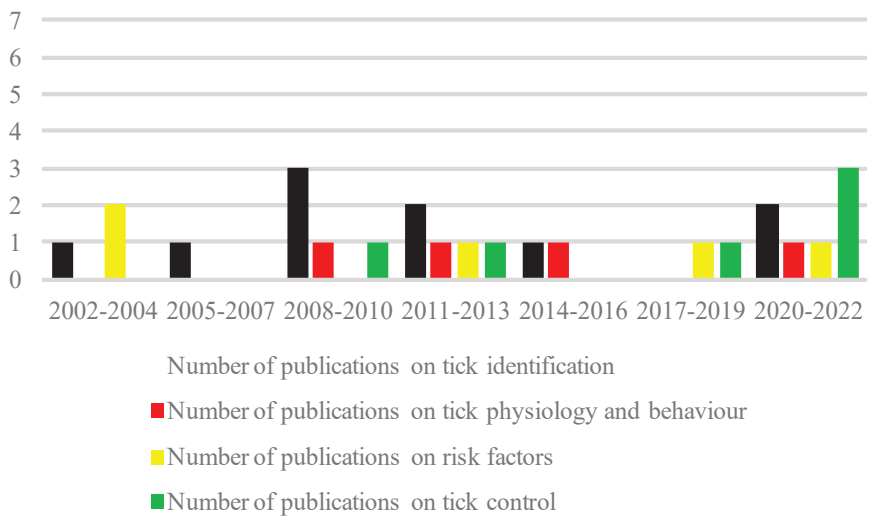


Figure 2. Number of qualifying publications during the study period (2002 to 2022)

Table 2. Cattle tick species, pathogens transmitted, and prevalence in South Africa

Tick species	Description	Pathogens transmitted	Average prevalence
<i>Rhipicephalus (Boophilus) spp.</i>	Bluish ticks with hexagonal basis capitum, short compressed and ridged palps, faint/absent anal groove	<i>Babesia bigemina</i> , <i>Babesia bovis</i> , <i>Anaplasma marginale</i> .	31.2%,7.8%, and 14.3% respectively
<i>Rhipicephalus appendiculatus</i>	Brownish, reddish-brown, or dark ticks with short palps and reddish-brown legs	<i>Theileria parva</i>	1.7%
<i>Amblyomma hebraeum</i>	Brightly ornamented ticks, with eyes and long, robust mouthparts	<i>Ehrlichia ruminantium</i>	42%
<i>Rhipicephalus evertsi evertsi</i>	Medium-sized, beady-eyed, dark brown ticks with reddish-orange legs	<i>Anaplasma marginale</i>	22%
<i>Hyalomma spp.</i>	Dark-brown-bodied ticks with numerous punctations on the scutum and long, banded legs.	<i>Anaplasma marginale</i>	5%

Source: Marufu (2008); Mapholi et al. (2022)

DISCUSSION

Physical, physiological, and behavioural characteristics of ticks

Ticks are obligate external parasites that feed on blood, belonging to the phylum Arthropoda and comprising the largest group within the Acarina order (Marufu, 2008). They are categorized into two main groups based on their behaviour and lifecycle as single-host or multiple-host (Manamela et al., 2001). Their geographical distribution varies across different climate zones; some thrive in cool and highly moist environments, while others are adapted to hot and dry climates. Ndlovu et al., (2009) reported an overall tick prevalence of 97.5% in cattle, with the most common tick species being *Rhipicephalus decoloratus*, *Rhipicephalus microplus*, *Amblyomma variegatum*, and *Hyalomma truncatum*. Table 2 shows the cattle tick species, pathogens transmitted and their prevalence in South Africa. Different tick species show varying preferences for attachment sites on the animals. For instance, *Amblyomma variegatum* is commonly found on the legs, ears, tail region, and head. *Hyalomma truncatum* prefers the tail, ear, and legs (Ndlovu et al., 2009). *Rhipicephalus decoloratus* and *Rhipicephalus pulchellus* tend to attach to the head, ear, and tail region (Ndlovu et al., 2009). *Amblyomma* ticks often cluster in large numbers and remain attached around the feet (Ndlovu et al., 2009) *A. hebraeum*, on the other hand, prefers hairless areas such as the axillae, genitalia, udder, and under the tail of the host. Various stages of tick life cycles occur on different parts of the animal's body. Larvae are often found on the face, neck, dewlap, and feet, while nymphs are commonly attached to the feet and legs, inguinal and perineal areas, sternum, and neck (Ndlovu et al., 2009). Adult ticks are usually found on larger animals like cattle and exhibit active host-seeking behaviour, climbing up the hooves and attaching themselves to their preferred feeding sites (Mapholi et al., 2017). Climate conditions play a significant role in favouring different life stages of ticks. For example, adult ticks of *A. hebraeum* are more abundant during the warm and wet summer months compared to the colder and drier late autumn and winter months (Horak et al., 2011).

Risk factors and mitigation strategies

Mapholi et al. (2017) investigated risk factors associated with tick infestations in cattle in the Northwest province of South Africa. The authors reported that age, breed, and grazing management practices were significant risk factors for tick infestations. The study emphasized the need for applying regular tick control measures and improved management practices to reduce the econom-

ic impact of tick infestations in the region. Masika et al. (1997) investigated the socio-economic factors associated with tick infestations in cattle in Zimbabwe and reported that farmers who owned larger herds, practiced transhumance, and did not use acaricides regularly were more likely to have tick-infested cattle. Eisen and Dolan (2016) discovered that farmers' age, education level, and herd size were significantly linked to tick-borne disease management practices. Monkwe et al. (2023) reported that farmers who own larger herds, have higher incomes, and are well educated were more likely to use tick control-related measures and had lower cattle tick infestation rates, while factors for tick infestation were found to vary based on tick species, cattle breed, geographic location, environmental factors, and husbandry practices.

Animal factors

Numerous studies have identified factors associated with tick infestations, which can differ depending on the tick species, region, and the animal species involved. Guo et al. (2019) indicated some animal-related factors that can contribute to tick infestations. Dzemo et al. (2023) added that factors such as hair coat type, skin thickness, and behavioural patterns influence the susceptibility of animals to ticks. Young animals such as calves or lambs are more susceptible to tick infestations due to their developing immune systems (Asrate and Yalew, 2012). In the same way, animals that are in poor health or have weakened immune systems are more susceptible to tick infestations and may suffer from more severe infestations and associated health problems. Several studies (Manamela et al., 2001, Mapholi, 2015 and Katswara and Mukaratirwa, 2021) reported that some animal breeds may exhibit greater resistance or tolerance to tick infestations. This resistance can be due to genetic factors, including innate immunity or traits that deter ticks, such as grooming behaviour or thicker skin. Indigenous cattle are reported to be resistant to most diseases including those that are transmitted by ticks (Dzemo et al., 2023). Hlatshwayo and Mbatii (2005) mentioned that, identifying, and promoting the use of genetically resistant or tolerant breeds can help in controlling tick infestations and other livestock diseases. Jongejan and Uilenberg (1994) reported that coat length and density are additional factors that contribute to creating favourable environments for ticks, particularly in animals with longer and denser fur. These types of coats provide more hiding spots for ticks, making it more difficult to detect and remove them manually. Dense coats also impede the effectiveness of acaricides. Cattle are known as bulk grazers due to their larger body sizes, which demand more forage to fulfil their nutritional requirements. As a result, cattle usually spend more time grazing to consume enough forage. During this

time, they also access tick-prone areas such as tall grass, wooded regions, or brushy vegetation, which increases the risk of tick infestations (Makwarela et al., 2023). Mapholi et al. (2022) noted that the behaviors of certain animals can enhance the likelihood of exposure to ticks. For example, animals rubbing against trees or walls can dislodge ticks from vegetation onto their bodies, increasing the chances of tick attachment. Animals that have been previously exposed to ticks may develop some level of immunity or resistance. However, this immunity can vary depending on the tick species and the specific immune response generated by the animal (Bryson et al., 2002).

Environmental factors

Environmental factors play a significant role in the prevalence and distribution of ticks, as well as their infestation rates in cattle (Rajput et al., 2006). A study by Hlatshwayo and Mbatl (2005) highlighted some environmental factors that increase the chances of tick infestations in communally raised cattle. Makgabo et al. (2023) stated that ticks are highly influenced by climate conditions with warm and humid environments being conducive to tick survival and development. Tick populations tend to increase during favourable seasons, such as spring and summer when temperature and humidity levels are optimal. In contrast, cold and dry conditions can suppress tick activity. The type and density of vegetation in an area can affect cattle tick infestation. Guo et al. (2019) stated that tick density was related significantly to vegetation index ranging from 1-5. Most ticks (15%) were observed along transects in areas with the highest vegetation index. Marufu et al. (2010) suggested that ticks are drawn to environments with tall grass, dense vegetation, and wooded areas, as these habitats provide ideal conditions for questing (waiting for a host) and seeking shelter. Overgrown pastures, areas with dense shrubs, and wooded edges near grazing areas provide suitable environments for ticks to thrive (Boyard et al., 2007). Al-Hosary et al. (2021) indicated that one of the environmental factors affecting the occurrence of ticks in cattle is grazing practices and habitat management. Continuous grazing systems can result in higher tick populations due to continuous exposure. Implementing rotational grazing can help to reduce tick infestations by allowing natural breaks in tick life cycles (Cruz-Gonzalez et al., 2023). Wildlife, such as deer or rodents, can act as reservoirs for ticks. This observation is supported by Keesing et al. (2013) who reported that cattle that graze in the areas where there is interaction with wildlife are exposed to a higher risk of tick infestations. Abbas et al. (2014) highlighted that microclimate conditions such as temperature, humidity, and

precipitation within an area can influence tick distribution since ticks are ectothermic organisms. Marufu et al. (2011) reported that high temperatures and relative humidity are favourable for tick survival, reproduction, and development. Warmth and moisture contribute to the questing behaviour of ticks, where they climb up vegetation to attach to the hosts. According to Sungirai et al. (2017) tick-refuge areas, such as tall grasses, brush piles, or shaded areas, provide shelter for ticks during adverse environmental conditions or when they are not actively questing for a host. These refuge areas can contribute to tick survival and increase the chances of infestations in cattle. The geographic location and regional prevalence of specific tick species influence the likelihood of tick infestations. Different tick species have specific habitat preferences and host ranges, which can vary across different regions.

Husbandry factors

According to a study by Ndlovu et al. (2009), the intensity and timing of cattle grazing can affect tick populations. Msimang et al. (2021) added to this observation by saying that longer grazing time can increase the chances of tick infestations. Overgrazing can reduce vegetation height, making it easy for ticks to climb on cattle. Implementing tick control measures can significantly reduce tick infestations. These measures may include the use of acaricides applied on cattle, treating premises with acaricides to reduce tick populations in the environment, or using acaricide-impregnated ear tags or collars to provide continuous protection against ticks (Byaruhanga et al., 2020). Ndlovu et al. (2009) and Asmaa et al. (2014) stated that providing suitable housing or resting areas for cattle can help reduce tick exposure. Sheltered areas or barns with concrete flooring can limit contact between cattle and tick-infested environments, reducing the chances of tick attachment. Spickett et al. (2011) recommended selecting cattle breeds or genetic lines that exhibit resistance or tolerance to tick infestations combined with guidance from veterinarians on appropriate tick control strategies, performance of regular health checks, and treatment of tick-related diseases when necessary. Bryson et al. (2002) indicated that the primary tick species affecting cattle are *Rhipicephalus (Boophilus) decoloratus*, *Rhipicephalus appendiculatus*, *Amblyomma hebraeum*, *Rhipicephalus evertsi evertsi*, and *Hyalomma marginatum rufipes*. These findings align with those of Yawa et al. (2018). Notably, small ruminants such as goats were infrequently subjected to tick control measures in rural farming communities, a pattern consistent with the observations made by Ndlovu and Combrink (2015). Consequently, small ruminants and untreated cattle served as reservoirs for ticks, contributing to

the re-infestation of treated animals. Tick management is essential to ensure the well-being and optimum productivity of cattle. Acaricides have traditionally played a significant role in integrated tick control strategies (Moyo and Masika, 2009). Proper application is critical for effectiveness, as misuse can contribute to acaricide resistance. Some farmers manually remove ticks from cattle using specialized tools. Although time-consuming, this method can be effective, especially for localized infestations. Implementing good farm management practices, such as rotational grazing and maintaining clean surroundings, can help reduce tick habitats and minimize infestations (Randela, 2005).

Mitigation strategies

Cattle tick infestations are a significant concern in South Africa due to their impact on livestock health and productivity. There are some common mitigations used in South Africa to control cattle tick infestations.

Chemical control

The primary method involves the use of acaricides, which are chemicals specifically designed to kill ticks. These can be applied to cattle through sprays, dips, or pour-on. The repeated use of a single acaricide commonly results in the rapid development of resistance (Ramudzuli, 2014). Regular monitoring of ticks for resistance to various acaricides can play a vital role in reducing the selection of resistant ticks. It is essential to educate livestock owners and farm workers on tick control and resistance management. Acaricides have different modes of action, so alternating their use can help reduce the selection for resistance. Due to the high cost of commercial acaricides, rural farmers rely on ethno-veterinary remedies such as *Ptaeroxylon obliquum*, *Aloe ferox*, *Lantana Camara*, *Tagetes minuta* as alternative tick control methods (Moyo et al., 2009). It is recommended to rotate acaricides such as pyrethroids with coumaphos (OPs) (Obaid et al., 2022).

Biological control

Introducing natural tick predators, like specific species of predatory mites or nematodes, can assist in controlling tick populations. For example, the fungus *Metarhizium anisopliae*, parasitoid wasps, *Bacillus thuriengensis*, nematodes as well as insectivorous birds have been used as a biological control agent against ticks (Rajput et al., 2006).

Pasture management

Maintaining pastures and grazing areas through practices such as mowing, controlled burning, and removing leaf litter can reduce tick habitats and population density. Implementing rotational grazing, where pastures are rested and rotated, can help break the tick life cycle and reduce the buildup of tick populations (George et al., 2004). Resting pastures after grazing can also allow for the natural desiccation of tick larvae and decrease the availability of hosts for ticks. Maintaining pastures by mowing them, clearing brush, and removing overgrown vegetation helps reduce tick habitats and decrease tick populations. Well-maintained pastures with shorter grasses and reduced vegetation density make it more challenging for ticks to quest and attach to cattle (Mapholi et al., 2017).

Tick-resistant cattle breeds

Breeding and selection of cattle breeds that show resistance or tolerance to tick infestations can reduce the need for chemical treatments. Selecting cattle breeds or genetic lines that exhibit resistance or tolerance to tick infestations can be beneficial. Some cattle breeds have inherent traits, such as grooming behaviour or thicker skin that make them less susceptible to ticks. Breeding for tick resistance can help improve overall resilience to tick infestations (Mapholi et al., 2022). Some indigenous cattle breeds in South Africa have developed natural resistance to ticks. Nguni cattle, Bonsmara, and Hereford cattle breeds have demonstrated high levels of resistance according to index determinations, indicating superior levels of natural immunity in these breeds (Spickett et al., 2011). Mapholi (2015) suggested the selective breeding of cattle with natural resistance to ticks, as certain breeds, like the Nguni, are recognized for their genetic resistance to tick infestations. Mapholi et al. (2022) suggested utilizing estimated breeding values for tick counts to select for tick resistance.

Tick monitoring and surveillance

Regular monitoring of tick populations and the occurrence of tick-borne diseases is crucial for guiding control strategies and identifying emerging resistance to acaricides. Randela (2005) and Spickett et al. (2011) emphasized that regular veterinary care and monitoring are essential for the early detection and management of tick infestations. Veterinarians are well-equipped to provide guidance on effective tick control strategies, conduct routine health checks, and treat tick-borne diseases when needed.

CONCLUSION

This systematic review and meta-analysis provide a comprehensive assessment of cattle tick infestations in South Africa, highlighting key environmental, management, and host-related risk factors that influence tick infestation rates. The findings underscore the need for targeted interventions to control tick populations and reduce their impact on cattle health and productivity. To enhance control efforts, it is essential to educate and train farmers on effective tick control methods, the proper application of acaricides, and the significance of integrated pest management (IPM). Additionally, continued research into new acaricides, vaccines, and alternative control methods is essential to combat tick resistance. Developing environmentally friendly, stable, cost-effective, and easily accessible acaricides, along with anti-tick vaccines, will be critical in sustaining long-term tick control strategies. By considering the identified risk factors and adopting integrated, context-specific management approaches, policymakers, veterinarians, and farmers can work together to enhance cattle health and productivity across South Africa.

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Author's Contribution:

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Competing interest

The authors declare that they have no conflict of interest.

Data availability statement

All data generated during this study are available through a request to the corresponding author.

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HEALTH ASSESSMENT OF BALKAN DONKEYS: HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS

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Abstract

The Balkan donkey (*Equus asinus asinus*), an endangered breed native to Serbia with significant historical importance, has not been extensively studied in terms of its health and physiology. As their numbers continue to decrease, studying their physiological traits is key to designing effective conservation strategies. This study evaluated the hematological and biochemical profiles of 70 clinically healthy Balkan donkeys, divided into three age groups: Group A (5-7 months), Group B (12-14 months), and Group C (4-7 years). The analysis revealed notable age-related differences ($p < 0.05$) in several key parameters. Younger donkeys (Groups A and B) showed heightened immune activity, as indicated by elevated white blood cell (WBC) counts, indicative of their developing immune systems. In contrast, adult donkeys (Group C) demonstrated stable immune profiles and elevated urea levels, likely linked to protein metabolism or hydration status. Consistent red blood cell (RBC) and platelet counts across all groups suggested efficient oxygen transport and coagulation. Higher phosphorus levels in younger donkeys suggested metabolic needs related to growth. Meanwhile, stable liver (ALT) and kidney (creatinine) function across all age groups underscored the breed's resilience to environmental and physiological challenges.

This study provides the first comprehensive dataset on the hematological and biochemical health of the Balkan donkey, providing valuable insights for veterinary diagnostics, conservation efforts, and breed management. By establishing baseline health data, this research not only aids in

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preserving this endangered breed but also enhances our understanding of donkey physiology in demanding ecological conditions.

Key words: Balkan donkey, endangered species, hematology, biochemistry, conservation

PROCENA ZDRAVSTVENOG STATUSA BALKANSKIH MAGARACA: UTVRĐIVANJE VREDNOSTI HEMATOLOŠKIH I BIOHEMIJSKIH PARAMETARA KRVİ

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Kratak sadržaj

Balkanski magarac (*Equus asinus asinus*) ugrožena i istorijski značajna rasa poreklom iz Republike Srbije, je nedovoljno proučena u pogledu zdravlja i fiziologije. Kako se brojnost populacije smanjuje, dublje razumevanje njegovih fizioloških parametara postaje ključno za razvoj strategija očuvanja prilagođenih ovoj rasi. Ova studija procenila je hematološke i biohemijske parametre kod 70 klinički zdravih balkanskih magaraca podeljenih u tri starosne grupe (grupa A: 5-7 meseci, grupa B: 12-14 meseci, grupa C: 4-7 godina), kako bi se istražili trendovi povezani sa starošću i obezbedili osnovni zdravstveni podaci za ovu rasu.

Utvrđene su značajne razlike u vrednostima pojedinih parametara kroz starosne grupe ($p < 0,05$). Kod mlađih magaraca (grupe A i B) utvrđene su povišene vrednosti leukocita (WBC) što ukazuje na pojačanu imunološku aktivnost, odražavajući razvoj njihovog imunološkog sistema. Stabilne vrednosti eritrocita (RBC) i trombocita u svim grupama ukazale su na efikasnu funkciju transporta kiseonika i koagulacije. Odrasli magarci (grupa C) pokazali su stabilizovane imunološke profile. kod odraslih su utvrđene povišene nivoe uree, što može biti povezano sa metabolizmom proteina ili statusom hidracije. Viši nivoi fosfora kod mlađih magaraca ukazali su na

metaboličke potrebe povezane sa rastom. Stabilne vrednosti ostalih biohemijskih parametara kroz sve grupe naglasile su otpornost ove rase na ekološke i fiziološke izazove.

Ova studija pruža sveobuhvatni skup podataka o hematološkim i biohemijskim parametrima zdravlja balkanskog magarca, nudeći ključne uvide za veterinarsku dijagnostiku, očuvanje i upravljanje ovom vrstom. Uspostavljanjem osnove za buduća istraživanja, ovaj rad ne samo da doprinosi očuvanju ove ugrožene rase već i obogaćuje naše razumevanje fiziologije magaraca u izazovnim ekološkim uslovima.

Ključne reči: Balkanski magarac, ugrožene vrste, hematologija, biohemija, očuvanje, fiziologija

INTRODUCTION

The global population of donkeys is estimated to be around 50 million, including a diverse range of breeds and species, many of which are classified as endangered because of decreasing numbers and limited genetic diversity (Kugler et al., 2008; FAO, 2011; Norris et al., 2021). There are about 189 recognized breeds (FAO, 2011). Donkeys have been important to rural economies, especially in developing countries. They have been widely used for transportation, in agricultural, and as carrier animals. With modernization of agriculture, the role of donkeys has shifted from traditional labor to tourism, milk production, and productions of skincare products. Donkey milk has gained considerable attention for its potential health benefits (nutritional benefits and hypoallergenic qualities). The growing demand for donkey milk products, especially in Europe, highlights its rising importance in the health and wellness industries (Martini et al., 2021).

The Balkan donkey (*Equus asinus asinus*) is a traditional breed of domestic donkey that has flourished in the mountainous areas of Serbia and Montenegro. This breed that descends from the African wild donkey has played a vital role in rural communities, mainly serving as a pack animal because of its resilience in enduring harsh, dry conditions (Beja-Pereira et al., 2004). The Balkan donkey is now considered an endangered breed, with small populations scattered across remote areas, making it difficult to gather consistent health data (Kugler et al., 2008).

Evaluating health of this endangered breed is of the great importance. Determination of species-specific reference values for hematological and biochemical parameters are crucial, as these can differ based on age, breed, and environmental factors (Ivanković et al., 2002; Vučićević et al., 2011). There

is limited information available on the health status of the Balkan donkey, as most research focuses on other domestic donkey breeds. This study seeks to address this knowledge gap by evaluating the hematological and biochemical parameters in Balkan donkeys, establishing physiological reference ranges, and comparing the findings with previous regional studies (Stanišić et al., 2015).

MATERIAL AND METHODS

Animal study

This study evaluated the hematological and biochemical blood profiles of 70 non-working donkeys, aged between 5-7 months and 7 years. They were sampled between June 2023 and June 2024 at Special Nature Reserve “Zasavica”, Serbia and divided into three groups: group A (5-7 months old), group B (12-14 months old) and group C (adults 4-7 years old). All the donkeys exhibited no visible clinical signs of illness or health issues during the examination prior to blood sampling.

All the donkeys were housed under identical conditions in the Special Nature Reserve “Zasavica,” with a consistent feeding regimen maintained throughout the seasons. Environmental and activity-related variations were minimized, ensuring that the observed differences primarily reflect age-related physiological changes. When the donkeys are kept outdoors, they graze on the grass available in the pasture. When housed indoors, the donkeys are fed dried clover, dried corn leaves, corn, along with a supplement of barley and sunflower seeds

Clinical assessment of donkeys and sampling

A total of 70 animals were included in the study. Clinical examinations of donkeys and blood samples were carried out by veterinarians. No significant clinical abnormalities were detected. Blood samples were drawn from the jugular vein into two tubes - 10 mL plain and EDTA-containing evacuated blood collection tubes (BD Vacutainer). Complete blood counts were carried out within 12 h of sampling.

Laboratory analysis

All samples were transported promptly to the laboratory of Scientific Veterinary Institute “Novi Sad”. Prior to biochemical analyses, the samples were

centrifuged at 1,500 g, for 10 minutes. Two aliquots of each serum sample were stored in sterile vials and frozen at -200°C until further analysis. Biochemical parameters were assessed by semi-automatic biochemistry analyzer RT-1904C (Rayto life and analytical sciences, Shenzhen, China), using BioSystems S.A. reagents (BioSystems S.A, Barcelona, Spain). The assessed parameters included triglycerides (glycerol phosphate oxydase/peroxydase method), aspartate aminotransferase (IFCC without pyridoxal phosphate method), alanine aminotransferase (IFCC without pyridoxal phosphate method), total serum protein (Biuret method), albumin (Bromocresol green dye-binding method), globulin (Glob), creatinine (Jaffe method), urea (Urease- kinetic method), calcium- cresoftalein (o-cresoftalein method), magnesium (Xylidyl Blue method), phosphorus (Phosphomolybdate/UV method), cholesterol (Cholesterol oxydase/peroxidase method) and glucose (glucose oxydase/peroxidase method).

Blood samples in EDTA evacuated tubes were analyzed using an automated cell counter (Veterinary haematology analyser BC-2800Vet, Shenzhen Mindray Animal Medical Technology Co.LTD., Czhina). Parameters assessed by the automated counter included counts of erythrocytes (RBC), total leucocytes (WBC) count with differential blood analysis (lymphocyte %, neutrophils %, and monocyte %), platelet (PLT) count, haemoglobin (Hb) concentration and haematocrit (Hct).

Data analysis

Statistical analyses were carried out using GraphPad software v.10 (GraphPad Software Inc., La Jolla, CA, USA). Data for both biochemical and hematological parameters were presented as mean $\pm$ standard deviation (SD). A one-way analysis of variance (ANOVA) was conducted to determine the significance of differences among the three age groups (Group A: 5-7 months old, Group B: 12-14 months old, Group C: 4-7 years old). For parameters showing significant differences ($p < 0.05$), post-hoc Tukey's HSD tests were applied to identify pairwise group differences.

RESULTS

The biochemical and hematological parameters of the Balkan donkeys were analyzed across three age groups: Group A (6 months), Group B (12 months), and Group C (4-7 years). The results are presented in two main sections-biochemical parameters and hematological parameters. The data, expressed as means $\pm$ standard deviations, highlight statistically significant

differences between age groups, where applicable. To simplify the findings, boxplots were utilized to illustrate the variability in key parameters, while bar graphs were used to summarize the overall trends. Comprehensive tables (Table 1 and Table 2) provide numerical details, including ANOVA results and post-hoc analyses, offering a clear and organized way to interpret the data.

I Biochemical parameters results

Table 1. This summary presents biochemical parameters in Balkan donkeys categorized into three age groups: Group A (6 months old), Group B (12 months old), and Group C (4-7 years old). The data is shown as mean $\pm$ standard deviation (SD). ANOVA *p*-values are provided to indicate the significance of differences between the groups, with further details on significant differences obtained from post-hoc Tukey's HSD tests. The parameters are organized into categories including liver function, kidney function, electrolytes and minerals, lipid profile, protein profile, and metabolic parameters.

Parameter	Group A (Mean $\pm$ SD)	Group B (Mean $\pm$ SD)	Group C (Mean $\pm$ SD)	ANOVA <i>p</i> -value	Significant Differences
AST (U/L)	253.5 $\pm$ 30.4	240.91 $\pm$ 25.6	300.3 $\pm$ 35.2	0.006	Group C > A, B
ALT (U/L)	3.57 $\pm$ 0.81	3.16 $\pm$ 0.64	3.0 $\pm$ 0.78	0.133	None
Creatinine (mmol/L)	106.2 $\pm$ 11.2	103.8 $\pm$ 10.4	102.2 $\pm$ 9.3	0.752	None
Urea (mmol/L)	5.49 $\pm$ 1.16	6.29 $\pm$ 0.98	5.30 $\pm$ 1.02	0.302	None
Calcium (mmol/L)	3.75 $\pm$ 0.45	3.64 $\pm$ 0.50	3.6 $\pm$ 0.37	0.641	None
Magnesium (mmol/L)	0.79 $\pm$ 0.16	0.76 $\pm$ 0.11	0.76 $\pm$ 0.14	0.780	None
Phosphorus (mmol/L)	2.45 $\pm$ 0.72	2.08 $\pm$ 0.65	1.7 $\pm$ 0.62	0.002	Group A, B > C
Triglycerides (mmol/L)	0.71 $\pm$ 0.22	0.82 $\pm$ 0.34	0.92 $\pm$ 0.25	0.059	None
Cholesterol (mmol/L)	2.37 $\pm$ 0.84	2.08 $\pm$ 0.68	2.4 $\pm$ 0.72	0.090	None
Total Protein (g/L)	80.16 $\pm$ 14.34	82.13 $\pm$ 11.97	82.12 $\pm$ 9.19	0.864	None
Albumin (g/L)	33.64 $\pm$ 9.35	34.17 $\pm$ 7.88	38.99 $\pm$ 4.07	0.161	None
Globulin (g/L)	46.51 $\pm$ 9.04	47.95 $\pm$ 7.81	43.13 $\pm$ 8.17	0.397	None
Glucose (mmol/L)	5.36 $\pm$ 1.20	4.77 $\pm$ 1.22	5.30 $\pm$ 1.14	0.513	None

Protein Profile

To evaluate the protein profile, we measured total protein, albumin, and globulin levels (Table 1, Figure 1). Group C showed slightly higher total protein levels compared to Groups A and B, but no statistically significant differences were observed between the groups ($p > 0.05$).

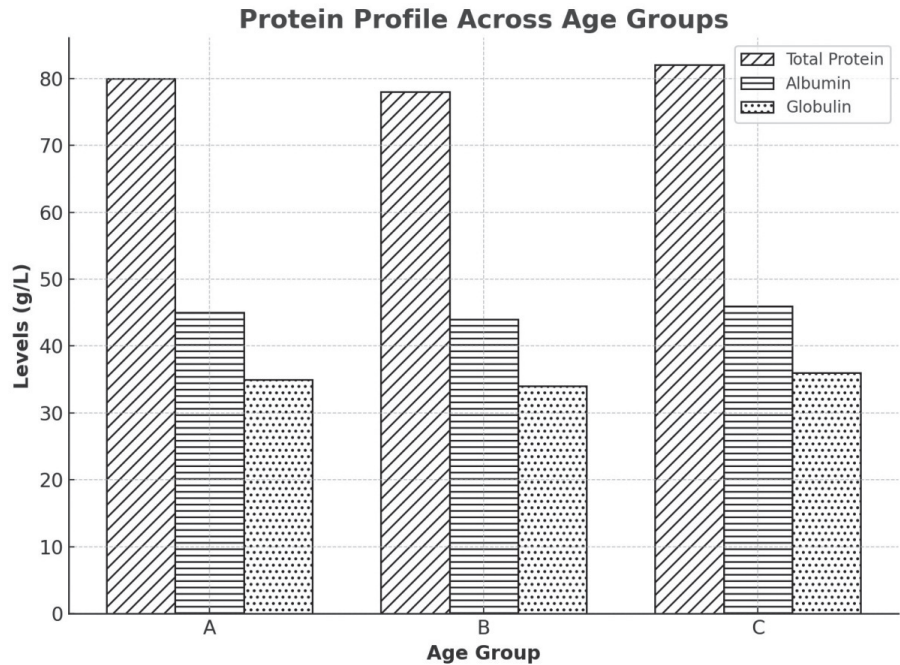


Figure 1. Protein profile across groups

Electrolytes and Minerals

Phosphorus levels showed significant differences among the age groups ($p < 0.05$). Groups A and B had notably higher phosphorus levels compared to Group C, as shown in Figure 2. On the other hand, calcium and magnesium levels remained consistent across all groups, with no significant differences observed ($p > 0.05$) (Table 1).

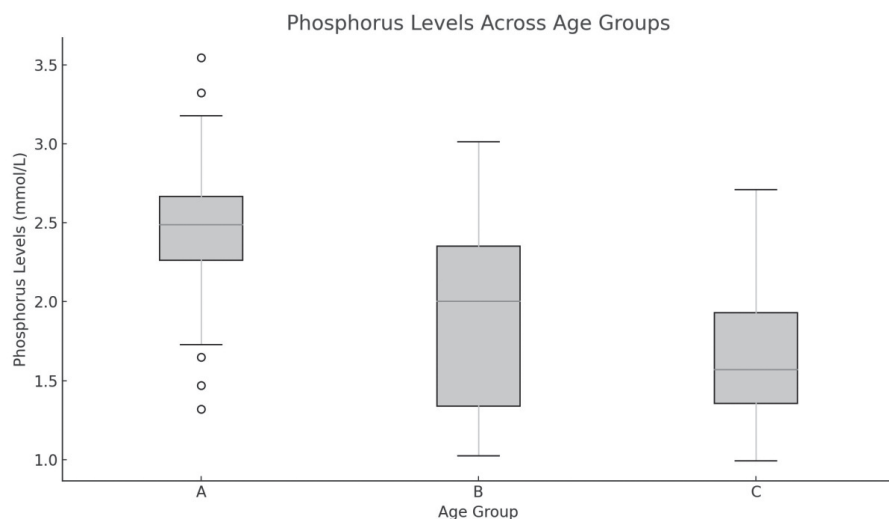


Figure 2. Distribution of phosphorus levels across groups.

Lipid Profile

As part of the lipid profile, triglycerides and cholesterol levels were measured (Table 1). Although there were some variations among the groups, the differences were not statistically significant ($p > 0.05$). Triglyceride levels were slightly higher in older donkeys, although this trend was not considered statistically significant.

Metabolic Parameters

Glucose levels were examined to assess metabolic function (Figure 3). While no significant differences were observed between the groups ($p > 0.05$), younger donkeys, particularly those in Group A, showed greater variability, including a few outliers.

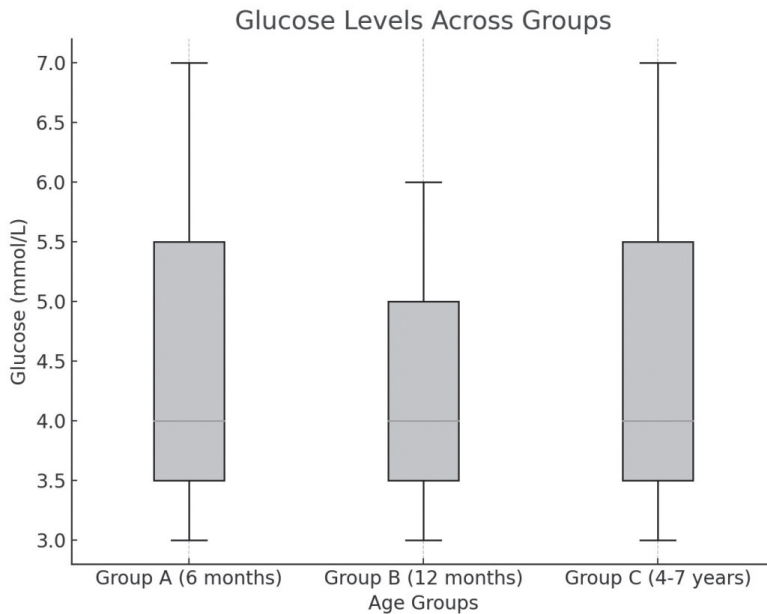


Figure 3. Glucose Levels across Groups. The boxplot illustrates the distribution of glucose levels across different groups. While no significant differences were found ($p > 0.05$), Group A displayed a higher level of variability.

Kidney Function Parameters

Creatinine and urea levels were measured to assess kidney function (Table 1). The creatinine levels remained consistent across all groups, showing no statistically significant differences ($p > 0.05$). In contrast, urea levels showed significant differences ($p < 0.05$), with Group C exhibiting higher levels compared to Groups A and B (Figure 4).

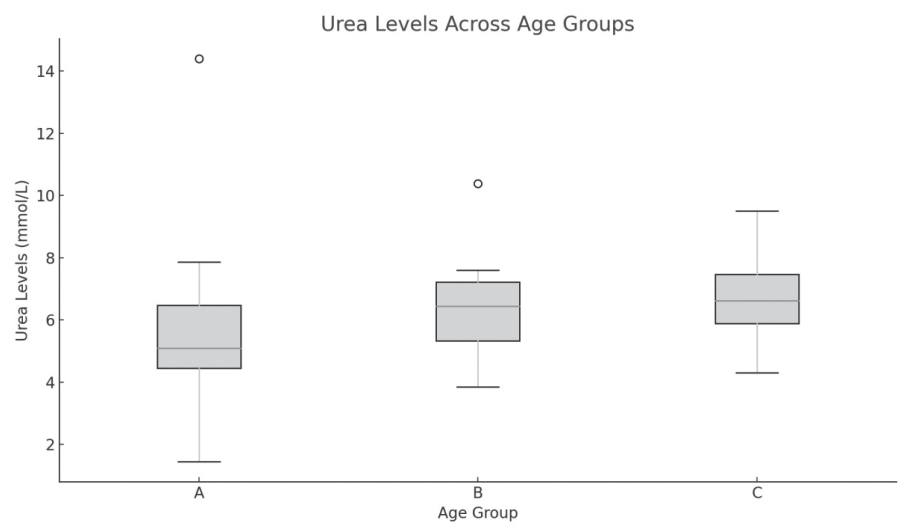


Figure 4. Urea Levels Across Groups; Boxplot displaying urea levels. Group C exhibited significantly higher urea levels compared to Groups A and B ($p < 0.05$).

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured across the three age groups (Figure 5). AST levels demonstrated significant differences among the groups ($p < 0.05$), with Group C showing higher levels than Groups A and B, as depicted in Figure 1. In contrast, ALT levels remained consistent across all groups ($p > 0.05$) and are summarized in Table 1.

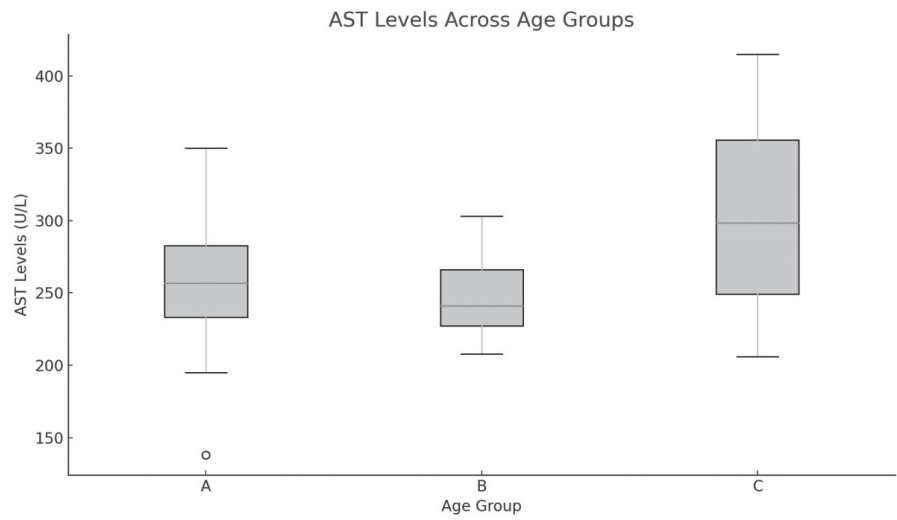


Figure 5. AST Levels Across Groups. Boxplot illustrating AST levels among the groups. Significant differences ($p < 0.05$) were observed, with Group C showing higher levels compared to Groups A and B.

II Hematology Results

Table 2. Summary of hematology parameters in Balkan donkeys across three age groups. Data are presented as mean $\pm$ standard deviation (SD). The ANOVA p -values indicate the significance of differences among the groups, with any significant differences further detailed based on post-hoc Tukey's HSD tests. The parameters measured include white blood cell count (WBC), lymphocytes, monocytes, neutrophils (granulocytes), red blood cell count (RBC), hemoglobin (Hgb), hematocrit (Hct), and platelets (Plt).

Parameter	Group A (Mean $\pm$ SD)	Group B (Mean $\pm$ SD)	Group C (Mean $\pm$ SD)	ANOVA p -value	Significant Differ- ences
WBC	20.31 $\pm$ 2.71	18.77 $\pm$ 3.02	13.46 $\pm$ 3.02	0	
Lymphocytes	53.21 $\pm$ 13.65	61.74 $\pm$ 7.20	54.43 $\pm$ 10.86	0.2711	A>B,C

Parameter	Group A (Mean ± SD)	Group B (Mean ± SD)	Group C (Mean ± SD)	ANOVA <i>p</i> -value	Significant Differ- ences
Neutrophils (granulo- cytes)	42.62 ± 12.96	34.43 ± 6.70	38.32 ± 13.59	0.4426	
Monocytes	4.16 ± 0.87	3.81 ± 0.66	4.20 ± 0.59	0.45	
RBC	7.51 ± 0.87	6.72 ± 0.37	6.29 ± 0.85	0.0047	
HGB	120.12 ± 7.40	111.00 ± 4.47	119.60 ± 14.13	0.2016	C < A, B
HCT	36.44 ± 2.67	33.49 ± 1.35	36.22 ± 3.98	0.1519	
Platelets	643.75 ± 321.34	594.00 ± 131.05	704.33 ± 206.93	0.5633	

White Blood Cell (WBC) Parameters

White blood cell (WBC) counts and their differential parameters- lymphocytes, monocytes, and neutrophils were analyzed in the three groups. Overall WBC counts demonstrated no significant differences between the groups ($p > 0.05$). However, lymphocyte levels were significantly higher in Group A compared to Groups B and C ($p < 0.05$, Figure 6). Neutrophil counts also varied, with Group C showing lower levels than Groups A and B ($p < 0.05$, Figure 7). Monocyte levels, on the other hand, remained stable across all groups ($p > 0.05$).

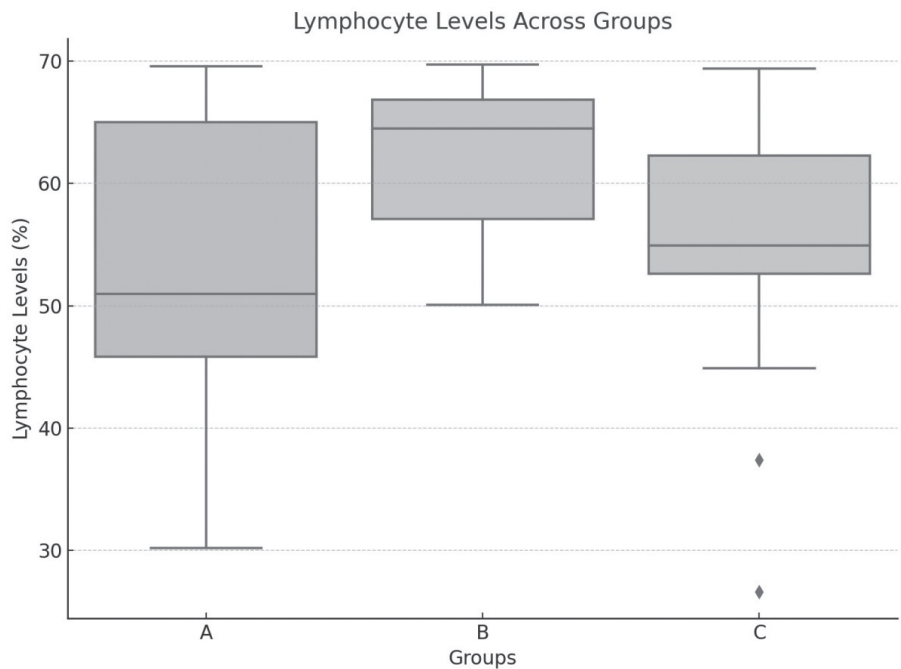


Figure 6. Lymphocyte levels across groups

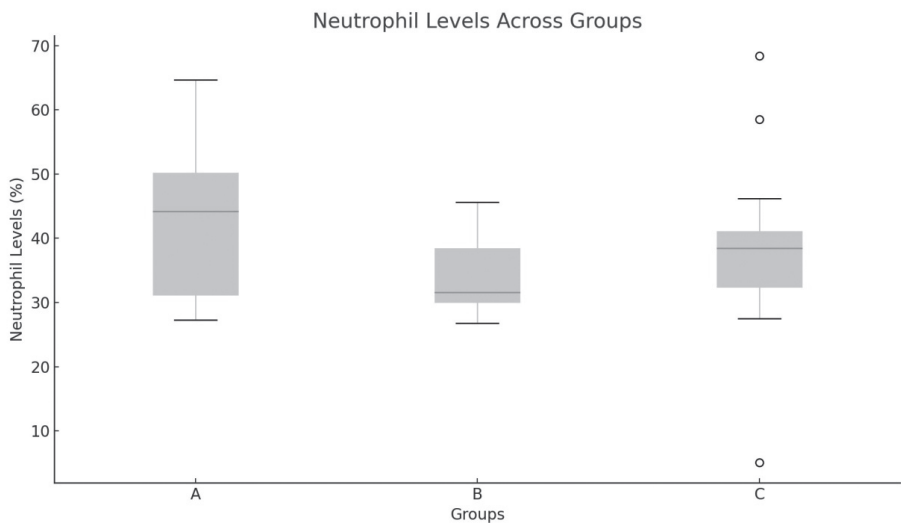


Figure 7. Neutrophiles levels across groups

Red Blood Cell (RBC) Parameters

The study evaluated red blood cell indices, including RBC count, hemoglobin (Hgb), and hematocrit (Hct). RBC counts showed no significant differences between the groups ($p > 0.05$). However, Group C had slightly lower levels compared to Groups A and B. Hemoglobin levels, on the other hand, were significantly lower in Group C than in the other groups ($p < 0.05$, Figure 8).

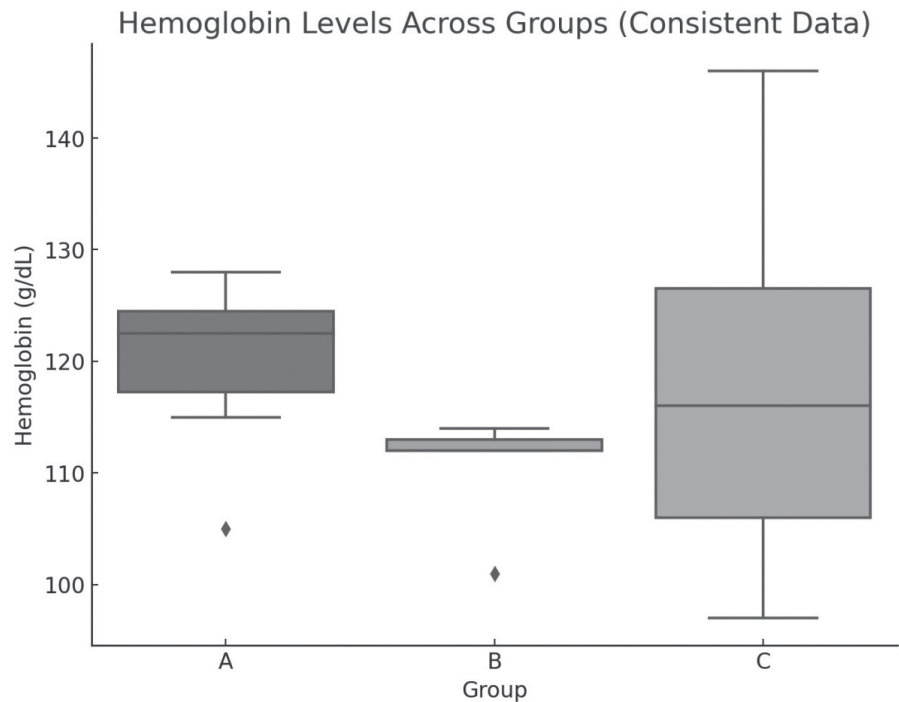


Figure 8. Hemoglobin levels revealed significant differences ($p < 0.05$).

Hematocrit values exhibited a similar trend to hemoglobin, showing significantly lower levels in Group C ($p < 0.05$) (Figure 9).

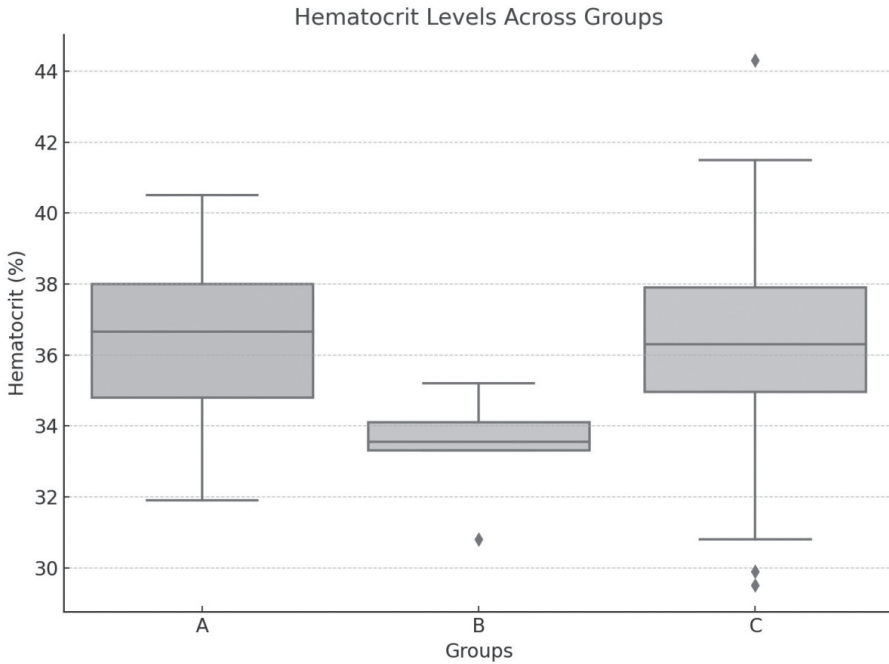


Figure 9. Hematocrit levels showed significant differences ($p < 0.05$).

Platelet counts

Platelet (Plt) counts were analyzed across the three groups (Figure 10). No significant differences were observed ($p > 0.05$). However, Group C exhibited a greater variability in platelet levels.

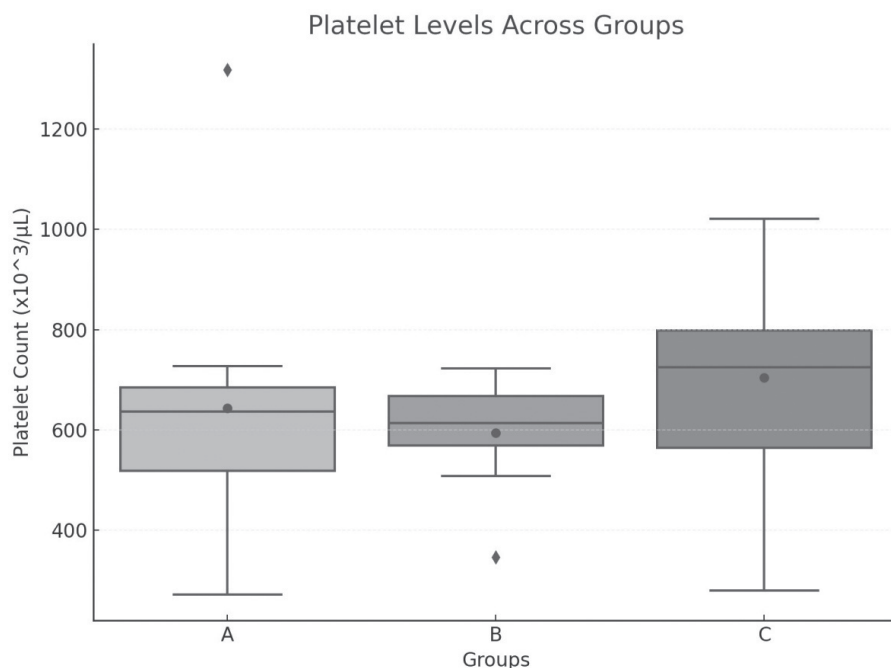


Figure 10. Platelet Levels Across Groups Boxplot showing the distribution of platelet counts among groups. No significant differences were observed ($p > 0.05$), though Group C exhibited greater variability. Group A: 5-7 months, Group B: 12-14 months, Group C: 4-7 years

DISCUSSION

Our study provides important insights into the physiological health of the endangered Balkan donkey (*Equus asinus asinus*). We concentrate on key areas, including liver and kidney function, metabolic activity, electrolytes, and hematological parameters. These findings are crucial for conservation efforts and contribute to the limited knowledge about this unique breed. By establishing baseline physiological values, this research fills critical gaps in the current literature and enhances our understanding of the adaptive mechanisms in these animals. The stable alanine aminotransferase (ALT) levels observed across all age groups in this study indicate good liver health and minimal metabolic stress. This is in line with findings from other studies on donkey populations, such as those by Ivanković et al. (2002) and Stanišić et al. (2015). In contrast, variability in aspartate aminotransferase (AST) levels, particularly in

adult donkeys, has been noted in research on Miranda's donkey breed by Silva et al. (2024). These fluctuations are likely due to temporary factors like physical activity or environmental changes and may reflect physiological adaptability rather than health concerns. Similar results have been reported in European donkey breeds by Burden et al. (2016), highlighting the resilience of donkey liver function across diverse conditions. Urea and creatinine levels across the age groups were within expected ranges, showing only minor variations influenced by age, diet, and hydration. Lower creatinine levels in younger donkeys (Groups A and B) likely reflect their smaller muscle mass and active growth, consistent with findings by Vučićević et al. (2011). In contrast, higher urea levels in adult donkeys, as reported by Stanišić et al. (2015) and Goodrich and Webb (2024), may result from increased protein metabolism or mild dehydration linked to age-related metabolic demands or environmental factors. These results highlight the importance of proper nutrition and hydration, especially in conservation settings like the Zasavica reserve, to maintain optimal renal health across all age groups. Electrolyte levels, such as calcium, phosphorus, and magnesium, remained stable among the groups, suggesting that muscle and nerve function was well-balanced in the population studied. The higher phosphorus levels observed in younger donkeys likely indicate their increased needs for skeletal growth and development, a pattern also noted in research on Balkan donkeys by Stanišić et al. (2015) and Croatian donkey populations by Ivanković et al. (2002). Minor fluctuations in calcium levels can likely be attributed to dietary factors and environmental conditions, as highlighted in studies of Croatian donkey breeds by Ivanković et al. (2000). Similar observations were made by Silva et al. (2024) in Miranda's donkeys, where age and diet had a significant impact on electrolyte levels. These findings highlight the significance of diet and environmental adaptation in preserving mineral balance across various life stages.

There were clear age-related metabolic changes in glucose, cholesterol, and triglyceride levels. Glucose levels varied greatly among the three groups, likely due to dietary factors or stress. In adult donkeys, cholesterol levels rose, indicating metabolic adjustments, a trend that was also observed in different breeds of donkeys as reported by Caldin et al. (2005), Trimboli et al. (2020) and Silva et al. (2024). Lower triglyceride levels in younger donkeys may indicate less fat intake during their growth periods. These observations are in line with broader research, including the work of Burden et al. (2016), which highlights metabolic adaptations across different age groups. Our primary focus was to examine age-related differences. We noticed that sex can also influence certain biochemical parameters. However, due to highly imbalanced sex ratio among the sampled donkeys, an accurate statistical analysis specifically addressing

sex-related effects was not feasible. Despite this limitation, our findings suggest notable metabolic differences between males and females. Female donkeys had significantly higher total protein, albumin, calcium, and urea levels, indicating potential differences in protein metabolism and mineral regulation. Male donkeys exhibited higher triglycerides, cholesterol, ALT, and glucose, pointing to variations in lipid metabolism, carbohydrate processing, and liver function. These findings highlight the importance of considering both age and sex in assessing donkey health. Regarding the impact of gender to the analyzed parameters, Silva et al., 2024 observed significant differences between groups (females versus males). This study supports our findings, demonstrating that gender significantly influences certain biochemical parameters in donkeys. Recognizing these differences is essential for accurately assessing their health and ensuring proper management and care.

Hematological Parameters

Hematological analysis showed notable age-related trends, especially in white blood cell (WBC) counts. Neutrophil levels were highest in Group A, indicating the increased innate immune activity commonly seen in younger animals as they adjust to environmental challenges. Differential WBC counts showed a peak in lymphocyte levels in Group B, suggesting enhanced adaptive immune activity during early adulthood. Monocyte levels remained consistent across all groups, aligning with their function in tissue repair and immune monitoring. Younger donkeys in Groups A and B displayed higher overall WBC levels, likely due to active immune development and responses to environmental pathogens. These results are in line with the findings of Stanišić et al. (2015), who noted age-related variations in WBC counts in Balkan donkeys, and Sedlinská et al. (2016), who observed similar patterns in younger donkeys from Czech and Slovak populations. Conversely, the lower WBC counts seen in Group C (adults) indicate a stable and efficient immune system, typical of immunological maturity in equids. The red blood cell (RBC) parameters, including hemoglobin (HGB) and hematocrit (HCT), showed little variation among age groups, indicating a stable capacity for oxygen transport in the donkeys studied. The slightly elevated RBC and HGB levels in younger donkeys (Group A) likely reflect their increased oxygen demands during growth, which aligns with the observations of Laus et al. (2015), Burden et al. (2016) and Sedlinská et al. (2016) who noted similar trends in healthy donkeys and age-related physiological adaptations in equids. The consistent HCT levels across all groups further highlight the adaptability and resilience of the red blood cell system in meeting metabolic needs throughout different life stages.

Platelet counts showed no significant differences among the age groups, suggesting consistent thrombopoietic activity and coagulation function throughout the population. The minor variability noted in Group A could be due to physiological changes occurring during early growth stages. This finding is consistent with the research by Stanišić et al. (2015), which indicated stable platelet parameters and established reference ranges for Balkan donkeys, reinforcing the idea of hematological stability across various age groups.

CONCLUSION

This study provides valuable insights into the physiology of the Balkan donkey breed, contributing to the existing body of literature on donkey health and conservation. Our results emphasize liver and kidney function, stable metabolic parameters, and age-related trends in blood health that showcase the remarkable adaptability of these animals. The increased white blood cell counts in younger donkeys indicate active immune responses during their growth, while the consistent red blood cell and platelet parameters across different ages highlight stable physiological characteristics that are clearly not influenced by age. This research marks a significant advancement in understanding the physiological health of the endangered Balkan donkey breed, with a focus on liver and kidney function, metabolic health, and blood parameters across various age groups. Although the findings offer valuable baseline data and insights into the adaptability of these animals, they do not provide enough information to establish definitive reference values for the breed. Future studies should further explore how environmental factors, seasonal shifts, and genetics influence metabolic adaptations in donkeys, contributing to better conservation strategies and improved veterinary care for this endangered breed. These preliminary findings establish a strong foundation for future studies focused on developing breed-specific reference values. They also highlight the need for continued research and customized conservation strategies that address the unique nutritional, environmental, and physiological needs of the Balkan donkey. By building on this foundation, future research can enhance veterinary diagnostics and contribute to the long-term preservation of this endangered breed.

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Author's Contribution:

SS, MŽ – the idea of the publication, organisation of work, writing the manuscript. MŽ, VG – laboratory analysis for all the samples.

SS, MŽ, VG, MŽB, DL, SL, DB reviewed the manuscript and participated in the final draft of the manuscript.

Competing interest

There is no competing interest existing. The manuscript is not influenced by any personal or financial relationship with people or organizations.

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CULTURAL AND BIOCHEMICAL IDENTIFICATION OF ANTIBIOTIC-RESISTANT BACTERIA IN THE EARS OF DOGS AND PATIENTS WITH OTITIS EXTERNA

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Abstract

Otitis externa is a common ear condition in humans and dogs caused by bacteria, fungus, yeasts, and ectoparasites. The aim of the study was molecular identification and sequencing of isolated antibiotic-resistant bacteria. The study consisted of 250 swab samples, with 100 samples from human patients and 150 from dogs. The samples were collected from October 2023 to January 2024 in Diyala Province. The isolates were identified using Vitek 2 depending on colony's color, form, and odor on study agar. Biochemical tests were performed to identify the bacteria. DNA extraction and PCR were performed to confirm the identity of the bacterial isolates identified by the VITEK system. While VITEK provides preliminary identification based on biochemical tests, while molecular techniques such as PCR allow for more definitive confirmation of the species, particularly for identifying antibiotic-resistant strains. The study revealed that *Staphylococcus* spp. was the most common infection in humans, affecting 26.3% of cases, followed by *Pseudomonas*, *Proteus*, *Escherichia coli*, *Streptococcus*, *Klebsiella*, and *Corynebacterium*. Dog samples exhibited the highest infection rate of 26.7%, predominantly caused by *Staphylococcus* spp., followed by *Streptococcus*, *Pseudomonas*, *Escherichia coli*, *Proteus*, *Klebsiella*, *Pasteurella* species, and *Corynebacterium* respectively. Eight antibiotics were tested against these bacterial isolates using the disc-diffusion method, with all isolates

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showing susceptibility to Gentamycin, Ciprofloxacin, Tetracycline, and Amikacin. The investigation found two *Staphylococcus aureus* isolates OK 560669,1 and OK 9560670,1. Two GenBank entry numbers OK 560673,10 and OK 560674,1 were *Escherichia coli* isolates. Two *Pseudomonas aeruginosa* isolates with GenBank entries OK 560672,1 and OK 560671,1, were detected.

Key words: Bacterial isolates, Otitis externa, Dogs and humans, Molecular and phylogenetic, Antibiotic susceptibility testing

BIOHEMIJSKA IDENTIFIKACIJA BAKTERIJA REZISTENTNIH NA ANTIBIOTIKE U UHU PASA I KOD PACIJENATA SA ZAPALJENJEM SPOLJAŠNJEG UHA (OTITIS EXTERNA)

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Kratak sadržaj

Otitis externa je često oboljenje uha kod ljudi i pasa, izazvano bakterijama, gljivicama, kvascima i ektoparazitima. Cilj studije je bila molekularna identifikacija i sekvenciranje izolovanih bakterija otpornih na antibiotike. Analizirano je 250 uzoraka briseva, od kojih je 100 uzoraka bilo od ljudi, a 150 od pasa. Uzorci su prikupljeni od oktobra 2023. do januara 2024. godine u provinciji Dijala. Izolati su identifikovani pomoću Vitek 2, u zavisnosti od boje, oblika i mirisa kolonija na nutritivnom agaru. Biohemijski testovi su izvršeni radi identifikacije bakterija. Ekstrakcija DNK i PCR su sprovedeni kako bi se potvrdio identitet bakterijskih izolata identifikovanih VITEK sistemom. Dok VITEK pruža preliminarnu identifikaciju na osnovu biohemijskih testova, molekularne tehnike poput PCR-a omogućavaju precizniju potvrdu vrste, posebno u identifikaciji sojeva otpornih na antibiotike. Studija je pokazala da je *Staphylococcus* spp. bio

najčešći uzročnik infekcije kod ljudi, prisutan u 26,3% slučajeva, a zatim su sledili *Pseudomonas*, *Proteus*, *Escherichia coli*, *Streptococcus*, *Klebsiella* i *Corynebacterium*. Uzorci pasa pokazali su najvišu stopu infekcije od 26,7%, pri čemu je *Staphylococcus* spp. bio najzastupljeniji, nakon čega su sledili *Streptococcus*, *Pseudomonas*, *Escherichia coli*, *Proteus*, *Klebsiella*, *Pasteurella* spp. i *Corynebacterium*. Osam antibiotika je testirano protiv ovih bakterijskih izolata metodom difuzije diska, pri čemu su svi izolati pokazali osetljivost na gentamicin, ciprofloksacin, tetraciklin i amikacin. Istraživanje je otkrilo dva izolata *Staphylococcus aureus*, OK 560669,1 i OK 9560670,1. Dva GenBank broja unosa, OK 560673,10 i OK 560674,1, odnosila su se na *Escherichia coli* izolate. Otkrivena su i dva izolata *Pseudomonas aeruginosa* sa GenBank brojevima OK 560672,1 i OK 560671,1.

Ključne reči: Izolati bakterija, *Otitis externa*, ljudi i životinje, molekularna i filogenska, testiranje osetljivosti na antibiotik

INTRODUCTION

Inflammation of the external ear, known as otitis externa, is a common inflammatory condition in dogs which often occurs due to yeast and bacterial infections (Bradley et al., 2020). The severity of external otitis ranges between acute or chronic inflammation, and it can affect the inner ear and lead to the development of an inflammatory condition, causing a range of signs and symptoms. Researchers have conducted many studies to better understand how otitis externa occurs in dogs (O'Neill et al., 2021; Griffin, 2020). A pneumatic otoscope is the most accurate and reliable tool, offering higher specificity for diagnosis compared to a regular otoscope. However, a tympanometer and other methods can assist in the diagnostic process when an otoscope is unavailable (Griffin, 2020). Both will yield decreased TM mobility on tympanometry or pneumatic otoscopy (Minnat et al., 2021; Lilenbaum, et al., 2000). According to Sood et al. (2002) and Daneshrad et al. (2002), between 3% and 5% of the population might be affected. Ninety percent of individuals experience their initial attack on one side, typically occurring seven years after the onset of ear illness, and it is more common in those with a prior history of the condition. Middle ear infections can be viral, bacterial, fungal, or a result of coinfection. The most common bacterial organisms causing otitis media in humans are *Streptococcus pneumoniae*, followed by non-typeable hemophilic influenza (NTHi) and *Moraxella catarrhalis* (Hu et al., 2021). Following

the introduction of the conjugate pneumococcal vaccines, the pneumococcal organisms have evolved to non-vaccine serotypes. There are more common viral pathogens that cause otitis media, including respiratory syncytial virus (RSV), coronavirus, influenza virus, adenovirus, human metapneumovirus, and picornavirus (Bulut et al., 2007). Otitis media is diagnosed clinically by observing the signs presented by the body after a visual physical examination. An otoscope is then used, along with an assessment of the patient's medical history and the apparent symptoms, to make a thorough diagnosis. There are many diagnostic tools, including the pneumatic endoscope, tympanometer, and acoustic reflectometer, which help doctors to diagnose a case of middle ear infection (Shand and Campe, 2016). Otitis media begins as an inflammatory process after infection of the upper respiratory tract with a viral disease that affects the nasal mucosa, pharynx, middle ear mucosa, and Eustachian tube. From an anatomical point of view, the middle ear passages are narrow (Atila et al., 2021). The edema resulting from the inflammatory process closes the narrow part of the Eustachian tube, and this leads to reduced ventilation. As a result, a chain of consecutive events causes an increase in negative pressure in the middle ear, which in turn leads to an increase in secretions from the inflamed mucous membrane. This buildup of mucous secretions creates an environment that promotes the growth of bacterial colonies and viral infections in the middle ear (Dilesh, 2017). The proliferation of these microbes in the middle ear leads to suppuration, eventually resulting in noticeable purulence within the middle ear space. Tu et al., (2014) clinically demonstrated a bulging or erythematous tympanic membrane along with purulent middle ear fluid. Tang et al. (2022) described chronic serous otitis media (CSOM), which is characterized by thick, amber-colored fluid in the middle ear space and a retracted tympanic membrane upon otoscopic examination. In addition to heightened sensitivity to loud sounds, an acute otitis externa patient's symptom may worsen with exposure to warm water, taking a bath, wearing hearing protection, or experiencing any form of trauma (Beers and Abramo, 2004). Overall, this study aims to contribute to our understanding of antibiotic resistance in otitis externa and provide insights into the prevalence, distribution, and genetic basis of antibiotic resistance genes in these bacterial isolates. The samples from dogs and humans are used together in the study as both groups are investigated for the presence of antibiotic-resistant bacteria causing otitis externa (Tang et al., 2022). The research highlights the prevalence of these bacteria in both populations, indicating a shared concern regarding antibiotic resistance in veterinary and human medicine. This connection is relevant and significant, as it underscores the importance of understanding antibiotic resistance across species, which can have implications for treatment and public

health (Dilesh, 2017). The aim of the study is molecular identification and sequencing of isolated antibiotic-resistant bacteria from dogs and human.

MATERIAL AND METHODS

Sample collection

This study involved the collection of 250 swab samples, which were divided into three groups: 100 ear swabs from individuals (50 from males and 50 from females), and 150 swab samples from the ears of dogs. The control group included 150 swab samples, divided into 75 female swab samples and 75 male samples. The samples were collected in accordance with ethical guidelines and verbal approval from the Ethics Committee of the Technical Institute. They were collected in Baqubah city, Diyala Governate, Iraq, for the period from October 2023 to January 2024. Furthermore, the patient's ear condition appeared to worsen in warm water, after bathing, while wearing hearing protection, or following an injury. Additionally, the patient's ear condition made them more sensitive to loud noises, as noted by (Beers and Abramo, 2004). The samples were grown on the blood Agar and MacConkey Agar, and chemical tests were performed to isolate and preliminary identify bacteria (Riedel et al., 2018). In addition, biochemical tests were conducted using the (VITEK 2) device to determine the bacterial species. The bacterial culture was transferred to Mueller Hinton Agar for drug sensitivity testing.

DNA extraction from the genome

The procedures were carried out according to the protocol required of the producer (Anatolia/Turkey). The Deoxyribonucleic acid (DNA) that was acquired was stored at a temperature of -20 °C to ensure its preservation and facilitate its future utility and processing. Following the instructions in the Pres-toTM Mini gDNA Bacteria Kit, genomic DNA was extracted from bacterial growth. A Nanodrop spectrophotometer was used to examine the extracted DNA, measuring DNA content (ng/L) and DNA purity by reading the absorbance at (260/280 nm) (Lee, et al., 2010; Mohammad et al., 2024).

Primer selection and 16S rRNA amplification preparation

The sequences of molecular duplication primers and the size of amplified fragments related to three different genes were determined in *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. In *Staphylococcus au-*

reus, the Arc primer was used with the sequence TTGATTCACCAGCGGTATT for the forward primer and AGGTATCTGCTTCAATCAGCG for the reverse primer, yielding an amplified fragment 570 base pairs long. For *Pseudomonas aeruginosa*, the AroE primer was applied with the sequence ATGTCACCGTGCCGTTCAAG for the forward primer and TGAAGCAGTCGGTTCCTTG for the reverse primer, generating an amplified fragment of 1,500 base pairs in size. Finally, in *Escherichia coli*, the ArcA primer with the sequences GAAGACGAGTTGGTAACACG and CTTCCAGATCACCGCA-GAAGC were used for the forward and reverse primers, respectively, resulting in a duplication of a similar size of 1,500 base pairs (Nee, 2018). The selection of these primers was driven by their ability to amplify specific genes associated with pathogenicity and antibiotic resistance in *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. Understanding these genes is crucial for developing effective diagnostic tools and treatment strategies for otitis externa infections, especially in light of the increasing issue of antibiotic resistance (Lee, et al., 2010).

PCR amplification

To obtain the best results in PCR amplification, a specific set of chemicals was used in a total volume of 25 μ L: these include 12.5 μ L of Go Taq® basic mix from Promega, USA, plus 2 μ L of primers, 2 μ L of template DNA, 8.5 μ L of nuclease-free water. The amplification protocol was carried out using a Mastercycler (Eppendorf) and included the following steps: the first step involved preheating the specimens at 94 °C for one minute. The first step involves heating for thirty-five cycles at the same temperature, followed by a temperature reduction to 63 °C for annealing. The process then proceeds with an extension at 72 °C for the same duration. The final step consists of a single extension cycle at 72 °C for ten minutes.

Agarose gel electrophoresis

The PCR amplified products were tested by electrolysis on a 1.5% agarose gel, the gel was stained with Safe Red, and the products were photographed using ultraviolet light with the help of the Imagemaster VDS device produced by the American Pharmacia Biotech. 25 μ L of PCR positive products were used for sequence analysis, with the BLAST algorithm from the National Center for Biotechnology Information (NCBI) used to evaluate the results. The improved protocol for PCR and gel electrophoresis provides valuable information about genetic composition (Ligozzi et al., 2010; Sami Awayid and Qassim Mohammad, 2022).

Ethical approval

Ethical approval was obtained from the Research Ethics Committee at the Southern Technical University, Baqubah Technical Institute.

Statistical analysis

Patients' demographics and cross-tabulations were calculated using the Statistical Package for the Social Sciences for Windows, version 2017 (SPSS, Armonk, NY: IBM Corp). Pearson's chi-square test and Pearson's correlation coefficient were used to assess the association between variables from the two tests. Statistical significance was set at $p \leq 0.05$ and $p \leq 0.01$.

RESULTS

Table 1. Primer selection and 16S rRNA amplification

Bacteria	Genes	Primer sequence	Size (bp)	Reference
<i>Staphylococcus aureus</i>	Arc	F: TTGATTACACGCGCGTATT R: AGGTATCTGCTTCAATCAGCG	570bp	Sastalla et al., 2017
<i>Pseudomonas aeruginosa</i>	AroE	F: ATGTCACCGTGCCGTTCAAG R: TGAAGGCAGTCGGTTCCTTG	1500bp	Embaby et al., 2014
<i>Escherichia coli</i>	ArcA	F: GAAGACGAGTTGGTAACACG R: CTTCCAGATCACCGCAGAAGC	1500bp	Beutin et al., 2005

Table 2. The research provides data on the numerical distribution and proportional representation of bacterial samples.

The objective is to isolate	People	Dog	Sum
<i>Staphylococcus</i> spp.	20(26.3 %)	39(26.7%)	59(26.6%)
<i>Streptococcus</i> spp.	8(10.5 %)	17(11.6%)	25(11.3%)
<i>Pseudomonas</i> spp.	16(21.1 %)	29(19.9 %)	45(20.3%)
<i>Escherichia coli</i>	11(14.5 %)	15(10.3 %)	26(11.7%)
<i>Proteus</i> spp.	13(17.1 %)	16(10.9%)	29(13.1%)
<i>Klebsiella</i> spp.	7(9.2 %)	11(7.5 %)	18(8.10%)
<i>Pasturella</i> spp.	0(0%)	8(5.5%)	8(3.6%)

The objective is to isolate	People	Dog	Sum
<i>Corynebacterium</i> spp.	1(1.3%)	6(4.1%)	7(3.2%)
<i>Enterobacter</i> spp.	0(0%)	5(3.4%)	5(2.3 %)
Total	76(34.2%)	146(65.8%)	222(100%)

Table 3. Sensitivity of isolates Bacteria to antibiotics

Antibiotic		<i>Klebsiella</i> spp.	<i>Staphylococcus</i> spp.	<i>Escherichia coli</i>	<i>Proteus</i> spp.	<i>Pseudomonas</i> spp.
Gentamycin	(GEN10µg)	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive
Amoxicillin	(AML25µg)	Resistance	Resistance	Resistance	Resistance	Resistance
Ampicillin	(AMP10µg)	Resistance	Resistance	Resistance	Resistance	Resistance
Azithromycin	(AZ15µg)	Resistance	Resistance	Resistance	Resistance	Resistance
Chloramphenicol	(C30µg)	Resistance	Resistance	Resistance	Resistance	Resistance
Lincomycin	(L2µg)	Resistance	Resistance	Resistance	Resistance	Resistance
Doxycycline	(DO30µg)	Resistance	Resistance	Resistance	Resistance	Resistance
Amikacin	(AK30µg)	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive
Tetracycline	(TET30µg)	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive
Ciprofloxacin	(CIP5µg)	Sensitive	Sensitive	Sensitive	sensitive	Sensitive

Table 4. Single (conventional) PCR detection results of clinical samples from dogs and patients with otitis externa.

Samples	Pathogens	Proportion of positive samples (%)	Concordance rate (%)
Dogs ear swabs 9 (3 of each pathogen)	<i>E. coli</i>	3/3 (100)	100
	<i>P. aeruginosa</i>	3/3 (100)	100
	<i>S. aureus</i>	3/3 (100)	100
Patient ear swabs 6 (2 of each pathogen)	<i>E. coli</i>	2/2 (100)	100
	<i>P. aeruginosa</i>	2/2 (100)	100
	<i>S. aureus</i>	2/2 (100)	100

Distribution of bacterial isolates based on sample origin

The research provides data on the numerical and relative distribution of bacteria samples, with the aim of isolating bacterial species in humans. As shown in (Table 2.), The results show the isolation of 20 samples (26.3%) of *Staphylococcus* species, 8 samples (10.5%) of *Streptococcus* species, 16 samples (21.1%) of *Pseudomonas* species, and 11 samples (14.5%) of *Escherichia coli*, 13 samples (17.1%) of *Proteus* species, 7 samples (9.2%) of *Klebsiella* species, and one sample (1.3%) of *Corynebacterium auris*. The samples isolated from dogs included 39 samples (26.7%) of *Staphylococcus* species, 17 samples (11.6%) of Streptococci, 29 samples (19.9%) of *Pseudomonas*, 15 samples (10.3%) of *Escherichia coli*, and 16 samples (10.9%) of *Proteus*, 11 samples (7.5%) of *Klebsiella*, 8 samples (5.5%) of *Pasteurella* species, and 6 samples (4.1%) of *Corynebacterium*. As for *Enterobacter* species, no samples were isolated from people, but 5 samples (3.4%) were isolated from dogs. These results show the distribution and relative representation of bacterial samples out of a total of 222 samples. Of these, 76 samples (34.2%) were taken from people, while a higher number, 146 samples, were collected from dogs (65.8%). These numbers highlight the diversity of bacterial species isolated and their importance in the natural environment for both people and animals.

Antimicrobial susceptibility testing of bacteria

The study shows bacterial sensitivity to a group of antibiotics among laboratory isolates of different bacterial species, including *Klebsiella* spp., *Staphylococcus* spp., *Escherichia coli*, *Proteus* spp., and *Pseudomonas* spp.. The results indicate that all tested isolates were sensitive to gentamycin (GEN 10µg), with *Klebsiella*, *Staphylococcus*, *Escherichia coli*, *Proteus*, and *Pseudomonas* demonstrating a sensitive response. On the other hand, complete resistance to amoxicillin (AML 25 µg), ampicillin (AMP 10 µg), azithromycin (AZ 15 µg), chloramphenicol (C 30 µg), lincomycin (L 2 µg), and doxycycline (DO 30 µg), without exception, was recorded for all bacteria tested. Furthermore, isolates show sensitivity to both amikacin (AK 30 µg) and tetracycline (TET 30 µg) across all bacterial species tested. In addition, the data show that the antibiotic ciprofloxacin (CIP 5 µg) was effective against all types of bacteria. All bacterial isolates tested in the study were found to be susceptible to amikacin (AK 30 µg), Tetracycline (TET 30 µg), and Ciprofloxacin (CIP 5 µg). Therefore, there were no bacteria that exhibited a high level of resistance to these antibiotics.

Isolate bacterial deoxyribonucleic acid (DNA) and extract its genetic material

For each type of a swab, the proportion of positive samples and the concordance rate were determined for three pathogens: *E. coli*, *P. aeruginosa*, and *S. aureus*. The 9 dog ear swabs analyzed (3 for each of the three etiologies) all yielded 100% positive results for each of the three etiologies, demonstrating a concordance rate of 100%. As for the patients' ear swabs, which numbered 6 (2 for each of the three causes), the percentage of positive samples was 100% for each of the causes, and the concordance rate was also 100%. These data confirm the effectiveness of detection and the strength of the association between the samples and the three causes in both groups.

16S rRNA gene amplification

The study included five *E. coli* isolates, three from dogs and two from human patients. The use of specific PCR primers resulted in efficient amplification of the 16S rRNA gene of *E. coli*, as indicated by the results shown in Figure 1, which showed that this gene has a molecular weight of 1500 base pairs.

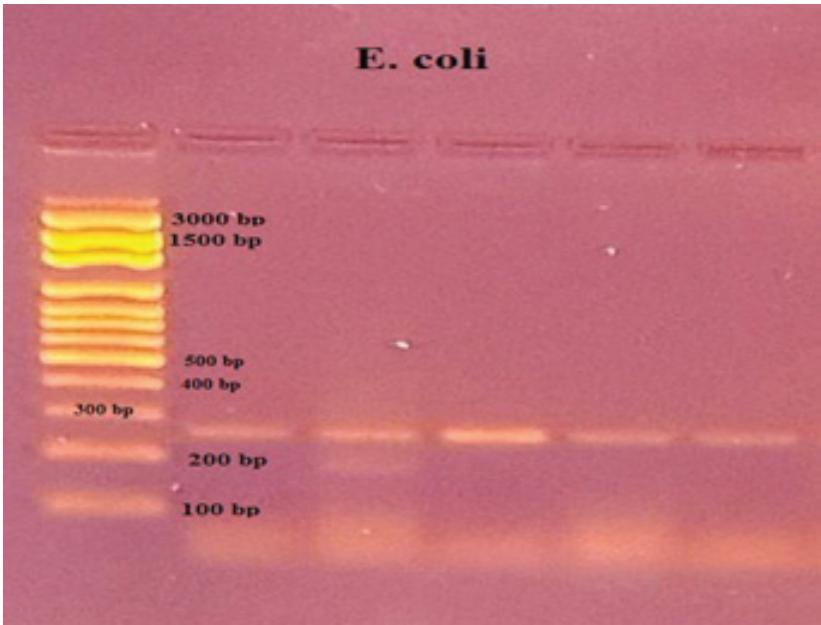


Figure 1. PRISMA flow diagram according to Renald et al. (2023)

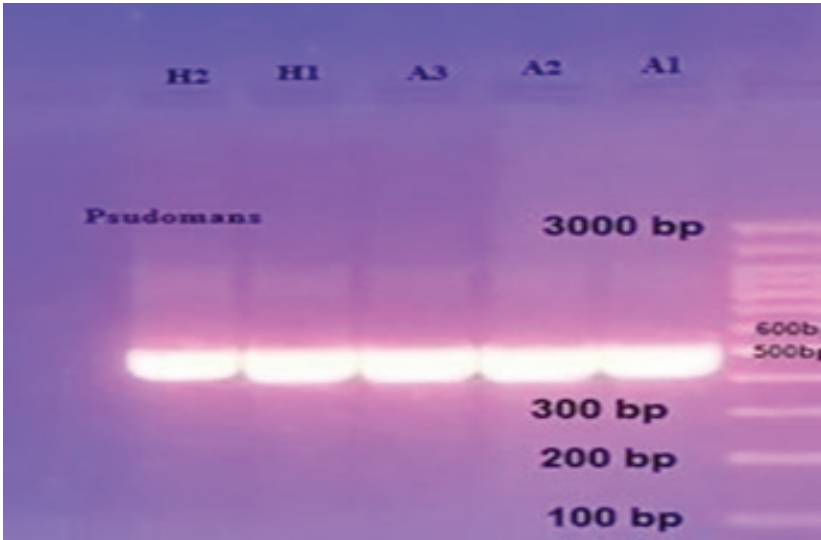


Figure 2. The DNA genome of *Pseudomonas aeruginosa* isolates

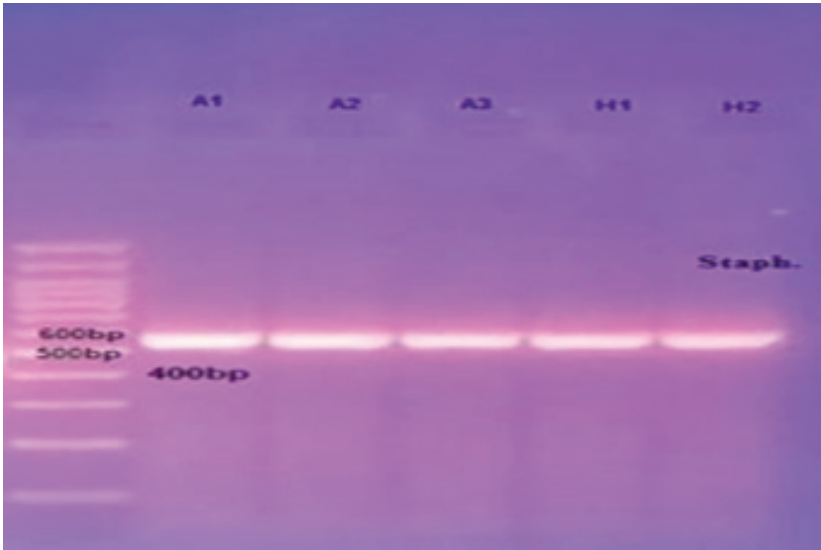


Figure 3. The DNA genome of *Staphylococcus aureus* isolate

Phylogenetic tree analysis

The present study employed phylogenetic tree analysis using partial ribosomal RNA (16S) gene sequences to identify and classify local *Staphylococcus* spp. strains. This study presents a recent submission of locally isolated Iramogre *Staphylococcus* spp. clinical specimens. The findings demonstrate the successful identification of two *S. aureus* isolates, each assigned unique Gen-Bank entry numbers: OK 560669,1 and OK 9560670,1 (Figure 4). Our findings include the successful identification of 2 isolated *E. coli* strains, each assigned a unique Gen-Bank entry number: OK 560673,1 and OK 560674,1 (Figure 5). In contrast, it is important to highlight a recent submission of indigenous Iraqi *Pseudomonas* clinical isolates, which has been documented during the course of the present investigation. This submission has yielded 2 distinct *Pseudomonas aeruginosa* isolates, each assigned with unique Gen-Bank entry numbers: OK 560672,1 and OK 560671,1 (Figure 6).

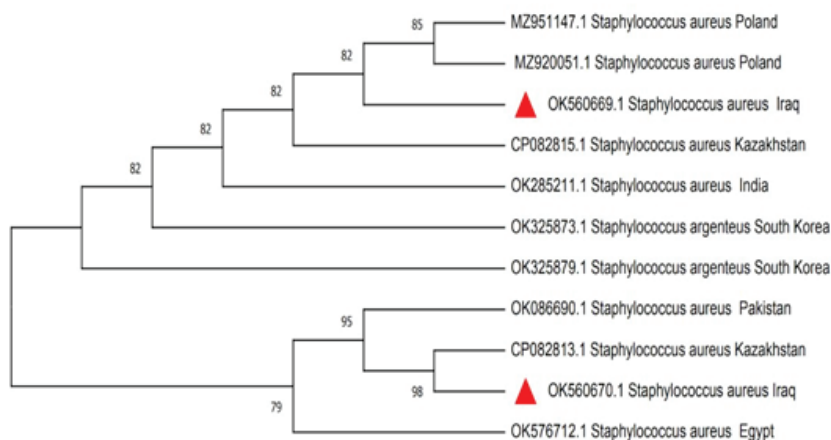


Figure 4. The recognition of local *Staphylococcus* spp. isolates were performed by phylogenetic-tree analysis utilizing partial sequences of the 16-S ribosomal Ribonucleic Acid (RNA) genes.

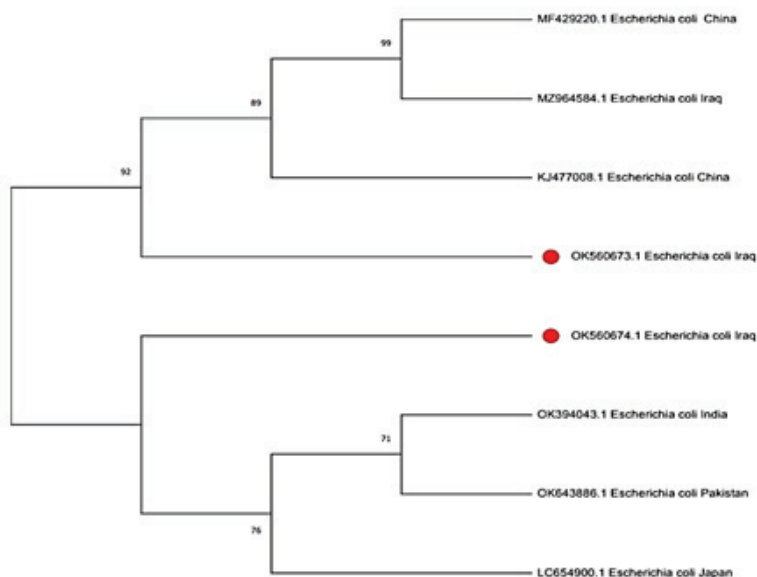


Figure 5. The identification of local *E. coli* strains was performed by phylogenetic-tree analysis utilizing the partial sequence of the 16-S ribosomal ribonucleic acid (RNA) genes.

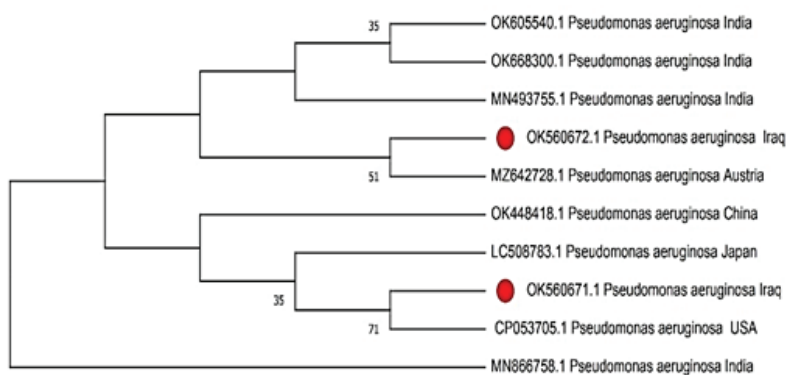


Figure 6. The identification of local *Pseudomonas* strains (*Pseudomonas aeruginosa*) was performed by phylogenetic-tree analysis utilizing the partial sequence of the 16-S ribosomal ribonucleic acid (RNA) genes.

DISCUSSION

The study revealed a high overall infection rate of 90% in dogs, while in patients, the infection rate was slightly lower at 68%. Among the patient cases, 68% were associated with *acute otitis externa*, highlighting the prevalence of this condition. This finding is consistent with the studies by Turner et al. (2023) and Salim et al. (2023). In the auditory canal, various bacteria were identified, including both Coagulase-positive and -negative strains. *Staphylococci* and *Streptococci* species were frequently found, while *Corynebacterium* strains and coliform germs were less commonly detected. On the other hand, the presence of other types of bacteria, *Pseudomonas* spp. and *Proteus* spp., was detected infrequently in the specific area of the outer ear. It is worth noting that the mere presence of microorganisms is not conclusive evidence of *otitis externa*, as indicated by (Deneva et al., 2020 and Szewczuk et al., 2020). The results confirmed that bacterial infection is complicated in the case of ear infection, and therefore it is necessary to make an accurate diagnosis and appropriate treatment for the condition (Rawson et al., 2023). The presence of specific bacterial species in the auditory canal indicates knowledge of the species associated with ear infections, which helps in finding therapeutic methods that target the specific type of bacteria, which facilitates the treatment of the disease, and this is consistent with the findings of Chen et al. 2024. These differences in prevalence were found to be statistically significant, with a p -value of ≤ 0.05 that is compatible with (Petrov et al. 2013). The observed outcomes might potentially be associated with the production of biofilm by *Staphylococcus* spp. and *Pseudomonas* spp. bacteria, leading to the persistence of infection even when appropriate treatment measures are implemented. For any antimicrobial medication to be effective in treating the infection, it must be able to destroy the biofilm. The study has provided evidence that *Pseudomonas aeruginosa* and *S. aureus* are frequently acknowledged as the predominant causative agents responsible for the development of *acute otitis externa* (Ghapanvari et al., 2022). This study aligns with our findings, where *Staphylococcus* spp. was frequently isolated from the auditory canal of dogs with otitis (Pirashanna and Rajapaksha, 2021). In another study conducted on dogs with ear infections, it was noted that bacterial infection often occurs as a secondary infection in about 50% of cases, given that the main infection is caused by pathogenic fungi. The prevalence of *Staphylococcus* spp. among the analyzed dog specimens was determined to be 26.7% (39 out of 146 samples), while *Pseudomonas* species had a prevalence of 19.9% (29 out of 146 samples). Among the dog samples analyzed, *Staphylococcus* spp. showed a very high prevalence of 26.7% (39

out of 146 samples), followed by *Pseudomonas* species at 19.9% (29 out of 146 samples). *Proteus* species accounted for 10.9% (16 out of 146 samples), while both *Streptococcus* species and *Escherichia coli* were found in 10.3% (15 out of 146 samples) each. *Klebsiella* species were present in 7.5% (11 out of 146 samples), while *Pasteurella* species were detected in 5.5% (8 out of 146 samples). *Corynebacterium* species and intestinal bacteria species were less prevalent, reaching 4.1% (6 out of 146 samples) and 3.4% (5 out of 146 samples), respectively (here it is explained which bacteria had a higher percentage during diagnosis. and insulation) (Paterson, 2013). This study is consistent with Bajwa (2019), which identifies prevalent perpetuating factors such as bacteria like *Staphylococcus* and *Pseudomonas*, as well as *Malassezia* yeast. The presence of this infection in the middle ear may also act as a perpetuating factor, causing repeated external ear infections if it spreads to the tympanic bulla. In dogs with recurrent *otitis externa*, the primary cause of therapy failure is often perpetuating factors. To authenticate the identity of the *Staphylococcus* spp., *Pseudomonas* spp., and the *E. coli* strain that were isolated during the current investigation, it was imperative to conduct a sequencing analysis of the 16srRNA gene (Karnad et al., 2020). This alignment has also been successfully documented for the two *Staphylococci* isolates. Sequencing using the UPGMA tree in MEGA version 10.0 revealed that the *S. aureus* strain, with accession numbers OK9560670.1 and OK560669.1, shows 98% identity with the two *S. aureus* strains CP086690.1 and CP082813.1, which were isolated in Pakistan, Kazakhstan, and local Iraqi clinical isolates of *Staphylococci* obtained during the current study. This sequence arrangement also successfully documented the two *Staphylococci* isolates. Likewise, the application of the UPGMA tree in MEGA version 10.0 revealed that an *E. coli* strain with accession numbers OK560673.1 and OK560674.1 showed 98% concordance with closely related strains identified through NCBI-Blast, specifically *E. coli* strain OK560673.1 from China and OK394043, isolated in Pakistan, respectively. Thus, this analysis provides a noteworthy record of two *E. coli* isolates, each bearing GenBank accession numbers OK 560673.1 and OK 560674.1. In contrast, the current study found two successful cases of two strains of *Pseudomonas aeruginosa*, OK560672.1 and OK560671.1, which were isolated from local Iraqi medical samples (Rudra et al., 2022). Utilizing the UPGMA-tree method in the MEGA 10.0 versions, sequence alignment analysis revealed the genetic relatedness of these (*Pseudomonas aeruginosa*) strains, as indicated by their respective accession numbers: OK560672.1 and OK 98% of the variations between Iraqi bacterial isolates and isolates from other countries may be attributed to environmental factors and varying levels of isolation (Shehab et al., 2020).

CONCLUSION

S. aureus, *P. aeruginosa*, and *E. coli* are the most prevalent bacterial pathogens in humans and dogs, posing a significant health threat. Prevention measures, responsible antibiotic use, accurate diagnosis, effective treatment, and clear infection prevention guidelines are crucial. The study found isolates' sensitivity to gentamycin, ciprofloxacin, tetracycline, and amikacin was favorable, while resistance to azithromycin, chloramphenicol, and ampicillin was high, suggesting limited treatment options. High rates of β -lactamase resistance pose a significant challenge to antibiotic treatment, necessitating research and innovation to counteract this. Advocating responsible antibiotic use and strong infection control measures is crucial. The study confirmed the identification of *S. aureus*, *P. aeruginosa*, and *E. coli* isolates from Iraq using molecular techniques, enhancing knowledge of bacterial diversity and potential infection control strategies.

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Author's Contribution:

AK, SQ and SJ made contributions to the conception and design of the study, were involved in data collection and drafted the manuscript. AK, SQ and HI carried out the molecular diagnostic tests and prepared the alignment of nucleotide sequences and conducted the molecular genetic analysis. AK carried out the serological tests and data analysis and performed the statistical analysis. AK, SQ and SJ revised the manuscript critically and together prepared the final draft of the manuscript, etc. All authors read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

Data availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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ACTIVATED CHARCOAL DERIVATED FROM BIOCHAR AS A FEED ADDITIVE FOR GOATS: A RUMINAL ANALYSIS

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Abstract

Biochar, which is the product of biomass pyrolysis, has been suggested as a feed supplement to improve understanding of the rumen microbial ecosystem. The study aims to investigate the potential of activated charcoal to favorably modify rumen fermentation and reduce methane emission by measuring ruminal parameters, enzymes, and microbial communities. Thirty goats (weighing 14.35 ± 1.40 kg) were randomly put into three groups: a control diet group and groups with diet supplemented with activated charcoal at 50 mg/kg and 150 mg/kg. Each group comprised ten replicate pens. The basal diet contained 40% barley and 60% alfalfa hay, and diets were similar to isocaloric and isonitrogenous. The trial lasted 60 days following a 14-day adaptation. The biochar was obtained from pyrolysis of a river plant (*Ceratophyllum demersum*), impregnate with zinc chloride (ZnCl_2), and went through the carbonization process at 700 °C. Rumen fluids were sampled, filtered, and stored at -80 °C in liquid nitrogen to analyze eukaryotic diversity and enzyme activity; the rest was stored at -20 °C to measure fermentation parameters. The results showed that supplementation with activated biochar did not affect pH values and acetate/propionate molar ratio ($P = 0.021$). Ruminal ammonia ($\text{NH}_3\text{-N}$), Total Volatile Fatty Acids, Acetate, and Butyrate (%) were significantly ($p < 0.05$) higher at 50 mg and 150 mg activated charcoal groups, compared with control group. Supplementation with activated biochar increased the activities of CMCase, Xylanase, Pectinase, and α -amylase in the rumen, but β -glucosidase and

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protease were not affected. These changes were associated with improved growth performance in the goats. Overall, the interplay of biochar's physical and chemical characteristics plays a major role in different living systems by influencing the different metabolic enzymes and processes, availability of different nutrients, and environmental conditions. Dietary supplementation with activated biochar increased total bacteria, *Fibrobacter succinogenes*, *Rummonococcus albus*, and *Ruminococcus flavefaciens*) and methanogenic, but did not affect protozoa. In conclusion, this study would further promote biochar applications in ruminal fermentation processes.

Key words: Goats, Biochar, Activated charcoal, Ceratophyllum Demersum, Microbial community, PCR

AKTIVNI UGALJ KAO ADITIV U HRANI ZA KOZE: ANALIZA SADRŽAJA RUMENA

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Kratak sadržaj

Aktivni ugalj, koji predstavlja proizvod pirolize biomase predložen je kao dodatak ishrani, u cilju boljeg razumevanja mikrobioma rumena. Cilj ovog rada je ispitivanje potencijala aktivnog uglja da povoljno utiče na fermentaciju u rumenu i tako dovede do smanjenja emisije metana, merenjem parametara rumena, enzima, i mikrobioma. Trideset koza (težine 14.35 ± 1.40 kg) nasumično je podeljeno u tri grupe: kontrolna grupa i grupe sa dodatkom aktivnog uglja 50 mg, i 150 mg/kg u obroku. Svaka grupa sastojala se iz 10 bokseva za ponavljanje. Bazalna ishrana saržala je 40% ječma i 60% lucerke, a ishrana je bila ujednačena po izokaloričnom i izonitrogenom sastavu. Ispitivanje je trajalo 60 dana, koje je usledilo nakon perioda adaptacije od 14 dana. Aktivni ugalj dobijen je prirolizom iz rečne biljke *Ceratophyllum demersum*, impregniran je cink hloridom ($ZnCl_2$), a proces karbonizacije je izvršen na 700 °C. Tečnosti iz rumena su uzorkovane, filtrirane i

skladištene na -80 °C u tečnom azotu kako bi se analizirala raznovrsnost mikrobioma i aktivnost enzima; ostatak je čuvan na temperaturi od -20 °C kako bi se izmerili parametri fermentacije. Rezultati su pokazali da suplementacija aktivnim ugljem nije uticala na pH vrednosti i odnos molarne mase acetata i propionata ($P = 0.021$). Ruminalni amonijak ($\text{NH}_3\text{-N}$), ukupne isparljive masne kiseline, acetat, i butirat (%) bili su značajno viši ($p < 0.05$) u grupama sa dodatkom od 50 mg i 150 mg aktivnog uglja u ishrani u poređenju sa kontrolnom grupom. Suplementacija aktivnim ugljem dovela je do povišenih nivoa aktivnosti CMCaze, ksilanaze, pektinaze, i α -amilaze kod rumena, dok na β -glukosidazu i proteazu nisu imali uticaja. Ove promene nisu povezane sa poboljšanim prirastom koza. Načelno, međudejstvo fizičkih i hemijskih karakteristika aktivnog uglja ima ključnu ulogu u različitim živim sistemima, utičući na različite metaboličke enzime i procese, dostupnost različitih nutrijenata i uslove sredine. Suplementacija aktivnim ugljem dovela je do povećanog ukupnog broja bakterija, *Fibrobacter succinogenes*, *Rumonococcus albus*, i *Ruminococcus flavefaciens* kao i metanogene, ali nije uticala na protozoe. Ovo istraživanje bi dodatno promovisalo primenu aktivnog uglja u procesima fermentacije u rumenu.

Ključne reči: koze, biougalj, aktivni ugalj, Ceratophyllum Demersum, mikrobna zajednica, PCR

INTRODUCTION

Dietary manipulation (feed additives) is critical for increasing the profitability of livestock industry through improving fermentation in the rumen and enhancing nutrient absorption efficiently, hence optimizing the health and production of ruminants. There is documented evidence that the use of biochar as a dietary supplement for both humans and animals for the treatment of digestive disorders (O'Toole et al., 2016). Biochar acts as a rumen modifier, particularly in reducing enteric CH_4 emissions. Schmidt et al., 2019 report that it may reduce methane emissions up to 20% in cow feed diet that contains 6 g biochar per kg dry matter (DM) (Leng et al., 2012). In addition, biochar's enhancing mechanisms, including immobilization effect (Yang et al., 2022), electron-mediating properties in biological redox reactions, good conductivity beneficial to electron transfer (Nguyen et al., 2021), biochar's alkalinity for improvement of digester buffering capability, adsorption effect (Wang et al., 2019), and contribution to microbial growth (Lü et al., 2019).

Biochar is a byproduct of biofuels industry, it is non-toxic and can be produced through pyrolysis or gasification processes under average temperatures

of 700 °C and low oxygen levels (Toth and Dou, 2016), and undergoes an activation process in order to increase surface of the substance to adsorption of a larger quantity of molecules (Soo et al., 2013) by a series of physical (CO₂, steam) or chemical (salt, organic and inorganic acid treatment) processes. The sources of biochar and pyrolysis conditions determine the characteristics of activated charcoal.

Ceratophyllum demersum (CD) is a submerged plant diffused in quiet streams and ponds whose increased growth might cause waterway clogging and acts as a secondary environmental pollutant (Yang, et al., 2022). Biochar production is an emerging strategy in waste biomass management, offering a new approach to enhance animal health and improve feed efficiency (Schmidt et al., 2017).

Activated charcoal has been used as a feed additive in ruminant diets due to promoting digestive health through removing toxins, supporting rumen ecology, and improving feed conversion efficiency (Wang et al. 2019). Biochar can inhibit butyric acid formation and enhance the quantity of lactic bacteria and provide a biofilm habitat for microbiota to proliferate (Pereira et al., 2014). In addition, biochar also possesses electron-mediating properties that support biological redox reactions in the rumen, enhancing energy conversion efficiency in livestock and helping to reduce greenhouse gas emission (Leng et al. 2012). Since most biochar is alkaline, it can act as a pH buffer in the rumen, resulting in improved livestock weight gains under high-energy diets (Schmidt et al. 2019). Several studies have been carried out to study the impact of biochar supplemented diet on ruminants (Al-Azzawi et al. 2021).

Despite its many potential benefits, further research is needed to fully understand the effectiveness of biochar in animal production, as current knowledge is limited and existing studies often report conflicting results. Therefore, the aim of this study was to deepen the understanding of microbial ecology and their functional roles in the rumen, while also exploring the potential of activated charcoal derived from biochar as a feed additive for ruminant livestock.

MATERIAL AND METHODS

Ethical approval

The protocol used in this study was approved by the ethics committee on animal experimentation of the Faculty of Veterinary Medicine, University of Baghdad (no.1632/P.G.12 August. 2023).

Biochar Preparation

The biochar used in the experiment was obtained from pyrolysis of the river plant *Ceratophyllum Demersum*. The biomass was harvested from water surface of Tigris River, Iraq, from May, to July. The roots were cut off, discarded, and washed with distilled water. The samples were air-dried under sunlight for 48 hours by spreading them on sheets and turning them over multiple times to ensure even drying. The biomass was tightly packed into smaller barrels, which were then inverted into larger barrels. The space between the two barrels was filled with wood and ignited. The larger barrel was heated from below with a gas heat source until heating up to a peak temperature of either 350 or 550 °C for 3 h. The biomass was then impregnated with zinc chloride (ZnCl_2), and it went through the carbonization process at temperatures of 700 °C (Romanos et al., 2011).

Experimental procedure

This study was carried out from September up to November 2023, on Al-Zubaydiaa agricultural farms for goat breeding (private sector), Wasit, Iraq. Thirty male local-breed goats, aged between 8 and 11 months with an initial body weight of 14.35 ± 1.40 kg, were randomly assigned to three experimental diets using a completely randomized design ($n = 10$). Experimental diets contained the following:

1. Control (C), a basal diet of alfalfa hay and barley (60:40);
2. Activated charcoal (AC1), basal diet + 50 mg/kg activated charcoal (0.5%) and
3. Activated charcoal (AC2) basal diet + 150 mg/kg activated charcoal (1.5%), rations were an isocaloric and isonitrogenous (Table 1).

The experimental period lasted for 60 days. Goats were individually penned in adjacent pens with dirt floors, measuring 2.5×2.5 m, with free access to feed and water. Before the beginning of the experiment, the goats were fed experimental diets for 14 days as an adaptation period and weighed for three days at 8 am to fix the primary weight. The goats were fed 3% of their body weight twice a day (at 08:00 and 16:00). All goats were treated against ecto and endo parasites and vaccinated against clostridiosis before the beginning of the experiment.

Sampling schedule

Feed samples (50 g) were taken for each group in the morning. At the end of the experiment, feed samples representing 10% of the total weight were collected in a labeled polythene bag and stored at 10 °C until analyzed. Rumen fluids were sampled on days 0, 30, and 60 from each animal (after 2 hours of morning feeding) by using a stomach tube fitted to a vacuum pump to siphon fluid through a tube (length: 1 m, diameter: 1.5 cm). In order to prevent saliva interference, rumen pH was measured by using a pH electrode (Model PB-20, Sartorius, Germany). Ten and 5 mL of rumen fluids were collected from each goat, filtered and strained through four layers of compressed gauze, transferred to sterile tubes, and stored at -80 °C in liquid nitrogen for analysis (eukaryotic diversity and enzyme activity); the other was stored at -20 °C for measurement of fermentation parameters.

Chemical analysis

Feed samples were analyzed for dry matter (DM), organic matter (OM), crude protein (CP), and ether extract (EE) according to AOAC (2006), while neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the procedure by Van Soest, (1991). Using a Leco CHN1000 analyzer and coal reference material (Leco Corp., St. Joseph, MI, USA), the AC was analyzed by combustion. For further analysis, 0.1 g of AC was mixed with 10 ml of deionized water and shaken at 250 rpm for 24 hours, after which the supernatant was collected. The Tristar II 3020 (Micromeritics) was done to determine the surface area of the AC. Ammonia nitrogen ($\text{NH}_3\text{-N}$) and the total volatile fatty acids (TVFA) were evaluated according to AOAC (2006) and Martínez et al. (2010) respectively.

Rumen content samples were thawed at room temperature and placed in an ultrasonic beater at 4 °C, subjected to 4 cycles of 30 seconds each, with 30 second rest periods in between. The samples were then centrifuged at $20,000 \times g$ for 15 minutes at 4 °C to separate cellular debris and enzymes (Martin and Michalet-Doreau, 1995). The supernatant was collected, and subsequent processing was done by the kit (R&D Systems, Minneapolis, MN, USA), to measure activities of carboxymethyl cellulase (CMCase), xylanase, pectinase and β -glucosidase. The activity of lipase and amylase was measured using commercially available reagent kits (NanJing JianCheng Bioengineering Institute, Nanjing, China).

Protease activity was measured using visual-light spectrophotometry at 680 nm. After reaction mixture preparation (1 mL of casein and 4 mL of protease enzyme), the mixture was incubated at 38 °C for 4 h. Then, the reac-

tion was stopped by adding trichloroacetic acid (10%), the sample centrifuged (3500×g for 15 min). The 1 mL of supernatant was mixed with 5mL 0.4M Na₂CO₃ with 1 mL Folin-Ciocalteu's reagent. The mixture was then incubated for 15 minutes before measuring.

Rumen microbial community analysis

The manufacturer provides the guidelines of the protocol. The DNA extraction using QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA, USA), and Real-time PCR to determine the population of total bacteria, *Fibrobacter succinogenes*, *Rumonococcus albus*, and *Ruminococcus flavefaciens*, methanogens, and protozoa was used. The primers for detecting the ruminal bacteria 16S rRNA gene sequences were used through CFX 96 system to amplify 16S rDNA (Table 1).

Table 1. The PCR primer used for quantification of rumen microorganism.

Microbes	Primer	
	Forward	Reverse
General bacteria	5'-CGGCAACGAGCGCAACCC-3'	5'-CCATTGTAGCACGTGTGTAGCC-3'
<i>Fibrobacter succinogenes</i>	5'-GTTCGGAATTACTGGGCGTAAA-3'	5'-CGCCTGCCCTGAACTATC-3'
<i>Ruminococcus albus</i>	5'-CCC TAA AAG CAG TCT TAG TTC G-3'	5'-CCT CCT TGC GGT TAG AAC A-3'
<i>Ruminococcus flavefaciens</i>	5'-CGAACGGAGATAATTGAGTTTACTTAGG-3'	5'-CGGTCTCTGTATGTTATGAGGTATTACC-3'
Methanogenic archaea	5'-TTCGGTGGATCDCARAGRGC-3'	5'-GBARGTCGWAWCCGTAGAATCC-3'
Protozoa	5'-GCTTTCGWTGGTAGTGATT-3'	5'-CTTGCCCTCYAATCGTWCT-3'

The amplification reaction was carried out in a final volume of 25 µL, consisting of 12.5 µL of Maxima SYBR Green qPCR Master Mix, 8.5 µL of RNase-free distilled water, 2 µL of DNA elution, and 1 µL of species-specific forward and reverse primers. Amplification involved one cycle at 95 °C for 15 min for initial denaturation, then 39 cycles of 95°C for 30 s followed by 30 s at annealing temperature, and 20 s at 72°C for an extension.

Statistical analysis

All analyses were computed by SPSS software version 21.0 (Mean ± SE) and the obtained data were analyzed. Statistical comparisons were made using one-way ANOVA of the generalized linear model (GLM), using the following

model: $y_{ij} = \mu + T_i + e_{ij}$, where y_{ij} is the dependent variable, μ is the overall mean, T_i is the treatment effect, and e_{ij} is the experimental error. Duncan's multiple range test to compare differences between groups was used. The ($p < 0.05$) level was adopted for statistical significance.

RESULTS

Chemical analysis of diets and activated biochar

The analysis of the experimental diets was similar (Table 2). The chemical composition of the activated charcoal is shown in Table 3.

Table 2. Ingredients and chemical composition of treated diets

Ingredient	g/kg, as-fed basis	Treatment		
		C	AC1	AC2
Barley		40	39.75	39.25
Alfalfa hay		60	59.75	59.25
Activated charcoal		---	0.5	1.5
Chemical composition, % DM				
DM		89.3	89.1	89.7
CP		14.9	14.86	14.8
EE		5.46	5.3	5.1
NDF		35.6	34.9	34.7
ADF		27.2	26.5	26.1
Metabolizable energy (Mcal/kg DM)		2.37	2.31	2.3

Table 3. Physical properties and chemical composition of activated charcoal

Surface area (m ² /g)	516
Bulk density (kg/m ³)	387
Pores volume (cc/g)	9.21 × 10 ⁻²
pH	8.91

Ruminal fermentation parameters

The effect of supplemented AC in diet on ruminal fermentation is presented in Table 3. The AC did not affect ruminal pH ($P = 0.018$) and sampling time and the highest value was obtained at 60 d (6.7) in the AC2 group, while the lowest levels were at 0 d (6.45) in control group.

The $\text{NH}_3\text{-N}$ concentration in rumen was increased ($P = 0.0048$) in the AC1 and AC2 diets relative to the control diet. The sample time was an effect significantly ($p < 0.05$) which gradually increased over 30 and 60 days in all treated groups, while no change was observed between 0.5 and 1.5% AC.

The results show that the supplementation of AB significantly ($p < 0.05$) increased TVFA (mmol/dL) concentration during experimental feeding. Unlike this, TVFA levels tended to increase in AC1 and AC2 groups than control group. The acetate, propionic, and butyric acid concentration reveal significant ($p \leq 0.05$) increases with the higher level of AC inclusion, unlike acetic acid to propionic acid ratio (A/P) was not affected by the different levels of AC.

The activity of ruminal enzymes (CMCase, xylanase, pectinase, and α -amylase) was increased with high inclusion of AC. The activity of α -amylase in group AC1 was not significantly different from control ($p > 0.05$). However, the activities of these enzymes were significantly higher in AC2 group, whereas protease and β -glucosidase activity was not affected by supplement of AC (Table 4).

Table 4. Effect of activated charcoal on Rumen enzymes activity (mean $\pm$ SE) of goat

Parameter	Days	Treatment			p-value
		C	AC1	AC2	
CMCase (U/mL)	0	71.21 $\pm$ 1.23	71.43 $\pm$ 1.90	71.42 $\pm$ 1.11	0.072
	20	72.64 $\pm$ 1.97 ^c	75.18 $\pm$ 4.32 ^b	77.12 $\pm$ 3.55 ^a	0.018
	60	74.41 $\pm$ 4.01 ^c	81.86 $\pm$ 3.45 ^b	84.42 $\pm$ 2.18 ^a	0.020
Xylanase (U/mL)	0	10.99 $\pm$ 1.72	10.05 $\pm$ 1.75	10.07 $\pm$ 1.03	0.120
	30	13.31 $\pm$ 2.12 ^c	15.22 $\pm$ 1.43 ^b	18.20 $\pm$ 1.37 ^a	0.004
	60	14.1 $\pm$ 1.83 ^c	18.42 $\pm$ 3.19 ^b	22.12 $\pm$ 1.33 ^a	0.003
Pectinase (U/mL)	0	43.73 $\pm$ 3.52	44.22 $\pm$ 1.09	43.13 $\pm$ 1.06	0.110
	30	42.48 $\pm$ 4.52 ^c	50.25 $\pm$ 3.31 ^b	52.76 $\pm$ 2.66 ^a	0.004
	60	40.18 $\pm$ 2.56 ^c	51.91 $\pm$ 1.88 ^b	53.21 $\pm$ 0.88 ^a	< 0.001

Parameter	Days	Treatment			p-value
		C	AC1	AC2	
β -glucosidase (U/ mL)	0	42.62±3.04	42.09±3.22	42.41±3.05	0.310
	30	44.61±4.32	44.87±4.77	45.38±3.62	0.250
	60	44.90±1.01	44.88±1.98	45.98±3.19	0.070
α -amylas (μ g / min.mL ⁻¹)	0	20.95±0.75	20.92±0.32	20.89±0.34	0.051
	30	21.09±0.33 ^b	22.39±0.84 ^b	24.15±0.34 ^a	< 0.001
	60	21.45±0.53 ^b	21.55±0.43 ^b	24.99±0.54 ^a	0.003
Protease (μ g / min.mL ⁻¹)	0	3.43±0.86	3.65±0.56	3.47±0.39	0.060
	30	3.51±0.69	3.63±0.22	3.42±0.73	0.140
	60	4.13±0.55	4.22±0.44	4.21±0.35	0.170

*The different lowercase letters refer to significant differences between different treated groups at ($p \leq 0.05$).

Table 5. presents the effect of supplement AC on the rumen microbial. Results show a significant ($p \leq 0.05$) increase in total bacteria count in the treated groups compared with the control group after 30 days of feeding. On the 60th day, group AC2 showed a significant ($p \leq 0.05$) increase in total bacteria count ($P = 0.036$) compared with other groups.

Table 5. Effect of activated charcoal on rumen microbial population (mean $\pm$ SE) of goat

Parameter (Log10 copy No/g)	Days	Treatment			p-value
		C	AC1	AC2	
Total bacteria	0	8.49 $\pm$ 0.39	9.06 $\pm$ 0.76	9.27 $\pm$ 0.61	0.063
	30	8.91±0.54 ^b	10.52±0.82 ^a	10.89±0.52 ^a	0.001
	60	9.01±0.71 ^c	10.04±0.65 ^b	11.6±0.46 ^a	0.036
<i>Fibrobacter succinogenes</i>	0	3.07±0.13	3.02±0.88	3.31±0.83	0.230
	30	3.20 $\pm$ 0.38 ^b	3.61 $\pm$ 0.16 ^a	3.8±0.63 ^a	0.026
	60	3.23±0.43 ^b	4.01±0.24 ^a	4.20 $\pm$ 0.11 ^a	0.001

Parameter (Log10 copy No/g)	Days	Treatment			p-value
		C	AC1	AC2	
<i>Rumonococcus albus</i>	0	7.61 ± 0.21	7.45 ± 0.18	7.27 ± 0.229	0.060
	30	7.82 ± 0.42	7.87±1.01	7.79±1.20	0.140
	60	7.81±0.37 ^b	7.90±0.75 ^b	8.38±0.24 ^a	0.040
<i>Ruminococcus flavefaciens</i>	0	4.42±0.18	4.57 ±0.07	4.66±0.62	0.120
	30	4.72± 0.22 ^b	4.90±0.91 ^b	5.29±0.74 ^a	0.002
	60	5.03±1.10 ^b	5.1±0.65 ^b	5.32±0.31 ^a	<0.001
Methanogenic archaea	0	3.71 ± 0.52	3.62±0.86	3.66±0.82	0.110
	30	4.2± 0.76 ^b	4.3±0.34 ^{ab}	4.72±0.57 ^a	0.041
	60	4.5± 0.85 ^b	4.62±0.74 ^b	5.13±0.66 ^a	0.037
Protozoa	0	3.63 ± 0.42	3.49 ± 0.22	3.51 ± 0.32	0.063
	30	3.57±0.16	3.50±1.32	3.52±0.89	0.071
	60	3.55±0.54	3.51±0.52	3.55±0.64	0.055

*The different lowercase letters refer to significant differences between different treated groups at ($P \leq 0.05$).

In addition, the AC supplement showed significantly ($p \leq 0.05$) increased population count of *Fibrobacter succinogenes*, *Rumonococcus albus*, and *Ruminococcus flavefaciens* in the AC2 group compared with other groups. Regarding the methanogenic archaea count, it increased significantly in the AC2 group compared to the other groups ($P = 0.041$). The inclusion of AC had no effect on the total protozoa count.

DISCUSSION

Rumen pH is a key indicator of rumen health, with a normal range of 5.50 to 7.50. Activated charcoal supplementation did not significantly affect pH levels but tended to result in slightly higher values compared to the control group. These results were consistent with Saleem et al. (2018) who found no significant link between the addition of pine activated charcoal and pH in RUSITEC system. Garillo et al. (1994) revealed that the ruminal pH in goats tended to increase slightly after 4 h of feeding on diets containing 0.3% and 0.6% of AC. Sun et al., (2021) report that activated charcoal, being alkaline in nature, has rich oxygen functional groups on the surface, which can produce a certain

amount of carboxyl, which can maintain a neutral environment. This result is in contrast with Wang et al. (2019) and Cabeza et al., (2018) who reported ruminal pH decreases after feeding activated charcoal.

The inclusion of AC increased ruminal $\text{NH}_3\text{-N}$ concentration, which is consistent with Leng et al. (2012) who observed a significant increase in $\text{NH}_3\text{-N}$ in yellow cattle after feeding them 0.6% activated charcoal. The increase in ruminal $\text{NH}_3\text{-N}$ may be attributed to numerical increase in protozoal or an increase in protein digestion. This explanation is in line with Doreau and Ferlay, 1995. However, Garillo et al., (1995) revealed decreased $\text{NH}_3\text{-N}$ in Suffolk ewe after feeding 0.3% AC. Pereira et al. (2014) in vitro and Cabeza et al., 2018 proved that there was no effect of $\text{NH}_3\text{-N}$ when using different sources of activated charcoal.

Volatile fatty acids (VFA) are the end product of fermentation and indicate relative fermentability. Dietary AB supplementation tended to significantly ($p < 0.05$) increase total VFAs in the treated groups compared to the control group, which is consistent with increased AB levels. A similar result was reported by Pereira et al. (2014) in vitro when using activated charcoal from silage and hay. Garillo et al. (1994) observed an increase in ruminal total VFA when goat diet contained 0.6% biocarbon. In contrast, an increase in VFAs is consistent with an increase in the molar proportion of acetate, propionic, and butyric. In contrast, Pereira et al. (2014) found increased TVFA and acetate concentrations consistent with an increased level of activated charcoal, whereas the amount of propionate was decreased and butyrate was not affected. Terry et al. (2019) and Teoh et al. (2019) found that activated charcoal did not significantly affect rumen VFA. Cabeza et al. (2018) reported that the concentrations of TVFA and acetate were not affected by addition of 1.16 and 11.6 %. McFarlane et al (2017) reported that they did not observe any changes in VFA, acetate, propionate, and butyrate production.

The ruminal microbes play roles in digestive processes through their enzymes. The potential effects of AC on rumen enzymes could provide new information and deepen our understanding of the functions of the rumen microbiome, thereby improving management of the rumen microbiome. AC showed improved CMCase, xylanase, and pectinase activity compared with the control group ($p < 0.05$) throughout the experimental period. These results can be explained by an increase in the amount of surface area of activation activated charcoal, as well as enhanced growth of certain rumen microbial communities by forming micro-sites or formation of functional groups that can enhance microbial redox reactions (Schmidt et al. 2019). In addition, AC facilitates direct interspecies electron transfer (DIET) between microbial populations,

promoting more rapid microbial growth (Leng et al., 2012). A similar result was reported by Bagherpoor et al., (2023) in vitro when activated charcoal was given separately or in a mixture with probiotics. Activated charcoal may stimulate bacterial growth and enzyme activity by modulating biofilm formation and activity within the rumen (Lehmann and Joseph, 2015).

Our study revealed the activity of α -amylase increase ($p < 0.05$) in AC2 compared with other groups. These results may be attributed to the influence of AC on microorganisms based on their cell envelope structure — Gram-positive bacteria tend to be less effectively adsorbed by activated charcoal, whereas Gram-negative bacteria exhibit greater adsorption. Bagherpoor et al., (2023) observed that protease activity was not affected by the experimental treatments (probiotic and activated charcoal) in vitro. The effect of activated charcoal on extracellular enzyme activity is known to depend during interaction between the substrate and enzyme and could be affected by activated charcoal porosity or specific surface area (Lammirato et al., 2011). A previous study suggested that charcoal can form complexes with phenolic compounds, preventing hydrolysable tannins from inhibiting bacterial enzyme function and protein digestion, thereby potentially enhancing protein availability and protease activity (Van et al. 2006). The reason for these increases can be the improvement of the cellulolytic bacteria population. Therefore, we speculate that the increase in relative abundance of *Fibrobacter* and *Ophryoscolex* was responsible for the increase in the activities of xylanase and pectinase and the decrease in activity of β -glucosidase.

The rumen provides a relatively stable environment for microorganisms, with their composition being primarily influenced by the animal's diet. Total bacteria appear to be affected by AC, particularly at higher supplement levels, results show that the number of bacteria significantly ($p < 0.05$) increased in treated groups compared with control, with the increase in the level of inclusion. These results differ from a study by Teoh et al. (2019) which found that activated charcoal may have shifted microbial communities but does not significantly influence the total bacteria in the rumen when 800 mg/per day is added. Qomariyah (2021) reported activated charcoal addition significantly decreased bacterial population in the rumen. Therefore, we speculate that the increased activities of xylanase and pectinase, along with the unchanged β -glucosidase activity, contributed to the relative abundance of *Fibrobacter* species, suggesting that the rumen bacterial community may be strongly oriented toward fiber degradation.

Fsuccinogenes, *R. albus*, and *R. flavefaciens* are predominant cellulolytic bacteria in goat rumen. In the present experiment, we found that cellulolytic

bacteria increased numerically in treated groups compared with control. Similar findings were reported by Sirjani et al. (2022), who observed a significant increase in cellulolytic bacterial populations ($p < 0.05$) following the separate inclusion of a lactobacilli mixture and activated charcoal in the diet. The increase in cellulolytic bacteria may be due to a form of different amino acids for protein synthesis and bacterial growth through incorporating ammonia into carbon skeleton by synthesizing enzymes (such as glutamate dehydrogenase, glutamine synthetase, and glutamate synthetase). This interpretation is in line with Antonopoulos et al., (2003). Another cause for increase is a shift of reduction methane mitigation to more propionic production. A similar report by Mitsumori et al., (2012) shows increased abundances of *Prevotella spp.*, *Selenomonas spp.*, and *F. succinogenes* as a result of decreased methane mitigation in ruminants, shifting towards a more propionic production. Activated charcoal has characteristics that improve the growth and preserve cellulolytic bacteria. This effect may be attributed to the anaerobic nature of cellulolytic bacteria, as activated charcoal helps reduce oxygen levels in the rumen environment. Barboza et al. (2009) also reported that neutral pH values promote the growth and activity of cellulolytic bacteria, supporting the findings of our study. During the activation process, carbon develops a larger surface area, enabling it to adsorb bacteria into its porous structure and potentially release them later, facilitating microbial colonization and activity (Villarreal et al., 2015).

Regarding methanogenic archaea, the activated charcoal may increase the abundance of methanotrophs, inhibit methanogens, or absorb CH_4 produced by the rumen. This result is consistent with the findings of Leng et al. (2012) and Toth and Dou (2016), who proposed that activated charcoal reduces ruminal CH_4 production by modifying rumen microbial biofilms, decreasing methanogenic populations, and promoting the growth of methanotrophs.

The numbers of rumen protozoa increased numerically ($p > 0.05$) in treated groups, and this is due to the symbiotic relationship with methanogens. Similar observations were noted by Saleem et al. (2018) and Garillo et al. (1994) in goats. Machmüller et al., (2003) mention when protozoa are increased, the symbiotic methanogens also increases. The result is in disagreement with Terry et al. (2019) who found reduced protozoa counts in heifers fed pine activated charcoal at levels 0.5 and 1.0%.

CONCLUSION

Overall, this study demonstrates that adding activated charcoal to the goat diet is a simple and manageable intervention that can positively impact animal

health. It improves the rumen microbiota structure, alters fermentation processes, and enhances feed utilization efficiency, thereby potentially improving growth performance to some extent. The most important finding is that there was no adverse affected on ruminal ecology, total bacteria, and cellulolytic bacteria, while methanogenic archaea increased. In contrast, there was an increase in the activity of cellulose-degrading enzymes. However, further studies are needed to explore the impact of AB on the ruminal environment under different dietary conditions.

Author's Contribution:

A.M.M Mani designed the experiments. H.H Ghaza and A. Mani conducted the experiments. R.R. Al -Araji and A.MM Mani analyzed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Competing interest

The authors declare that there was no conflict of interest in carrying out this work.

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NUTRITIVE AND PHYTOCHEMICAL QUALITY AND PHYTOBIOTIC PROPERTY OF *PHYLLANTHUS AMARUS* (SCHUM. & THONN.) ORIGINATING FROM NIGERIA

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Abstract

Negative impact of antibiotics and synthetic antioxidants to livestock production including poultry are rising. Therefore, the current shift to phyto-biotics as alternative that can improve the productivity and quality of commercial animals by enhancing feed property and performance. However, the use of phyto-biotics in our environment is limited due to scarce research outputs. This study evaluates the nutrient quality, phytochemical constituents and phyto-biotic (antimicrobial and antioxidant) properties of *Phyllanthus amarus*, a non-toxic medicinal herb, using standard procedures and documented methods. The results revealed that *Phyllanthus amarus* contains high quality nutrients and rich medicinal phytochemicals in both essential oil and methanol extract which demonstrated significant ($p < 0.05$) antibacterial activity with selectable characteristics against *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* Enteritidis from poultry isolates. The plant's samples (both extracts) also inhibited the growth of *Eimeria tenella*, a protozoan that causes coccidiosis in chickens. Furthermore, two of the gas chromatography-mass spectrometry identified compounds 6-methoxy-4-methyl-5-((5-[3-(trifluoromethyl)phenol]plenty)oxy)-8-quinolinylamine and glyoxalbis (2,4-dinitrophenyl-hydrazone) were respectively identified to have high docking interactions with bacterial ATP synthetase and OxyR protein complex, involved in bacterial protein synthesis and oxidative stress

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defense. In addition, these compounds inhibited Keap1 complex which induces oxidative stress in chickens. Thus, 6-methoxy-4-methyl-5-((5-[3-(trifluoromethyl)phenol]plenty)oxy)-8-quinolinyamine and glyoxalbis (2,4-dinitrophenyl-hydrazone) could be optimized and studied as hits for discovery of phytobiotics with antibacterial and antioxidants properties for use in livestock, especially poultry.

Key words: Phytobiotics, Antibacterial, Antioxidant, *Phyllanthus amarus*, Phytochemical

NUTRITIVNI I FITOHEMIJSKI KVALITET I FITOBIOTSKE OSOBINE *PHYLLANTHUS AMARUS* (SCHUM. & THONN.) POREKLOM IZ NIGERIJE

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Kratak sadržaj

Neželjeno dejstvo antibiotika i sintetičkih antioksidanasa na stočarsku proizvodnju, uključujući živinarstvo, sve je izraženije. Zbog toga dolazi do prelaska na fitobiotike koji predstavljaju alternativu koja može poboljšati produktivnost i kvalitet komercijalnih životinja putem unapređenja svojstva i iskorišćenja hrane. Međutim, upotreba fitobiotika u našem okruženju je ograničena zbog nedostatka rezultata relevantnih istraživanja. Ova studija procenjuje nutritivni kvalitet, fitohemijska jedinjenja i fitobiotska svojstva (antimikrobna i antioksidativna) biljke pelin - *Phyllanthus amarus*, netoksične lekovite biljke, korišćenjem standardnih procedura i dokumentovanih metoda. Rezultati su pokazali da *Phyllanthus amarus* sadrži visokokvalitetne nutritivne materije i bogata lekovita fitohemijska jedinjenja u oba ekstrakta – etarskom ulju i metanolnom ekstraktu, koji su pokazali

značajnu ($p < 0,05$) antibakterijsku aktivnost sa selektivnim karakteristikama protiv *Escherichia coli*, *Staphylococcus aureus* i *Salmonella* Enteritidis izolovanih iz živine. Uzorci biljke (oba ekstrakta) takođe su inhibirali rast *Eimeria tenella*, protozoe koja izaziva kokcidiozu kod pilića. Pored toga, dva jedinjenja identifikovana gasnom hromatografijom sa masenom spektrometrijom — 6-metoksi-4-metil-5-((5-[3-(trifluorometil)fenol]plenti)oksi)-8-kinolinilamin i glikoksalbis (2,4-dinitrofenilhidrazon) — pokazala su visoke vrednosti molekulskog vezivanja sa ATP sintetazom bakterija i OxyR proteinskim kompleksom, koji su uključeni u sintezu proteina i odbranu od oksidativnog stresa kod bakterija. Pored toga, ova jedinjenja su inhibirala Keap1 kompleks, koji indukuje oksidativni stres kod živine. Shodno tome, 6-metoksi-4-metil-5-((5-[3-(trifluorometil)fenol]plenti)oksi)-8-kinolinilamin i glikoksalbis (2,4-dinitrofenilhidrazon) mogli bi biti optimizovani i dalje proučavani kao potencijalni kandidati za otkrivanje fitobiotika sa antibakterijskim i antioksidativnim svojstvima za upotrebu u stočarstvu, a posebno u živinarstvu.

Ključne reči: Fitobiotici, Antibakterijski, Antioksidant, *Phyllanthus amarus*, Fitohemijski

INTRODUCTION

Phyllanthus amarus (Schum. & Thonn.), a tropical herb popularly called *stonebreaker*, belongs to the Phyllanthaceae family (Onyesom et al., 2023). It is found in various regions of the world, especially in tropical Asia, Africa, and South America. *P. amarus* has been used in Nigeria traditional medicine for centuries to treat a variety of ailments, like jaundice, liver diseases, digestive disorders, malaria, and kidney stones (Okom et al., 2023). Its medicinal properties have recently drawn considerable scientific attention, with modern research validating many of its traditional applications, especially its antioxidant (Ghasemzadeh et al., 2012), anti-inflammatory (Ghosh et al., 2022), antimicrobial (Ghasemzadeh et al., 2012), and hepatoprotective (Ghosh et al., 2022) activities.

One of the main areas of focus in the current research is phytobiotic property of *P. amarus*. Phytobiotics are plant-based substances that boost animal health by enhancing gut function, increasing immunity and providing antimicrobial protection, and serving as natural alternatives to antibiotics. This is particularly relevant and important in modern livestock farming, where there is a growing demand for sustainable, antibiotic-free alternatives for disease

management, especially in poultry production (Chen et al. 2025). *P. amarus* has been reported to contain several bioactive compounds, including flavonoids, alkaloids, tannins, and saponins, all of which contribute to its medicinal properties (Rakhee and Regin, 2008), including phytobiotic (antioxidant, anti-inflammatory, and antimicrobial) potential.

Rising concerns over the use of synthetic antioxidants and antibiotics as growth promoters in livestock feed have driven the search for alternative solutions that can deliver comparable performance and benefits, without the associated negative effects. One promising alternative currently under investigation is the use of phytobiotics (Filazi and Yurdakok-Dikmen, 2019; Alagawany et al., 2021). Over 5,000 different phytobiotics have been discovered from various sources which include herbs, fruits, legumes, essential oils, vegetables and whole grains, and are being assessed for their capacity to improve overall health and growth performance of animals. It is important to note that phytobiotics have numerous desirable characteristics (minimal side effects, low residue levels, and absence of resistance development) (Dialoke et al., 2020; Li et al., 2021; Mandey and Sompie, 2021) which makes them a promising alternative for enhancing animal health and productivity (Kikusato, 2021).

Various sources of literature highlight numerous beneficial effects of phytobiotics—including antioxidative, antimicrobial, and anti-inflammatory properties, as well as the promotion of probiotic flora growth in the gut (Filazi and Yurdakok-Dikmen, 2019). These effects are largely attributed to the diverse bioactive compounds found in phytobiotic substances, such as terpenoids, flavonoids, alkaloids, phenols, glucosinolates, and glycosides (Ulrikh et al., 2018; Pashtetsky et al., 2020).

Interest in phytobiotics with natural antimicrobial and antioxidant activities in poultry industry has been increasing in recent years due to growing resistance to antibiotics and the ever more present risk of chronic diseases among consumers of poultry products from treated birds. Phytobiotics as natural antioxidants and antimicrobials are cheaper, safer, more available, easily accessible, and they significantly minimize oxidative reactions in products during storage and prevent metabolic diseases in animals including poultry (Pashtetsky et al., 2019). Ginger has gained increased attention due to its rich array of medicinal compounds, which have strong antioxidant and antimicrobial properties (Ghasemzadeh et al., 2012). Supplementation with ginger at 5.10 or 15 g/kg has been reported (Ademola et al., 2006) to enhance growth performance of broilers by stimulating appetite and improving digestive properties, thanks to the constituents gingerol and shogaol. Moreover, it has been demonstrated that the addition of 5 mg/kg of *Camellia sinensis* powder en-

hanced carcass characteristics, blood lipid profile and growth performance of broilers (Ndomou et al., 2018). It has also been found that *Annona muricata* leaves have antioxidant activity by scavenging free radicals and chelating metals (Baskar et al., 2007).

Currently there is not much information about the use of *P. amarus* as phytobiotic in Nigeria. Therefore, this study examined the nutrient quality, phytochemical constituents, antioxidant and antimicrobial properties of the methanol leaf extract and essential oil obtained from *P. amarus* grown in our environment (Abraka, Delta State, Nigeria). The data from this study should provide important baseline information for further research that may culminate in the use of the plant's compounds as phytobiotics in livestock, and poultry farming in particular.

MATERIAL AND METHODS

Plant Collection and Preparation of Dry Sample

Mature, fresh whole samples of *P. amarus* were collected in Abraka, Ethiope East LGA of Delta State, Nigeria. Abraka (Long 6.1023468 and Lat 5.7894321) is located at the tropical rain forest zone of Nigeria at 22.5 m above sea level with a monsoon climate. The relative humidity oscillates between 83 to 95% and the annual rainfall is about 252.67 mm with two seasons: wet and rainy (March – September) and dry (November – February), while the ambient yearly temperature varies between 25.65 °C and 33.46 °C. The soil is soft, clayey, containing a high percentage of fine particles with over 50% measuring less than 0.075 mm in size (Weather and Climate, 2024).

The plant samples were collected between June and August, 2023 and authenticated at Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo State (FHI 10978). After authentication, the leaves were plucked and washed with running tap water, and then rinsed with distilled water and dried at room temperature (26 - 31 °C) for 21 days. The dried leaf sample was grounded by a crusher (Moulinex, France) and sieved (pore diameter = 0.4 mm) to get fine powder which was stored in a desiccator for use.

Nutrient Analysis of the Dried Plant Sample

Proximate composition, mineral element and vitamin constituents of the plant (powder) were analyzed in order to assess the nutritive quality of the plant.

The proximate composition (protein, crude fibre, fat, carbohydrate, mois-

ture, ash and calorie) which constitute the various classes of nutrients present in samples were determined using the standard methods of the Association of Official Analytical Chemists, AOAC (1990). Moisture content was determined by drying the plant's powdered leaf sample to constant weight at 105 °C in a drying oven. Kjeldahl method was used to measure the crude protein content. The nitrogen content was multiplied by factor of 6.25 to estimate the protein content. Crude fat was quantified using the Soxhlet extraction method with petroleum ether as solvent. The acid-detergent fiber (ADF) method was used to access the crude fiber. The ash content was estimated by incinerating the plant powdered leaf sample in a muffle furnace at 550 °C. Then the carbohydrate content was calculated by difference, subtracting the sum of moisture, protein, fat, and ash content from 100 %.

Mineral elements (five macro: potassium (K), magnesium (Mg), calcium (Ca), phosphorus (P) and sodium (Na), and five micro: copper (Cu), manganese (Mn), chromium (Cr), zinc (Zn) and iron (Fe)) were determined using the standard procedures already documented by AOAC (1990). The powdered leaf samples were digested using a mixture of concentrated nitric and perchloric acids and the resulting solution was analyzed for metal content by atomic absorption spectrophotometry (AAS).

The content of vitamins (A, B₁, B₂, B₃, B₆, B₉, C, D, and E) in the plant's powdered leaf sample was analyzed by high performance liquid chromatography (HPLC) as reported by Achikanu et al. (2022).

Preparation of Plant's Methanol Extract and Essential Oil

Twenty grams (20 g) of the fine powdered sample was soaked in 100 mL of 95% methanol at room temperature (25 - 31 °C) for 72 h with frequent agitation. The extract was first filtered using Whatman filter paper (No. 42, 125 mm), followed by filtration through cotton wool. The resulting filtrate was evaporated to dryness in a hot water bath for 72 h and then the obtained methanol extract was refrigerated for further use.

The essential oil was extracted by hydrodistillation method (Begum et al., 2018).

Analysis of phytochemical constituents in Methanol Extract and Essential Oil

Quantitative phytochemical profiling was conducted using the prepared crude methanol extract and the oil sample in order to determine the amounts

of the various phytochemical constituents in the crude methanol extract and oil sample of *P. amarus*. Standard methods whose procedures have been documented (Ameen et al., 2021) were adopted for screening.

Determination of Antimicrobial Activity of Methanol Extract and Essential Oil

Determination of minimum inhibition concentration (MIC) was conducted in a 96-well plate with methanol extract (ME) and essential oil (EO) (Li et al., 2019) ranging from 5 – 2,500 µg/mL, and 100 µL of working inoculum solution (prepared from the poultry bacterial isolates – *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Enteritidis and *Eimeria tenella*) per well. Gentamycin was the positive control and methanol, the solvent, negative control. Following incubation at 37 °C for 48 h, the plate wells were visually examined to assess the lowest concentration at which colour changed to pink from purple, and this was recorded as the value of MIC (Drummond and Waigh, 2000). In general, MIC values ≤ 100 µg/mL indicate good activity (Eloff, 2004), values up to 320 µg/mL are considered reasonable, while values greater than 640 µg/mL are regarded as poor activity (Adamu et al., 2013).

The cytotoxicity levels of ME and EO of *P. amarus* against Vero monkey kidney cells were assessed by the MTT (3-[4,5- dimethylthiazol-2-yl] -2,5- diphenyltetrazolium bromide) reduction assay as described previously (Mosmann, 1983) with slight modifications (Elisha et al., 2017). The cell growth inhibition (i.e. cytotoxicity in percentage) was calculated by comparison with untreated cells. Plant extracts with cytotoxicity activity at 50% (CC₅₀) ≤ 20 µg/mL are regarded as toxic (Zirihi et al., 2005).

The selectivity indices (SI) of *P. amarus* ME and EO was determined by the ratio of cytotoxicity (CC₅₀) and MIC values of the tested bacteria in the same units of measure (SI = CC₅₀/MIC). SI > 1 indicates that extracts are more toxic to the bacterial cells than the host cell (Makhafola et al., 2012).

Gas Chromatography – Mass Spectrometry Analysis of Phyllanthus amarus Methanol Extract and Essential Oil

Gas chromatography-mass spectrometry (GC-MS) analysis of the ME and EO samples of *P. amarus* was performed using 7890 A GC system, 5675 C Inert MSD with triple-Axis detector as previously described (Ameen et al., 2021). The column, with a length of 30 m, an internal diameter of 0.2 µm, and a thickness of 250 µm, was treated with phenyl methyl siloxane. Other conditions of

the GC-MS include the following: ion source temperature (EI), 250 °C, interface temperature; 300 °C, pressure; 16.2 psia, out time, 1.8 mm, 1 µL injector in split mode having split ratio 1:50 with injection temperature of 300 °C. The column temperature started at 35 °C for 5 min and then changed to 150 °C at the rate of 4 °C/ min. The temperature was, however, increased to 250 °C at the rate of 20 °C/ min and held for 5 min. The total elution was 47.5 min. Ms Solution software provided by supplier was used to control the system and acquire the data, while the identification of the compounds was carried out by comparing the mass spectra obtained with those of the standard mass spectra from National Institute of Standard and Technology (NIST) database. The identity of the spectra above 95% was used to ascertain the name, molecular weight and structure of the components in the ME and EO samples of *P. amarus*.

In Silico Study

The GC-MS identified compounds in the ME and EO samples of *P. amarus*, and the validated targets important in bacterial protein synthesis and oxidative stress defense were virtually studied using the computer-aided drug design (CADD) (Naik et al., 2022).

The GC-MS identified phytochemicals from the *P. amarus* and a standard drug structure were downloaded from the PubChem data base. The structures were optimized using the Spartan 14 software and saved in pdb file format. A three-dimensional X-ray crystal structure of the validated protein targets (penicillin-binding protein, RNA polymerase, DNA gyrase, ATP synthetases, dihydrofolate synthase, dihydrofolate reductase, 30s subunit, 50s subunit, peptidoglycan) involved in bacteria protein synthesis, and proteins (OxyR, MarR, HypS, cysteine glutathione peroxidase, peroxiredoxins, thioredoxin reductase, extra cellular thioredoxins, glutaredoxins, catalase and SOD) operational in bacterial oxidative stress defense were retrieved from the RCSB PDB (Research Collaboratory for Structural Bioinformatics, Protein Data Bank, <https://www.rcsb.org/>) website. Using the Molegro Virtual Docker, incorrect bonds and side chain anomalies were rebuilt and optimized. After that, all potential active binding sites of the targeted phytocompounds were identified using the Molegro Virtual Docker.

Using the Molegro Virtual Docker, a receptor grid box was generated once the target protein active binding site has been predicted. The created ready-to-dock phytochemicals library was docked against each target protein to explore the ligand-protein interactions. After docking, the phytochemicals with the lowest molecular docking scores were selected and visualized using the Dis-

covery Studio Visualizer tool to analyze the protein–ligand complex structures.

The evaluation of drug-likeness, pharmacokinetics, and toxicity profile of drug candidates are significant steps in drug discovery process (Wu et al., 2020). The pharmacokinetics (absorption, distribution, metabolism, and excretion), toxicity, and some physicochemical properties of the top-scored phytocompounds from *P. amarus* GC-MS profile were assessed using Swiss-ADME and pkCSM-pharmacokinetic online web-based tools.

The molecular structures of the identified bioactive compounds were optimized by the DFT/Becke-3-Lee-Yang-Parr (B3LYP) method using a 6-31G* basis set (Haunschild et al., 2019). Then, the optimized structures were used to determine the frontier molecular orbital energies of the chosen bioactive compounds and the energy gap was calculated from the lowest unoccupied molecular orbitals (E-LUMO) and highest occupied molecular orbitals (E-HOMO). The molecular orbital energy diagrams were visualized in the Spartan 14 software.

Assessment of In Vitro Antioxidant Activities

The ability of *P. amarus* ME and EO to scavenge DPPH (2, 2- diphenyl-1-picrylhydrazyl) radical was evaluated in accordance with the Braca et al. (2002) method described by Ndomou et al. (2018). A total of 4.5 mL of 0.002% alcoholic DPPH solution was added separately to 0.5 mL of extract samples and standard solutions at varying concentrations (250, 500, 1,000, and 2,000 µg/mL), resulting in final product concentrations ranging from 25 to 200 µg/mL. The samples were kept at room temperature in the dark for 30 min and then the absorbance of the resulting solution was measured at 517nm using a spectrophotometer (BioMate, Germany). The absorbance of the samples, control, and blank was measured using distilled water as the reference. The antioxidant activity (AA) was calculated as follows:

$$AA\% = \left(\frac{((AbS_{control} - AbS_{sample}) \times 100)}{AbS_{control}} \right)$$

In addition, the efficient concentration 50 (EC₅₀) was determined according to the antioxidant activity.

The standard TBARS (Thiobarbituric acid reactive substances) method was adopted to determine the degree of lipid peroxidation inhibition in isolated rat liver homogenate by the ME and EO samples of *P. amarus* (Shabbir et al., 2013).

From freshly excised liver of rat, 10× homogenate was made in cold phosphate buffer saline (pH 7.4). Extract and each fraction were added to 100 µL of (15 mM) ferrous sulphate followed by addition of 3 mL of homogenate. After incubation for 30 min, 0.1 mL of this reaction mixture was combined with 1.5 mL of 10% TCA. After 10 minutes of incubation, the mixture was filtered, and the resulting supernatant was transferred into a tube containing 1.5 mL of 0.67% thiobarbituric acid (TBA) solution (in 50% acetic acid) and placed in boiling water bath for 30 min. Concentration of chromogen formed was measured at 535 nm. Anti-lipid peroxidation was assessed using the following formula:

$$\text{inhibition} = (\text{control} - \text{test}) / \text{control} \times 100$$

Statistics

The obtained data were expressed as Mean ± SEM for triplicate determinations. Statistical significance was determined by one-way ANOVA test using GraphPad prism 5- software. $P < 0.05$ was accepted as significant.

RESULTS

In order to evaluate the functional food characteristics of *P. amarus* and its possible utilization as nutraceutical, the nutritive quality of the powdered sample and phytobiotic properties of ME and EO from the herbal plant were analyzed. Nutritive quality was determined by investigating the proximate composition, mineral elements contents and vitamin constituents of the plant's dried powdered leaf sample.

Nutritive Quality

Proximate Composition

The results obtained from the analysis of proximate composition of the medicinal herb are shown in Figure 1.

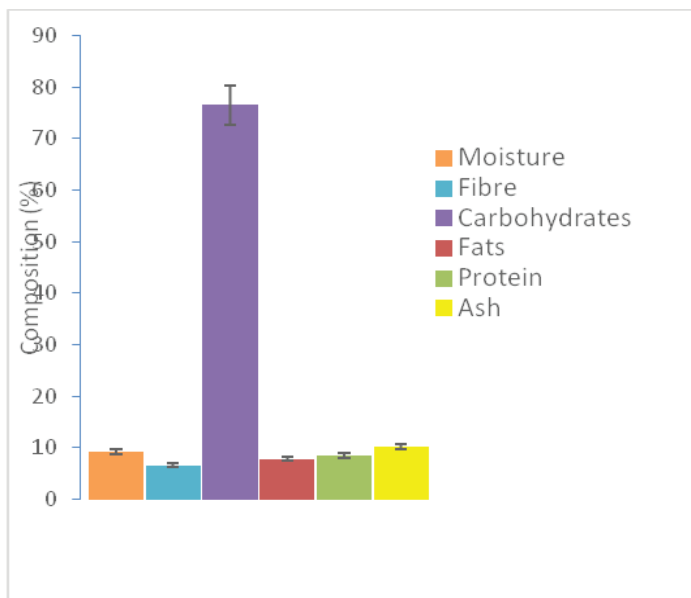


Figure 1. Proximate Composition of *P. amarus*
 Values are expressed as Mean $\pm$ SEM for triplicate determinations, and calorie content = 456.150.04 kCal/100 g

The data (Figure 1) show high amounts of carbohydrates, calories (energy), and ash, but moderate contents of protein, fibre, fat, and low concentration of moisture.

Mineral elements

Table 1. Mineral Contents of *P. amarus*

Minerals	Content (mg/g)
Magnesium	0.331 $\pm$ 0.002
Zinc	0.062 $\pm$ 0.001
Calcium	3.476 $\pm$ 0.012
Sodium	0.079 $\pm$ 0.003
Potassium	2.210 $\pm$ 0.005
Phosphorous	0.095 $\pm$ 0.004

Minerals	Content (mg/g)
Iron	0.173 ± 0.007
Copper	0.007 ± 0.001
Manganese	0.036 ± 0.002
Chromium	0.015 ± 0.013

Values are expressed as Mean ± SEM for triplicate determinations

Ten mineral elements were analyzed and include the following: Mg, Zn, Ca, Na, K, P, Fe, Cu, Mn and Cr with considerable good amounts of Mg, Fe, Ca and K, moderate concentrations of Na, Mn, Zn and P, but trace quantities of Cu and Cr.

Vitamin constituents

The nine (9) vitamins identified and quantified in this study include vitamin A, B₁, B₂, B₃, B₆, B₉, C, D, E, and are shown in Table 2.

Table 2. Vitamin Constituents of *P. amarus*

Vitamins	Constituents (mg%)
A	0.768 ± 0.001
B ₁	0.003 ± 0.0002
B ₂	0.001 ± 0.0003
B ₃	0.060 ± 0.002
B ₆	0.019 ± 0.003
B ₉	0.405 ± 0.001
C	7.05 ± 0.002
D	0.145 ± 0.001
E	1.615 ± 0.001

Data are represented as Mean ± SEM for triplicate determinations

The results (Table 2) show high proportion of vitamin C, moderate amounts of vitamins A, E, B₃, B₆, B₉, and D, and low levels of vitamin B₁ and B₂.

Phytochemical profile

Phytochemicals (plant-derived chemicals) play varied functions in plant defense and protection, human health, agriculture, industries, and environment. Several classes (flavonoids, alkaloids, saponins, phenols, triterpenoids, glycosides, tannins, carotenoids, etc.) of phytochemicals are recognized.

Figure 2 shows the quantitative phytochemical evaluation of the ME and EO of *P. amarus*

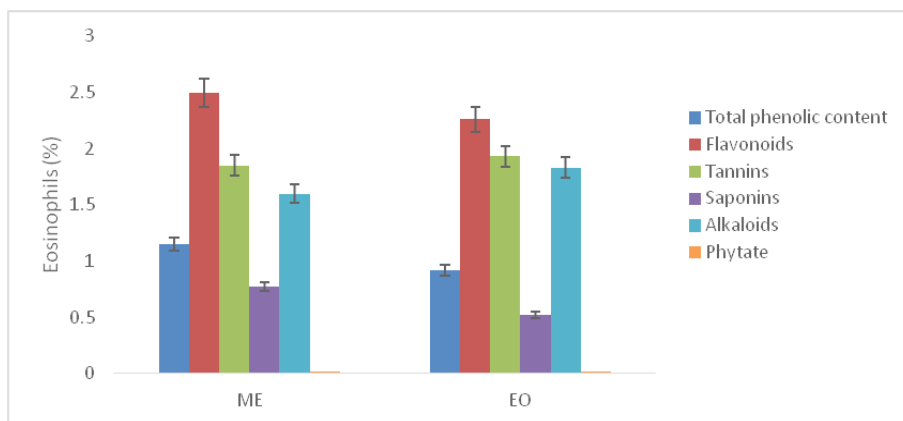


Figure 2. Quantitative Phytochemical Profile of *P. amarus*

Data represents mean values for n = 3 determinations

P. amarus leaf ME and EO contain good amounts of flavonoids, phenolics, tannins and alkaloids, but moderate concentration of saponins and trace level of phytate, an anti-nutrients.

Cytotoxicity, minimum inhibitory concentration and selectivity

The cytotoxicity, MIC and SI indices for the EO and ME samples of *P. amarus* against some poultry isolates (Bacteria: Gram-negative (*E. coli*, *S. Enteritidis*), Gram-positive (*S. aureus*) and protozoan (*E. tenella*)) were evaluated and results are presented in Table 3.

Table 3. Cytotoxicity MIC and SI of EO and ME of *P. amarus*

	ME	EO
CC₅₀ (µg/mL)	207	202
<i>S. aureus</i>		
MIC (µg/mL)	100	150
SI	2.07	1.35
<i>E. coli</i>		
MIC ₅₀ (µg/mL)	50	50
SI	4.14	4.04
<i>E. tenella</i>		
IC ₅₀ (µg/mL)	20.00	15.00
SI	10.35	13.47
<i>S. Enteritidis</i>		
MIC (µg/mL)	150	200
SI	1.38	1.01

CC₅₀ = Cytotoxicity at 50% inhibition. SI= CC₅₀ / MIC

Gentamycin MICs (µg/mL) (*S. aureus*: 12.5; *E. coli*: 6.25, *E. tenella*: 25.0; *S. Enteritidis*: 12.5).

Significant activity: MIC < 100 µg/mL; Moderate activity: 100 < MIC ≤ 625 µg/mL; Weak activity: MIC ≥ 625 µg/mL (Dao et al., 2020). SI > 1 indicates that extracts (ME and EO) are less toxic to the host cell than the bacteria (Makhafola et al., 2012; Elisha et al., 2017).

Results (Table 3) indicate that the ME and EO of *P. amarus* is safe and non-toxic since their CC₅₀ values exceeded 20 µg/mL. Also, their MIC values against *E. coli* showed significant activity (very active), but moderate with respect to the other organisms. However, their selectivity indices against all organisms reveal that the EO and ME of *P. amarus* are more toxic to the bacteria than the host, and hence, can be selected for further study.

GC-MS analysis

In order to identify the active antibacterial compounds in the EO and ME

samples of *P. amarus*, the GC-MS profile was established and sixty-six (66) compounds (31 in ME and 35 in EO) were identified.

In silico study

The 66 GC-MS identified compounds were docked against targets involved in bacterial protein synthesis and oxidative stress defense. Subsequently, the virtual properties of the selected hit compounds were studied by CADD.

Following the ADME-Toxicity and Density Functional Theory (DFT) assessments, the most reactive compounds with drug-like properties against each bacterial class of targets were identified. Therefore, 6-Methoxy-4-methyl-5-((5-[3-(trifluoromethyl)phenol]pentyl)oxy)-8-quinolinyllamine, found in both ME and EO was observed to particularly react with ATP synthase, an enzyme involved in bacterial protein synthesis. Then, glyoxalbis (2,4-dinitrophenyl-hydrazone), present only in the ME reacted the most with oxyR complex protein, operational in bacterial oxidative stress defense system.

The GC-MS characteristics (Figure 3a & b), docking profile (Table 4), ADME-Toxicity data (Table 5) and DFT analysis of the two identified most active compounds are stated as indicated.

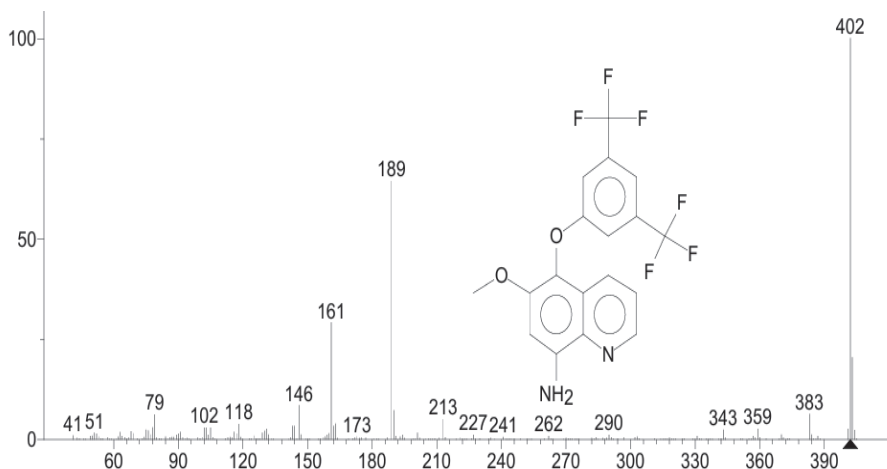


Figure 3a. GC-MS Chromatogram and structure of 6-Methoxy-4-methyl-5-((5-[3-(trifluoromethyl)phenyl]pentyl)oxy)-8-quinolinyllamine

Retention time (RT) = 12.215 min, Molecular weight (MW) = 418, Molecular formula (MF) = $C_{23}H_{25}F_3N_2O_2$, Peak area (PA) equivalent to concentration = 5.392%

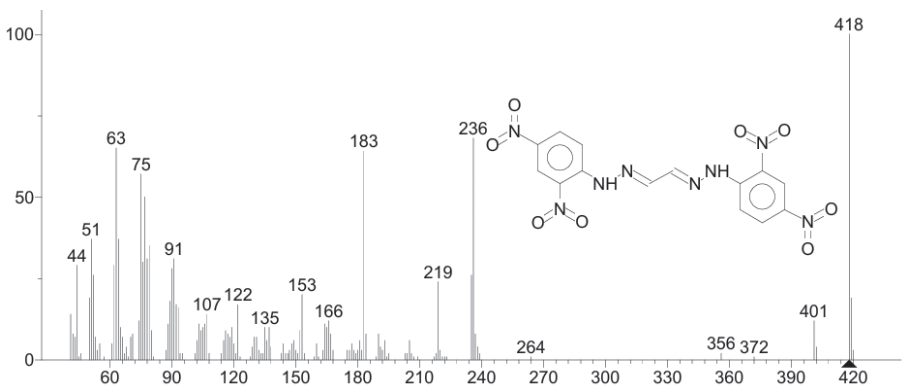


Figure 3b. GC – MS chromatogram and structure of glyoxalbis (2,4-dinitrophenyl-hydrazone)

RT = 12.310 min, MW = 418, MF = C₁₄H₁₀N₈O₈, PA equivalent to concentration = 15.518 %.

Table 4. The mole dock score of the two most active compounds

Compound	Compound ID	E-Total (kCal/mol)
6-Methoxy-4-methyl-5-(-[3-(trifluoromethyl)phenyl]pentyl)oxy)-8-quinolinylamine	609015	-198.064
Drug (Amoxicillin trihydrate)	21960594	-138.238
Glyoxalbis (2,4-dinitrophenyl-hydrazone)	3890919	-165.558
Drug (Allicin)	65036	-74.242

E-total are the mole dock scores for binding of compounds 1 and 2 to ATP synthetase, and compounds 2 and 4 to OxyR protein complex.

6-Methoxy-4-methyl-5-((5-[3-(trifluoromethyl)phenyl]pentyl)oxy)-8-quinolinylamine formed eight conventional hydrogen bonds, LYS80 (2.66 Kcal/mol), LYS80 (2.58 Kcal/mol), ARG99 (2.89 Kcal/mol), SER111 (1.81 Kcal/mol), ARG222 (2.68 Kcal/mol) ARG222 (2.54 Kcal/mol), SER111 (1.99 Kcal/mol), GLU101 (2.15 Kcal/mol). It also, formed three carbon hydrogen bonds, LYS80 (2.73 Kcal/mol), HIS110 (2.69 Kcal/mol), ALA261 (2.95 Kcal/mol). In addition, it formed seven hydrophobic bonds, PHE114 (3.66 Kcal/mol), PHE114 (3.78 Kcal/mol), ILE77 (4.23 Kcal/mol), ARH267 (3.55 Kcal/mol), PHE114 (4.77 Kcal/mol), ARG267 (5.01 Kcal/mol) with ATP syn-

thetases protein. The standard drug, Amoxicillin Trihydrate also interacted via fourteen hydrogen bonds, ARG99 (2.45 Kcal/mol), ARG99 (2.83 Kcal/mol), GLN116 (1.68 Kcal/mol), ARG267 (2.70 Kcal/mol), VAL216 (2.46 Kcal/mol), ASP47 (2.54 Kcal/mol), ASP195 (1.67 Kcal/mol), SER75 (2.67 Kcal/mol), VAL216 (2.99 Kcal/mol), ARG267 (2.29 Kcal/mol), GLU215 (2.22 Kcal/mol), ASP47 (2.37 Kcal/mol), GLN116 (2.33 Kcal/mol). It also, created two hydrophobic bonds, TYR194 (5.23 Kcal/mol), PHE114 (4.18 Kcal/mol) with ATP synthetases (PDB ID: 3reu) protein. Glyoxalbis (2,4-dinitrophenyl-hydrazone) (CID = 3890919) formed two GLU126, two GLU196, an ASP195, two GLY197, GLY223, two MET225, SER224, PRO99 at respective bond distance. A standard drug Allicin (CID = 65036) formed three hydrogen bonds with GLN217, THR218, THR218 at respective bond distances. It also formed three hydrophobic bond LYS114, and two TR192 at different distances as shown in Table 4 and Figures 4 a-d docking complexes.

Docking Complexes

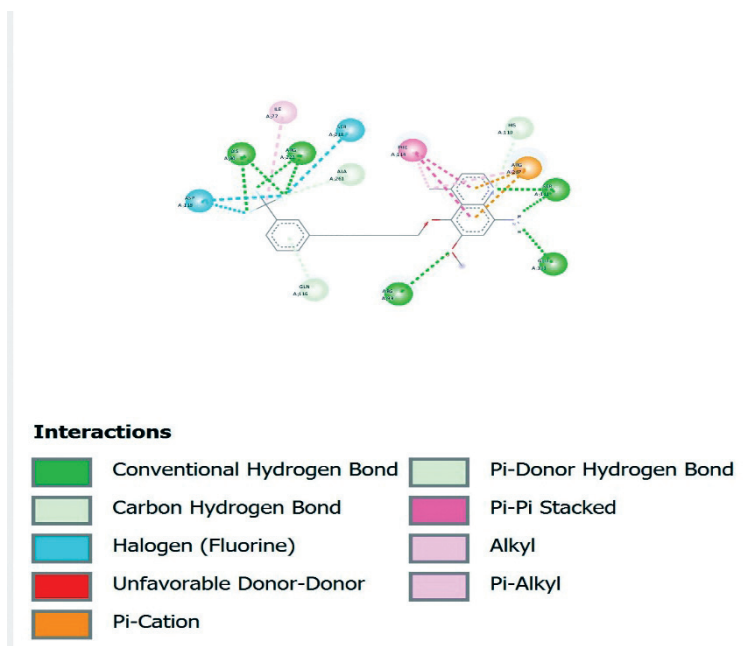


Figure 4a. 6-Methoxy-4-methyl-5-((5-[3-(trifluoromethyl) phenyl] pentyl) oxy)-8-quinolinylamine in complex with ATP synthetases

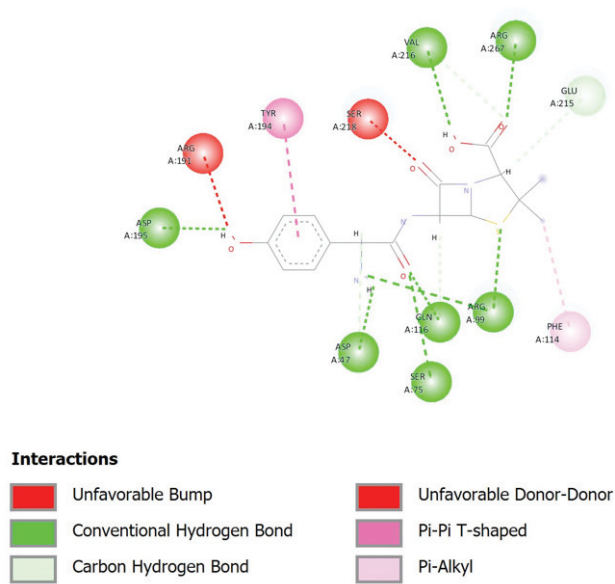


Figure 4b. 2D representation of Glyoxalbis (2,4-dinitrophenyl-hydrazone) in complex with OxyR protein.

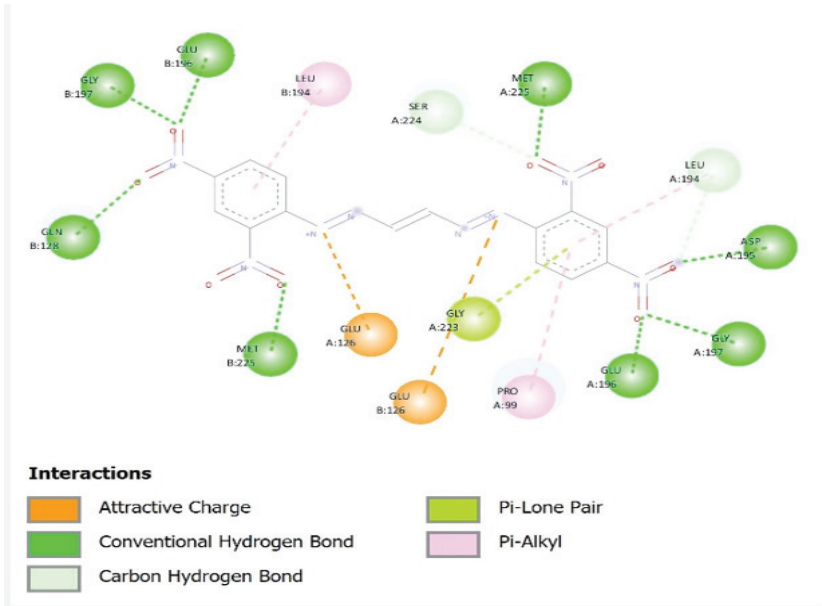


Figure 4c. Amoxicillin Trihydrate (Standard drug) in complex with ATP synthetases protein

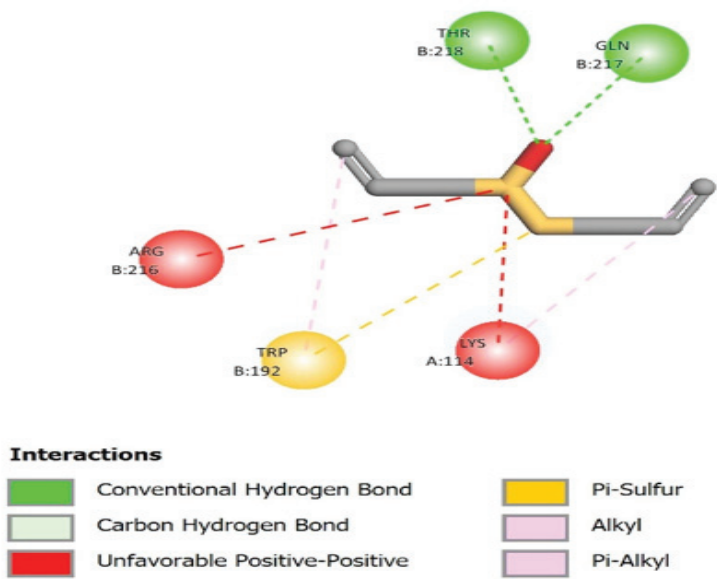


Figure 4d. 2D representation the standard drug (Allicin) in complex with OxyR protein

Analysis of Absorbtion Distribution Metabolism and Excretion (ADME) and Physicochemical Properties of the Active Compounds

Data obtained from the analyses are shown in Table 5.

Table 5. Physicochemical and pharmacokinetic (ADME) data for the selected most active compounds

Parameter	Compounds ID			
	609015	21960594	3890919	65036
Formula	C ₂₃ H ₂₅ F- N ₃ O ₂	C ₁₆ H ₂₅ N ₃ O ₈ S	C ₁₀ H ₈ N ₄ O ₄ S	C ₆ H ₁₀ OS
MW (g/mol)	418.45	419.45	280.26	162.27
Number of heavy atoms	30	28	19	9
Number of aro-matic heavy atoms	16	6	11	0

Parameter	Compounds ID			
	609015	21960594	3890919	65036
Fraction C _s P ₃	0.35	0.44	0.10	0.33
Number of rotatable bonds	9	5	3	5
Number of H-bond acceptors	6	9	5	1
Number of H-bond donors	1	7	1	0
Molar refractivity	112.81	103.73	71.40	45.88
TPSA (<i>A</i> ²)	57.37	185.95	141.73	61.58
Solubility class	Moderately soluble	Poorly soluble	Soluble	Very soluble
GI absorption	Low	No	High	High
BBB permeation	No	2	No	Yes
Violation of Lipinski's rule	0	1	0	0
Violation of Veber rule	0	0	1	0
Bioavailability score	0.55	0.17	0.55	0.55
Synthetic accessibility	3.03	4.50	3.23	3.60

Compound ID: 609015 (6-Methoxy-4-methyl-5-((5-[3-(trifluoromethyl)phenol]plenty)oxy)-8-quinolinyllamine), 21960594 (Amoxicillin anhydrate – drug), 3890919 (Glyoxalbis (2,4-dinitrophenyl-hydrazone), and 65036 (Allicin – drug)

The observed results presented (Table 5) for the pharmacokinetic (ADME) and physicochemical parameters indicate that in line with the Lipinski's rule of five, no compound violated the rule. The compounds are soluble and have high gastrointestinal absorption with relative ease of synthesis, but varied BBB (brain-blood barrier) permeation. However, the data for each bioactive compound compared well with the respective standard drug.

Toxicity

The toxicity profile of the two active compounds and their respective standard drugs are shown in Table 6.

Table 6. Toxicity profile of the top-scored active compounds

Parameter	Compound ID			
	609015	21960594	3890919	65036
AMES toxicity	NO	NO	YES	YES
Max. tolerated dose	0.04	0.85	0.48	0.74
hERG 1 inhibition	NO	NO	NO	NO
LD ₅₀ (mol/kg)	2.61	1.57	2.86	2.37
Hepatotoxicity	YES	YES	YES	NO
Skin sensitivity	NO	NO	NO	YES
<i>T. pyriformis</i> toxicity	0.42	0.29	0.95	0.90
Minnow toxicity	-3.85	5.62	3.08	1.24
Carcinogenicity	No	No	No	No

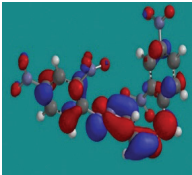
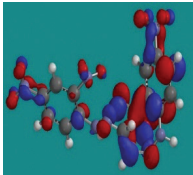
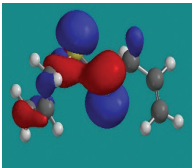
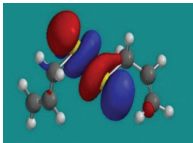
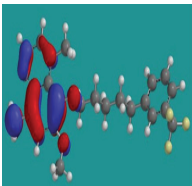
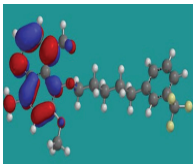
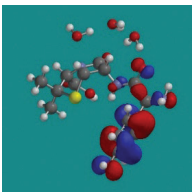
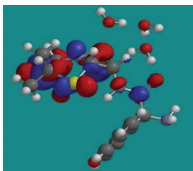
All the compounds are reported to be non-toxic with human tolerated dosage ranging from 0.04-0.85.

Compound ID: 609015 (6-Methoxy-4-methyl-5-((5-[3-(trifluoromethyl)phenol]plenty)oxy)-8-quinolinylamine), 21960594 (Amoxicillin anhydrate – drug), 3890919 (Glyoxalbis (2,4-dinitrophenyl-hydrazone), and 65036 (Allicin – drug)

Density Functional Theory

Molecular structure and reactivity are largely explained using the Frontier Molecular Orbital in organic chemistry (Rozeboom et al., 1981), while electronic properties of molecules can be described through the use of HOMO-LUMO bandgap energy (Feng et al., 2018). ENERGY gap between HOMO and LUMO helps to determine the stability of chemical bonds. Compounds with high energy gap have low chemical reactivity, while compounds with low energy gap have higher reactivity and lower stability (Miar et al., 2020). The two bioactive compounds are selected based on their energy gap, ΔE . The compounds' E-HOMO-E-LUMO diagrams and energy gap, ΔE values are shown in Table 7.

Table 7. HOMO-LUMO diagrams and energy gap, ΔE values of selected top-scored binding compounds and standard drugs.

COM- POUND ID	HOMO	EHO- MO	LUMO	ELU- MO	Energy Gap (ΔE).
3890919		-6.60		-3.2	3.4
65036		-6.40		-1.3	5.1
609015		-4.8		-1.0	3.8
21960594		-6.3		-0.7	5.6

Compound ID: 609015 (6-Methoxy-4-methyl-5-((5-[3-(trifluoromethyl)phenol]plenty)oxy)-8-quinolinyllamine), 21960594 (Amoxicillin anhydrate – drug), 3890919 (Glyoxalbis (2,4-dinitrophenyl-hydrazone), and 65036 (Allicin – drug)

Computational Antioxidant Activity of the Most Active Compounds

Along with antimicrobial activity, phytobiotics are expected to exhibit antioxidant activity in order to counteract the bacterial and metabolic-induced oxidative stress in the host (poultry products). So, the two antibacterial-identified compounds and a natural antioxidant used in poultry (curcumin) were

docked with the Keap1 protein complex, which promotes oxidative stress in chickens (Kovesi et al., 2024), and the docking results are shown in Table 8 and Figure 5a-c.

Table 8. The mole-dock scores of the two most active compounds against Keap1 protein complex

Active Compound	COM- POUND ID	E-Total (kCal/mol)
6-Methoxy-4-methyl-5-((5-[3-(trifluoromethyl)phenyl]pentyl)oxy)-8-quinolinylamine	609015	-136.665
Glyoxalbis (2,4-dinitrophenyl-hydrazone)	3890919	-157.752
Curcumin	969516	-161.582

6-Methoxy-4-methyl-5-((5-[3-(trifluoromethyl) phenyl] pentyl) oxy)-8-quinolinylamine and glyoxalbis (2,4-dinitrophenyl-hydrazone) had mole-dock score of -136.665k Cal/mol and -157.752kCal/mol, respectively. They both showed a lower mole-dock score compared with the standard drug, curcumin, which had -161.582 kCal/mol. Glyoxalbis (2,4-dinitrophenyl-hydrazone) has a better potential as Keap1 inhibitor as evidenced by the dock score relative to that of the standard, curcumin. This could guide further research into optimizing these compounds or exploring new derivatives that may enhance their binding properties.

Figure 5 indicates the interaction of 6-methoxy-4-methyl-5-((5-[3-(trifluoromethyl)phenyl]pentyl)oxy)-8-quinolinylamine (5a), glyoxalbis (2,4-dinitrophenyl-hydrazone) (5b) and the standard, curcumin (5c) in complex with (5FZN) Keap1 protein.

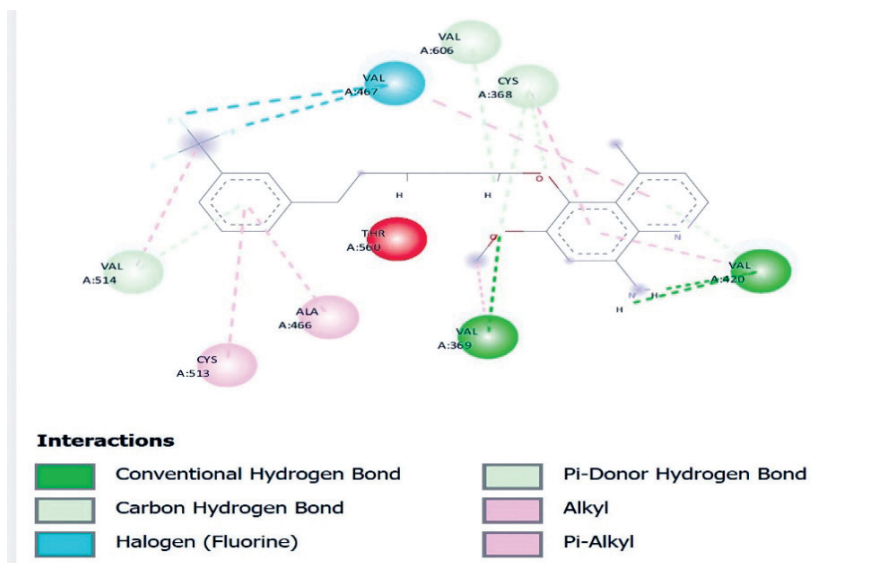
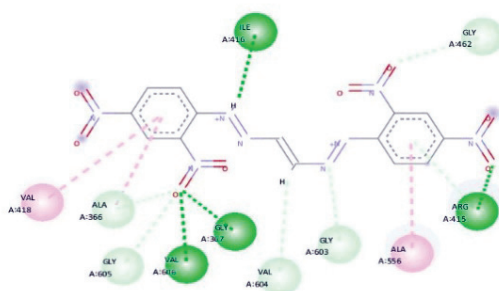


Figure 5a. Interaction of 6-methoxy-4-methyl-5-((5-[3-(trifluoromethyl)phenyl] pentyl)oxy)-8-quinolinyllamine in complex with (5FZN) Keap1 protein

6-Methoxy-4-methyl-5-((5-[3-(trifluoromethyl) phenyl] pentyl) oxy)-8-quinolinyllamine formed eight hydrogen bonds with VAL369 (1.84 Å²), VAL420 (2.34 Å²), VAL420 (2.68 Å²), CYS368 (2.31 Å²), CYS368 (2.99 Å²), VAL606 (2.74 Å²), VAL420 (2.95 Å²), and VAL514 (2.87 Å²). It also formed three halogen bonds, three VAL467 bonds at 3.28 Å², 3.30 Å², 3.60 Å² distance respectively. Lastly it formed eleven hydrophobic bonds, three VAL514 (at 4.78, 3.75, and 4.51 Å² distances), VAL369 (3.82 Å²), ALA466 (4.44 Å²), CYS513 (5.13 Å²), VAL514 (4.67 Å²), CYS368 (5.15 Å²), VAL420 (4.23 Å²), VAL420 (5.45 Å²), AND VAL467 (5.09 Å²) with Keap1 protein.



Interactions

	Conventional Hydrogen Bond		Pi-Donor Hydrogen Bond
	Carbon Hydrogen Bond		Pi-Alkyl

Figure 5b. Interaction of glyoxalbis (2,4-dinitrophenyl-hydrazone) in complex with (5FZN) Keap1 protein

Glyoxalbis (2,4-dinitrophenyl-hydrazone) formed ten hydrogen bonds, GLY367 (1.69 Å²), ARG415 (1.35 Å²), VAL606 (1.86 Å²), ILE416 (2.78 Å²), ILE416 (2.78 Å²), ALA366 (2.45 Å²), GLY462 (2.70 Å²), GLY603 (2.38 Å²), GLY605 (2.16 Å²), VAL604 (2.42 Å²) and ARG415 (2.83 Å²). It also formed four hydrophobic bonds, ALA366 (4.33 Å²), VAL418 (5.31 Å²), ARG415 (4.10 Å²) and ALA556 (3.79 Å²) with Keap1 protein.

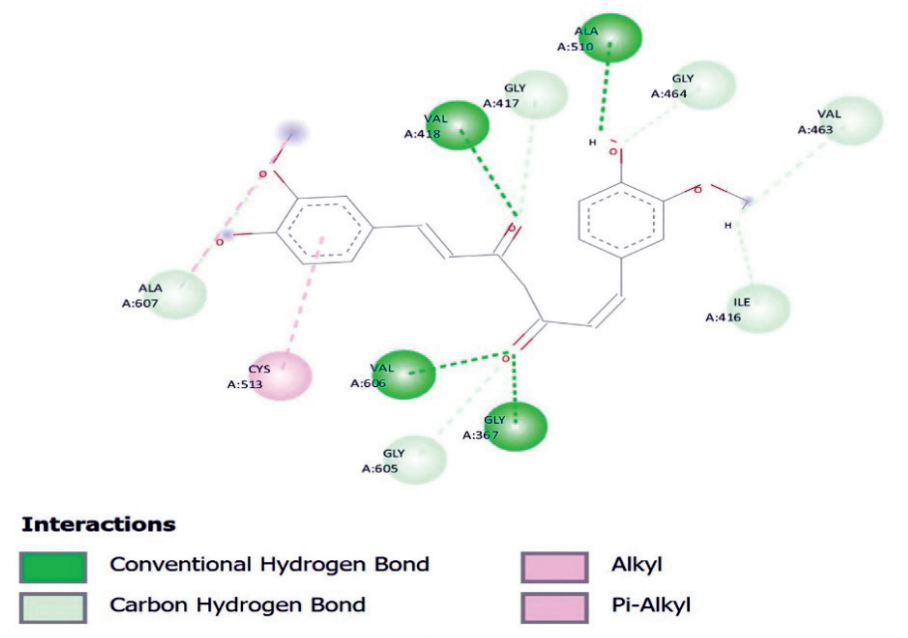


Figure 5C. Interaction of curcumin in complex with (5FZN) Keap1 protein

The standard drug, curcumin formed ten hydrogen bonds, GLY367 (1.58 Å²), VAL418 (2.11 Å²), VAL606 (1.87 Å²), ALA510 (1.81 Å²), GLY417 (2.68 Å²), GLY464 (1.64 Å²), GLY605 (2.51 Å²), ALA607 (2.29 Å²), ILE416 (2.52 Å²) and VAL463 (2.84 Å²). It also formed two hydrophobic bonds, ALA607 (3.59 Å²) and CYS513 (5.41 Å²) with Keap1 protein.

In Vitro Antioxidant Activity of ME and EO

In order to verify the virtual antioxidant property of the compounds in ME and EO samples of *P. amarus*, their *in vitro* 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging and lipid peroxidation inhibition were evaluated and results are shown in Table 9 and Figure 8 (a and b).

Table 9. *In vitro* antioxidant activity of ME and EO of *P. amarus*

Sample	IC ₅₀ (µg/mL)	
	DPPH	LPI
ME	11.10 ± 1.63 ^a	39.43 ± 3.42 ^a
EO	15.62 ± 1.56 ^b	60.60 ± 4.71 ^b
Vitamin C	10.68 ± 2.01 ^a	36.42 ± 3.61 ^a

Values are expressed as Mean ± SEM for triplicate determinations, and data bearing another superscript differ significantly ($p < 0.05$).

ME which had both active compounds, demonstrated the higher DPPH radical scavenging and lipid peroxidation inhibition activity which compared well with the standard drug, Vitamin C, but EO, containing only one of the bioactive compounds had lower DPPH scavenging and lipid peroxidation inhibition activity somewhat different from the standard drug. The values (Table 9) used to evaluate the samples' (ME and EO) antioxidant activity (DPPH and LPI) were obtained from the dose-response curve.

Low Mole dock scores indicate better binding affinity. So, from Table 8, glyoxalbis (2,4-dinitrophenyl-hydrazone) has stronger binding affinity to Keap 1, similar to the curcumin, the standard. However, the ME demonstrated more potent antioxidant activity (Table 9) than the EO. ME contains both compounds which may have acted in synergy to promote antioxidant activity demonstrated by the ME.

DISCUSSION

Photogenic feed additives (PFA) or phytobiotics are used in feed to promote health and growth performance of livestock, mainly because of their high quality nutrients and rich medicinal phytochemicals that have antimicrobial (Cheng et al., 2018) and antioxidant (Onyesom et al., 2015) properties.

This study shows that the leaves of *P. amarus* contain high quality nutrients (proximate, vitamins and mineral elements) and medicinal phytochemicals (high amounts of flavonoids, phenols, tannins and alkaloids, but low levels of phylate and anti-nutrients) necessary to enhance performance and maintain good health. These findings are in agreement with previous reports (Mazumder et al., 2006; Ameen et al., 2021; Achikanu et al., 2022). *P. amarus*, therefore, possesses adequate nutrients and medicinal secondary metabolites with the

potential for supporting growth, performance and good health.

P. amarus is globally distributed and it is a herb known for its therapeutic potential with long history of use in traditional system due to the significant medicinal attributes imparted by the phytochemical constituents. This study reveals the presence of such secondary metabolites (alkaloids, flavonoids, phenols, tannins and saponins) in the leaves of *P. amarus*. Ameen et al. (2021) reported that tannins content was the highest ($1.85 \pm 0.03\%$), followed by anthocyanins, alkaloids, phenolics, glycosides, saponins and triterpenoids ($0.13 \pm 0.10\%$) in the methanol leaf extract of *P. amarus*.

Moreover, this study shows that the leaf ME and EO of *P. amarus* exhibited notable antibacterial effects against certain poultry isolates (*E. coli*, *S. aureus*, *S. Enteritidis*) with selectable indices, SI > 1. The proposed antimicrobial mechanism of EOs is linked to the disruption of cytoplasmic membrane and cell wall which in turn increase cell permeability and reduce virulence. The MICs for the ME of *P. amarus* against *S. aureus* (100 µg/mL), and *E. coli* (50 µg/mL) have been reported (Dakpogan et al., 2019). In our study, the MICs of both ME and EO of *P. amarus* against *E. coli*, *S. aureus*, and *S. Enteritidis*, as well as the IC₅₀ for *E. tenella*, are presented in Table 3. Based on the classification of activity by Dakpogan et al. (2019), both ME and EO of *P. amarus* demonstrated strong antibacterial activity against poultry-derived *E. coli*. Against the other organisms, ME and EO showed moderate activity. Importantly, cytotoxicity data (CC₅₀ values) suggest no toxic effects on the host. The antimicrobial potential of *P. amarus* against multiple antibiotic-resistant bacteria has been studied (Dakpogan et al., 2019). Also, the anticoccidial activity of *P. amarus* has been evaluated (Akin-Osanaiye et al., 2011) and our results on the causative protozoan (*E. tenella*; Table 3) align with theirs. Ethyl oleate present in the ME of *P. amarus* has demonstrated pronounced antimicrobial activity and showed broad spectrum of MICs (Priya et al., 2013). It is important to note that quorum sensors play essential role in bacteria pathogenesis. So, several plant extracts have been screened to attenuate pathogenic bacteria (Mamza et al., 2012). These authors, therefore, postulated that the ME of *P. amarus* could potentially inhibit quorum sensing molecules in *P. aeruginosa* PA101 by interfering with the swimming motility, production of pyocyanin, and the lux: lecA expression. On this premise, the compounds of *P. amarus* can be used as antimicrobials for pathogenic bacteria.

To identify the antibacterial bioactive compounds in *P. amarus* and possibly speculate their likely mechanism of action, the GC-MS profiled compounds were studied virtually by the CADD method. Sixty-six (31 in ME and 35 in EO) compounds were identified with three overlapping. The GC-MS

analysis of compounds in the ethanol extract of *P. amarus* has been reported (Oshomoh and Uzama-Avenbuan, 2020). When compared with our GC-MS data for the ME, there were similarities in the forty-two (42) compounds identified by Oshomoh and Uzama-Avenbuan (2020). However, Mamza et al. (2012) identified nine (9) major compounds from the GC-MS profiling of the ethanol leaf extract of *P. amarus*, and based on Dr. Duke's phytochemical and ethnobotanical database, Mamza et al. (2012) speculated the activity of these nine compounds to include the following: antimicrobial, 8 (3,5-di-t-butyl phenol, hexadecanoic acid, 10-octadecanoate, 9-hexadecenal, glycerol-1,3-dipalmitate, 2,13-octadecadiene-1-ol, dioctyl ester and heptonoic acid), anti-oxidant; 1(3, 5-di-t-butyl phenol), antiinflammatory, 1(3, 5-di-t-butyl phenol), antifungal, 1(methyl 14-methylpentadecanoate). We also identified methyl 14-methylpentanoate, which has been suggested to possess antifungal properties (Oshomoh and Uzama-Avenbuan, 2020); however, its antifungal activity was not assessed in this study. Nonetheless, we identified two important compounds (Figures 3a & b) with different modes of antibacterial action (Table 4). 6-Methoxy-4-methyl-5-((5-[3-(trifluoromethyl)phenol]plenty)oxy)-8-quinolinyl- amine strongly bounded to bacterial ATP synthase (-198.063kCal/mol), but, that with the standard drug, amoxicillin trihydrate is -138.234kCal/mol. Bacteria ATP synthase has two (2) functional subunits; the F1F0-ATP synthase is the primary ATP synthase complex in bacteria that generates ATP from proton motive force (PMF), the FO subunit transports proton and generates PMF, while F1 forms ATP from ADP and Pi. Therefore, ATP synthase plays essential role in bacteria protein synthesis by provision of energy needed for translational events. Thus, it provides the energy (ATP) required for binding of ribosomes to mRNA, assembly of initiation complex, formation of peptide bond between amino acids during elongation phase of translation, assembly/disassembly of ribosomes and its translocation along mRNA.

Several researchers have reported the importance of ATP in bacteria ATP synthesis. Anisimova et al. (2018) demonstrated that the rates of ATP synthesis correlate with the rates of protein synthesis in *E. coli*. In addition, it has been shown that the initiation factors, IF 2 needs ATP to bind to ribosomes in order to initiate translation (Marshall et al., 2009). Marshall et al. (2009) have observed that the elongation factor EF-Tu utilizes ATP to hasten the formation of peptide bond during translation. Furthermore, the study on ribosome-associated protein RsfA by Pontes et al. (2016) indicated that ATP is essential and so, required for the assembly and disassembly of ribosomes from mRNA. Altogether, the inhibition of bacteria ATP synthase by 6-methoxy-4-methyl-5-((5-[3-(trifluoromethyl) phenol] plenty) oxy)-8-quinolinylamine could sig-

nificantly disrupt bacterial protein synthesis, thereby impairing their survival and leading to cell death.

In addition, we also observed that another *P. amarus* compound – glyoxalbis (2,4-dinitrophenyl-hydrazone) interacted strongly with oxyR protein complex (-165.558 kCal/mol) relative to the standard drug (inhibitor), alliin (-74.242 kCal/mol). OxyR, oxygen or oxidative stress regulator or repressor, is a transcriptional regulator protein which modulates the expression of genes engaged in protecting bacteria against free radicals, especially H_2O_2 and the consequent oxidative stress. OxyR plays vital role in bacterial response to oxidative stress. It responds to changes in bacterial cellular redox state, senses H_2O_2 , and activates the production of antioxidant enzymes (catalase, kat G, alkyl hydroperoxide reductase, ahpCF, and glutathione reductase, gor). OxyR regulates biofilm formation in response to oxidative stress in *E. coli* (Maksimova et al., 2023) and its mediated antioxidant defenses has been noted to contribute to bacterial tolerance of antibiotics (Li et al., 2021). Altogether, the two compounds may exhibit synergistic antibacterial action, and glyoxalbis (2,4-dinitrophenyl-hydrazone) could enhance bacteria tolerance of itself and other compounds, an action that could prevent bacterial resistance to these promising poultry antimicrobials.

Having identified the antimicrobial compounds in *P. amarus*, it is yet important to determine their antioxidant activity in poultry chickens, and this was accomplished by investigating docking with Keap1 which revealed scores of -136.665 kCal/mol and -157.752 kCal/mol for 6-methoxy-4-methyl-5-((5-[3-(trifluoromethyl)phenol]pentyl)oxy)-8-quinolinylamine and glyoxalbis (2,4-dinitrophenyl-hydrazone), respectively, but with the standard, curcumin, -161.582 kCal/mol (Table 6). Keap1 inhibitors have been studied in poultry chickens for their potential to improve performance and health. Keap1 inhibitors are known to activate Nrf2 pathway which enhance antioxidant defenses in chickens. Reports on curcumin's inhibition of Keap1 in poultry chickens are limited. However, curcumin (50mg/kg feed) inhibited Keap1 and activated Nrf2, reducing oxidative stress (Basiouni et al., 2023), lipid peroxidation and inflammation in broiler chickens (Wang et al., 2020). Curcumin is a natural compound found in turmeric.

The in vitro antioxidant activity of ME and EO of *P. amarus* was consistent with virtual screening results, which identified 6-methoxy-4-methyl-5-((5-[3-(trifluoromethyl)phenol]pentyl)oxy)-8-quinolinylamine and glyoxalbis (2,4-dinitrophenylhydrazone) as the most active compounds. Dakpogan et al. (2019) reported that *P. amarus* ethanol leaf extract demonstrated the most significant antioxidant activity ($IC_{50} = 5.83 \pm 0.05 \mu\text{g/mL}$ for DPPH radical scav-

enging) of the twenty (20) plants studied. The lipid peroxidation inhibition activity of ME compared well with the standard, and was found to be better than the EO and that of the ME of *Maytenus royleanus* leaves ($79.12 \pm 0.5 \mu\text{g/mL}$) (Shabbir et al., 2013). The identified compounds may have contributed synergistically to the antioxidant activity observed for the ME of *P. amarus*.

CONCLUSION

This study demonstrates that *P. amarus* from Nigeria possesses high nutraceutical value, exhibiting significant antimicrobial, antioxidant, and nutritional properties. The plant's high essential vitamins, protein content, and minerals, in addition to its potent antioxidant and antimicrobial properties, make it a promising source of phytobiotic in veterinary applications, particularly in poultry production.

The identification of the active compounds 6-methoxy-4-methyl-5-((5-[3-(trifluoromethyl)phenyl]pentyl)oxy)-8-quinolinylamine and glyoxalbis(2,4-dinitrophenylhydrazone) supports further investigation into their antimicrobial and antioxidant properties for potential applications in both animal health and human medicine. These compounds hold promise for future drug development aimed at combating oxidative stress, inflammation, and microbial infections in animals.

Observations from this study have wider implications for sustainable agriculture, providing a natural alternative to synthetic antibiotics in animal husbandry. Future research should therefore concentrate on investigating the synergistic effects of *P. amarus* with other phytobiotics, assessing its long-term impacts on various animal species, and optimizing its use in different production systems, which could contribute to both animal health and food security.

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Author's Contribution

OI designed the study, drafted the proposal and supervised the project. OET, ACO, OO and AJO conducted the different aspects of the laboratory investigation, overseen by EAT who managed the grant. The manuscript was prepared by OI and all authors approved its submission.

Competing interest

Authors declare that there is no conflict of interest.

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THE INFLUENCE OF SUPPLEMENTATION OF LICORICE POWDER (GLYCYRRHIZA GLABRA) TO BROILER DIETS ON GROWTH PERFORMANCE, HEMATOLOGICAL PARAMETERS AND SERUM LIPIDS

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Abstract

This study was designed to investigate the impact of dietary supplementation of licorice on growth performance, hematological, and biochemical parameters of broiler chickens. A local hatchery provided 192 one-day-old broiler chicks (Cobb500), which were randomly allocated into four dietary treatments. Each treatment was divided into four replicates, with each replicate containing 12 chicks housed in ground pens. The diet was supplemented with licorice powder (*Glycyrrhiza galabra*) at 0.0%, 0.25%, 0.5%, and 0.75%. The birds were provided with unrestricted access to food and water throughout the experiment. On day 42nd, blood samples were collected randomly from one bird in each replicate of dietary treatments. The results showed that broilers fed a diet supplemented with licorice powder experienced a significant improvement ($p < 0.05$) in body weight gain and feed conversion ratio compared to the control group. Nevertheless, the results also revealed that supplementation of licorice powder considerably decreased ($p < 0.05$) glucose, total cholesterol, triglyceride, low density lipoprotein and elevated high density lipoprotein, total protein, globulin and lowered albumin/globulin ratio, aspartate transaminase and alanine transaminase, in comparison with the control group. In conclusion, the proportion of 0.75% licorice powder in the diet resulted in an improvement in growth performance and health status.

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Key words: Chicken, licorice, medicinal plant, hematology, serum lipid profile

UTICAJ SUPLEMENTACIJE PRAHA SLADIĆA (GLYCYRRHIZA GLABRA) U ISHRANI BROJLERA NA RAST, HEMATOLOŠKE PARAMETRE I LIPIDE U KRVI

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Kratak sadržaj

Ova studija se bavi istraživanjem uticaja supletmentacije sladićem (*Glycyrrhiza galba*) u ishrani brojlera na njihov rast, hematološke i biohemijske parametre. Lokalna inkubatorska stanica je obezbedila 192 jednodnevna brojlerska pileta (hybrid Cobb500), koji su nasumično raspoređeni u grupe koje su imale četiri režima ishrane. Svaki režim je bio podeljen na četiri ponavljanja, pri čemu je u svakom ponavljanju bilo 12 pilića smeštenih u boksove na podu. U hranu je dodat prah sladića u količini od 0.0%, 0.25%, 0.5%, i 0.75%. Kokoške su imale neograničen pristup vodi i hrani tokom eksperimenta. Uzorci su uzeti 42. dana nasumično od po jedne kokoške, svake iz različitog režima ishrane. Rezultati su pokazali da su brojleri koji su hranjeni suplementiranom hranom značajno napredovali ($p < 0.05$) u dobijanju telesne mase i imali bolju stopu konverzije hrane u poređenju sa kontrolnom grupom. Pored toga, rezultati su takođe pokazali da je suplementacija prahom sladića znatno umanjila ($p < 0.05$) glukozu, ukupni holesterol lipoprotein niske gustine, ukupni protein, globulin i smanjila albuminsko/globulinski odnos, aktivnosti aspartat transaminaze i alanin transaminaze u poređenju sa kontrolnom grupom. Može se zaključiti da je dodavanje 0.75% praha sladića u ishranu brojlera dovelo do poboljšanja rasta pilića i njihovog opšteg zdravstvenog stanja.

Ključne reči: pilići, sladić, lekovito bilje, hematologija, serumski lipidni profil

INTRODUCTION

The advancements in poultry production over the recent years, particularly in broiler production, can be largely attributed to the modifications resulting from a range of research investigations conducted in different areas such as genetics, facility adaptations, automation of equipment, health, and nutrition.

The use of natural alternatives with similar beneficial effects like synthetic antibiotics has been intensified. The search for alternatives to antibiotics has led to the development of safe, naturally derived substances whose effects are influenced by the intestinal microflora. These alternatives include medicinal plants (Kairalla et al., 2022a; Kairalla et al., 2022b; Kairalla et al., 2023) and the supplementation of exogenous enzymes (Zamani et al., 2016; Zamani et al., 2017). Recently, various medicinal plants have been utilized to in order to enhance the growth, health, and physiological aspects of poultry (Grashorn, 2010). Addition of certain antibiotics to the diet has demonstrated an improvement in nutrient availability. However, in recent years, their routine usage has been banned in many countries (Hafez and Attia, 2020). One possible alternative to antibiotics is plant-derived substances such as essential oils (Abdulla et al., 2019), flavonoids, polyphenols, alkaloids, and tannins. The increase in the use of medicinal plants in poultry feed was a result of their beneficial properties, such as anti-inflammatory, antiseptic, soothing, antibactericidal, antifungicidal, antiviral, antioxidant capacity, growth-promoting effects, dietary palatability, intestinal function, immunostimulatory effects, stimulation of secretion of digestive enzymes, and nutrient absorption (Kairalla et al., 2022a; Kairalla et al., 2022b; Kairalla et al., 2023). Among these medicinal plants, licorice (*Glycyrrhiza glabra*) has been recognized for its numerous health-promoting properties. Moreover, licorice plays an important role due to its diverse antioxidant, anti-inflammatory, antidiabetic, anti-depressant, anti-obesity, anti-allergic, anti-carcinogenic, antiulcer, and liver protective properties. The chemical constituents of licorice possess potential therapeutic properties that may help prevent and manage a wide range of diseases and emerging health conditions in humans (Noreen et al., 2021). Therefore, the objective of the this study was to investigate the impact of dietary supplementation of licorice on growth performance, blood hematology, and blood lipids of broiler chickens.

MATERIAL AND METHODS

Ethical approval

The research involving animal use was conducted in accordance with all applicable national regulations and institutional policies, as approved by the

Sebha University Ethics Committee, 3751-SUEC, 2024. The current study was conducted at the Poultry Production Unit, Department of Animal Production.

Birds and experimental diets

In the present study, the total of 192 one-day-old broiler chicks (Cobb 500) were obtained from a local hatchery. The chicks were weighed individually and subsequently distributed into four equal groups, with 48 birds in each treatment. These groups were further arranged into four replicates, with 12 birds in each. The chicks were housed in ground pens (1.5 × 1.5 meters) and provided with wood shavings as bedding material. The birds were fed starter and finisher diets from 1 to 21 and 22 to 42 days of age, respectively (Table 1.). Food and water were *ad libitum* during the feeding trial. The birds were weighed individually, and the quantity of food consumed was recorded every week for all replicates. Feed conversion ratio (FCR) was calculated based on body weight gain (BWG). All chicks were vaccinated against infectious bronchitis disease and Newcastle disease, and Gumboro (infectious bursal) disease at 7 and 21 days, respectively. The diets were formulated based on (NRC, 1994). The experimental diets were supplemented with licorice powder as follows: 0%, 0.25%, 0.5%, and 0.75%.

Table 1. Ingredients and nutrient content of broiler meals (% as fed)

Ingredients (kg)	Starter 1 to 21 Day	Finisher 22 to 42 day
Yellow Corn	50.30	57.90
Soybean Meal 44%	31.10	28.15
Corn Gluten Meal	10.00	6.19
Di-calcium phosphate	2.20	2.05
Lime stone	1.10	1.00
Salt (NaCl)	0.25	0.25
Veg. oil	4.55	4.00
L-lysine	0.15	0.15
DL-Methionine	0.05	0.01
Vitamin and Mineral Premix*	0.30	0.30
Total	100	100

Ingredients (kg)	Starter 1 to 21 Day	Finisher 22 to 42 day
Calculated analysis		
Crude protein (%)	22.12	18.07
M.E (kcal/ kg)	2,980	3,158
C/P	134.71	175.76
Fat (%)	3.80	6.29
Crude fiber (%)	2.40	2.58
Calcium (%)	1.04	0.98
Available phosphorus (%)	0.44	0.38
Methionine (%)	0.53	0.42
Lysine (%)	1.15	0.99

* Each kilogram of Premix contained: Vit A 3350000 IU; Vit D3 760 000 IU; Vit E 6700 IU; Vit K3 335 mg; Vit B1 334 mg; Vit B2 1670 mg; Vit B6 500 mg; Vit B12 3.4 mg; Niacin 10000 mg; Ca.D. Pantothenate 3334 mg; Biotin 16.7 mg; Folic acid 334 mg; Trace minerals: Iron 13350 mg; Copper 3335 mg; Zinc 16700 mg; Manganese 25000 mg; Iodine 500 mg; Cobalt 84 mg; Selenium 100 mg; Additives: Ethoxyquine 600 mg; and Carrier (CaCO₃) up to 1 kg.

Blood parameters

On day 42, one bird was randomly chosen from each replicate in each dietary treatment. Blood samples were obtained as described by (Kairalla et al., 2023). These tubes were employed for the determination of packed cell volume (PCV), red blood cells (RBC), white blood cells (WBC), and hemoglobin (HB). Subsequently, all samples were subjected to centrifugation at 3000 g for 15 minutes. The resulting plasma was then transferred into Eppendorf tubes and kept at -20 °C for further analysis. The total protein, albumin, total cholesterol (Khatun et al., 2020), high density lipoprotein (HDL), low density lipoprotein (LDL), triglyceride, and glucose, as well as the enzymatic activities of alanine amino transferase (ALT) and aspartate amino transferase (AST) were measured by colorimetric methods using commercial kits (Kairalla et al., 2023).

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA). The collected data was subsequently analyzed using the general linear model procedure of the statistical analysis system (SAS 2003). In order to contrast the means of treatment at ($p < 0.05$), Tukey's test was performed.

RESULTS

Growth performance

Table 2 presents the impact of supplementation of licorice powder on the production efficiency of broiler chickens. The outcomes indicated that the inclusion of 0.5% and 0.75% licorice powder in the diets significantly enhanced ($p < 0.0001$) BWG, and FCR compared to the birds fed 0% or 0.25% licorice powder in their diets. Feed intake did not show any considerable differences among the dietary treatments or the control group at 0 - 21 days of age. Similarly, there were no significant variations in feed intake, BWG, or FCR among the dietary treatments and control group during the 22-42-day period. However, at the end of the experiment, broiler chickens that were fed diets supplemented with 0.5% and 0.75% licorice powder exhibited significantly improved BWG and FCR ($p < 0.0001$) in comparison with the control group or those fed 0.25% licorice powder.

Table 2. The effect of dietary supplementation of licorice powder on broiler performance

Traits	Licorice powder Level (%)				SEM ¹	<i>p</i> -value
	0	0.25	0.5	0.75		
<i>0 – 21days</i>						
Body weight gain (g/bird)	697.04 ^c	708.85 ^c	830.79 ^b	873.25 ^a	15.221	<0.0001
Feed intake (g/bird)	988.25	990.79	972.21	990.81	20.581	0.8341
FCR	1.41 ^a	1.40 ^a	1.17 ^b	1.13 ^b	0.0347	0.0001
<i>22 – 42 days</i>						
Body weight gain (g/bird)	1706.15	1704.11	1690.31	1715.22	17.192	0.725
Feed intake (g/bird)	3073.26	3079.12	3026.05	3096.79	28.729	0.377

Traits	Licorice powder Level (%)				SEM ¹	p-value
	0	0.25	0.5	0.75		
FCR	1.80	1.80	1.79	1.80	0.04182	0.754
<i>Overall (0 – 42 days)</i>						
Body weight gain (g/bird)	2403.04 ^c	2412.96 ^c	2521.10 ^b	2588.47 ^a	15.206	<0.0001
Feed intake (g/bird)	4061.50	4069.91	3998.26	4087.60	39.937	0.3900
FCR	1.69 ^a	1.68 ^{a b}	1.58 ^{a b}	1.57 ^b	0.02319	0.0149
Overall Live Body weight	2445.12 ^c	2455.59 ^c	2563.35 ^b	2631.80 ^a	15.212	<0.0001

^{a, b, c} Means within the same row with different superscript letters are significantly different at ($p < 0.05$).

¹SEM: Pooled standard error.

Hematological parameters

Hematological parameters analyses are presented in Table 3. Feeding a diet supplemented with licorice powder significantly reduced glucose levels ($p < 0.0001$) in comparison with control group. Birds consumed a diet incorporated with licorice powder did not show a considerable elevated levels of ($p > 0.05$) in WBC, RBC, hemoglobin, PCV and lymphocyte.

Table 3. Effect of licorice powder supplementation on hematological parameters

Parameters	Licorice powder Level (%)				SEM ¹	P-value
	0	0.25	0.5	0.75		
WBC's, 10 ⁶ /mm ³	20.03	20.62	20.24	20.26	0.4971	0.882
RBC's, 10 ⁶ /mm ³	2.92	2.94	2.90	2.84	0.0532	0.600
Hemoglobin, g/dL	8.29	8.32	8.30	8.30	0.0985	0.997
PCV, %	34.49	34.85	34.22	34.53	0.7409	0.945
Lymphocytes, %	54.10	53.98	54.76	54.42	1.1084	0.956
Glucose, mg/dL	116.88 ^a	93.30 ^b	95.33 ^b	94.65 ^b	1.4129	<0.0001

^{a, b} Means within the same row with different superscript letters are significantly different at ($p < 0.05$).

¹SEM: Pooled standard error.

Blood biochemistry

Table 4 indicates the impact of licorice powder supplementation on blood biochemistry. It is obvious that significant differences were found due to the experimental diets of growing birds on plasma total protein, globulin, A/G ratio, triglycerides and total cholesterol with its fraction (LDL and HDL) and liver functions (ALT and AST). In contrast to the control group, feeding licorice powder at 0.5% and 0.75% resulted in a substantial ($p < 0.05$) decline in ALT, AST, total cholesterol, triglycerides, albumin, and LDL. Broilers given a diet with licorice had substantially higher HDL levels ($p < 0.05$) than the control group. The birds in the group that were fed meals enriched with 0.5% or 0.75% showed a substantial elevated levels of total protein and globulin in comparison with the control group.

Table 4. Effect of licorice powder supplementation on serum lipid of broiler chickens

Parameters	Licorice powder Level (%)				SEM ¹	P-value
	0	0.25	0.5	0.75		
Total cholesterol (mg/dL)	119.74 ^a	121.24 ^a	111.01 ^b	110.24 ^b	1.263	<0.0001
Triglycerides (mg/dL)	66.16 ^a	65.11 ^a	53.12 ^b	52.67 ^b	1.461	<0.0001
LDL-cholesterol (mg/dL)	80.09 ^a	67.56 ^b	61.97 ^c	61.20 ^c	1.150	<0.0001
HDL-cholesterol (mg/dL)	37.40 ^b	36.79 ^b	46.30 ^a	47.84 ^a	1.295	<0.0001
Total protein (g/L)	3.03 ^c	3.09 ^c	3.44 ^b	3.86 ^a	0.062	<0.0001
Albumin(g/L)	1.18 ^{ab}	1.14 ^b	1.22 ^a	1.21 ^a	0.021	0.0106
Globulin	1.85 ^c	1.95 ^{bc}	2.22 ^b	2.65 ^a	0.070	<0.0001
A/G ratio	0.64 ^a	0.58 ^a	0.55 ^a	0.46 ^b	0.031	0.0004
ALT (IU/L)	43.36 ^a	41.93 ^a	35.42 ^b	35.85 ^b	1.141	0.0004
AST(IU/L)	54.44 ^a	55.08 ^a	46.26 ^b	46.50 ^b	0.87	<0.0001

^{a, b, c} Means within the same row with different superscript letters are significantly different at ($p < 0.05$).

¹SEM: Pooled standard error.

DISCUSSION

The observed increase in BWG and FCR can be attributed to the presence of licorice extracts, which are believed to enhance digestibility, stimulate the secretion of digestive enzymes, prevent oxidation of tissue, and alter gut microflora. Consequently, these improvements contribute to enhanced growth performance in the birds. Furthermore, the positive effects on performance can also be attributed to the bioactive components present in licorice, such as flavonoids and glycyrrhizin, which possess pharmacological and medicinal properties, as well as immunogenic and antioxidant activities. The synergistic effects of these components may improve growth performance and blood biochemistry in poultry, while also providing potential support to respiratory, digestive, and immune tissues (Alagawany et al., 2019). These findings are in line with previous studies conducted by Ocampo et al. (2016); Attia et al. (2017); Dogan et al. (2018); Beski et al. (2019); Rashidi et al. (2019); Alagawany et al. (2019); Ibrahim et al. (2020); Abo-Samaha et al. (2022), who similarly reported improvements in body weight of broiler chickens supplemented with licorice in comparison with the control.

Ocampo et al. (2016) stated that supplementing licorice in drinking water can play a crucial role in enhancing broiler growth. It also can play vital role in promoting the productive performance of broiler chickens under heat stress (Lashin et al., 2017). These findings may be due to the positive impact of licorice extracts which could improve feed palatability (Salary et al., 2014). Dhama et al. (2015) claimed that herbs have demonstrated powerful immunomodulatory and antiviral effects, and licorice extracts have a favorable influence on chicken immune systems, and therefore can be utilized to increase their immunological response and productivity. In contrast, several authors reported that no significant variation in body weight was found among birds that were given licorice through their feed (Sedghi et al., 2010; Hosseini et al., 2014; Dogan et al., 2018) or by drinking water (Moradi et al., 2014; Naser et al., 2017). Likewise, Moradi et al. (2014) found that varied amounts of licorice extract in drinking water - 0.1, 0.2, and 0.3% - had no impact on feed consumption in broiler chickens. The outcomes are in consistent with Salary et al. (2014); Ibrahim et al. (2020); Abo-Samaha et al. (2022), who reported that drinking water supplementation with licorice extracts enhanced feed intake in broiler chickens.

Regarding the FCR, the enhancement can be attributed to the favorable impact of supplementation with licorice extracts, which may enhance digestibility and regulate the populations of gut microbes. Consequently, this leads to an

increase in feed intake, FCR, and the growth performance of the avian species (Salary et al., 2014). These findings are consistent with those of Ibrahim et al. (2020) and Abo-Samaha et al. (2022), who reported that the inclusion of licorice influenced the feed conversion ratio (FCR). Additionally, Salary et al. (2014) documented that incorporating licorice extracts in the drinking water of broiler chickens led to a significant improvement ($p < 0.05$) in FCR during the starter phase. Conversely, some researchers have reported contradictory results, such as Khamisabadi et al. (2015) and Beski et al. (2019) carcass cuts and intestinal histomorphology of broiler chickens

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Glycyrrhiza glabra) to broiler chicks had no impact on FCR.

Meanwhile, these values fall within the normal range for farm animals. The results were in line with the observations of Moradi et al. (2014); Naser et al. (2017), who argued that total glucose levels were lower in birds fed a diet supplemented with licorice powder compared to the control group. Sedghi et al. (2010) suggested that the prebiotic incorporated with licorice in the feed had no considerable influence on the percentages of lymphocytes, heterophils, and monocytes, as well as the heterophil to lymphocyte ratio and red blood cell proliferation in broiler chickens. Additionally, the administration of 0.5, 1, and 2 g/kg of licorice in the diet did not affect the levels of monocytes, lymphocytes, and heterophils in the bloodstream, nor did it influence the heterophil to lymphocyte ratio or red blood cell proliferation (Sedghi et al., 2010). In addition, Abo-Samaha et al. (2022) mentioned that licorice contains active compounds which have antioxidant and anti-inflammatory properties. As a result, it has a beneficial impact on their growth performance. In contrast, Al-Daraji (2012) showed that high amounts of licorice (150 to 450 mg/L in drinking water) boosted broiler blood glucose levels. Blood indices, which include responsiveness to internal and external stimuli and stressors, are generally reliable indicators of overall health. None of the blood markers tested was abnormal. Furthermore, licorice supplements caused no toxicity or damage to the animals.

The outcomes indicated that the consumption of diets supplemented with licorice powder (0.75%) led to a noteworthy ($p < 0.05$) decrease in A/G ratio against the control group. These findings indicate an improved blood condi-

tion for the groups of birds fed diets enriched with licorice powder.

The findings align with the research by Sharifi et al. (2013), who demonstrated that the addition of licorice root (2 mg/kg diet) to broiler diets decreased blood components such as triglycerides, cholesterol, and LDL, while increasing HDL levels. This relation may be attributed to licorice's ability to suppress lipid peroxidation, lipoxygenase, and cyclooxygenase enzyme activity, as well as its alleviation of LDL oxidation. Higher concentration of total protein may also indicate the good health of the broiler chicks, which enhances their humoral immunological condition (Rezaei et al., 2014). Broiler chicks that consumed licorice in drinking water (0.1, 0.2, or 0.3 g/L) exhibited lower levels of LDL cholesterol and total cholesterol (Naser et al., 2017). Furthermore, the current results consistent with those of Salary et al. (2014), who reported a significant decrease in AST and ALT levels following the inclusion of licorice in broiler feed. The decline in the concentration of liver enzymes may be attributed to the mechanism of glycyrrhizin, which inhibits the progression of liver damage.

As a result of its high portions of flavonoids and ascorbate, dietary treatments with licorice powder enhanced HDL concentration and HDL/LDL ratio in blood (Naser et al., 2017). Furthermore, the addition of 0.4% licorice powder to broiler drinking water increased plasma HDL levels while simultaneously decreasing ALT levels (Salary et al., 2014). Pastorino et al. (2018) suggested that licorice had hepatoprotective qualities in terms of liver function enzymes (ALT and AST). The groups of birds fed licorice powder had the lowest levels of ALT and AST. The bioactive components of licorice may be responsible for improvements in the immunological and oxidative status of broiler chickens. As a result, the presence of saponins and phytosteroids in licorice powder may play a key role in reducing cholesterol levels and enhancing hepatic bile salt production in animals fed diets containing licorice powder (Shahryar et al., 2018).

CONCLUSION

In conclusion, supplementing the diet with 0.75% licorice powder is recommended for improving both the production efficiency and overall health status of broiler chickens.

Author's Contribution:

MAK contributed to the study design, conducted the feeding trial and sampling, and drafted the manuscript. MIA performed the statistical analysis and participated in writing and editing the manuscript. All authors have read and approved the final version of the manuscript.

Competing interest

The authors declare no conflict of interest related to the present study.

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INVESTIGATION ON ANTIMICROBIAL RESISTANCE RATES IN COMMENSAL *ESCHERICHIA COLI* ISOLATES FROM BROILERS ORIGINATING FROM BULGARIA (2020-2024)

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Abstract

The aim of the present study was to investigate the prevalence of resistance to several groups of antibiotics among commensal *Escherichia coli* bacteria isolated from broilers in Bulgaria. Between March 2020 and September 2024, a total of 510 cloacal swab samples were obtained from broilers on two poultry farms, one located in central Bulgaria and the other in the northern region of the country. Of the collected samples, 90 were taken from one-day-old broilers, 140 from 14-day-old broilers, and 280 from broilers aged 28 to 30 days. The total number of *Escherichia coli* isolates was 479, 89 of which from one-day-old broilers, 126 from 14-day-old and 264 from 28-30-day-old broilers. In addition, 12 samples were obtained from poultry litter, from which 10 *Escherichia coli* strains were isolated. The highest rates of resistance among commensal *Escherichia coli* from day-old birds were observed against ampicillin (49.4%), tetracycline (43.8%), followed by amoxicillin/clavulanic acid (34.8%). The strains resistant to the third-generation cephalosporins cefotaxime and ceftazidime were 7.9% and 3.4% respectively. The most prevalent resistance phenotype among the strains included resistance to aminopenicillins and tetracycline (28.1%), with the *tetA* gene being the most frequently detected (7.8%). The highest resistance rates were against ciprofloxacin (73.8%) in strains from 14-day-old broilers, while the highest resistance rate in isolates from 28-30-day-old birds was against ampicillin (65.6%). Tetracycline-resistant strains were 69.0% and 60.6%, respectively. The phenotypic profile including resistance to beta-

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lactams, tetracycline and ciprofloxacin predominated among 28.8% of the strains from both groups of broilers, with the highest prevalence for the *tetA* (26.4%) and *qnrS* (15.1%) genes. In the resistant strains isolated from poultry litter, the highest resistance rates were against ampicillin (100%), followed by tetracycline (80%), amoxicillin/clavulanic acid (70%) and ciprofloxacin (60%).

Key words: poultry, resistance, antimicrobial agents, commensal *Escherichia coli*

ISPITIVANJE STEPENA ANTIMIKROBNE REZISTENCIJE U KOMENSALNIM IZOLATIMA *ESCHERICHIA COLI* KOD PILIĆA BROJLERA IZ BUGARSKE (2020-2024)

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Kratka sadržaj

Cilj ovog rada je ispitivanje učestalosti rezistencije na nekoliko grupa antibiotika kod komensalnih bakterija *Escherichia coli* izolovanih kod brojlera u Bugarskoj. Između marta 2020. i septembra 2024., uzeto je 510 kloakalnih briseva od brojlera sa dve živinarske farme, jedne iz centralne Bugarske, a druge iz severnog dela zemlje. Od ukupnog broja uzoraka, 90 je od jednodnevnih pilića, 140 od brojlera uzrasta 14 dana, i 280 od pilića uzrasta 28 do 30 dana. *Escherichia coli* je izolovana iz 479 uzoraka: 89 od jednodnevnih brojlera, 126 od brojlera uzrasta 14 dana, 264 od brojlera uzrasta 28 do 30 dana. Dodatno, 10 *Escherichia coli* je izolovano iz 12 uzoraka prostrirke. Najviše stope rezistencije kod komensalnih *Escherichia coli* kod jednodnevnih pilića zabeležene su na ampicilin (49,4%), tetraciklin (43,8%), a zatim amoksicilin/klavulansku kiselinu (34,8%). Sojevi rezistentni na cefotaksim i ceftazidim, cefalosporine treće generacije, činili su 7,9% odnosno 3,4% svih izolata. Najzastupljeniji fenotip rezistencije među sojevima obuhvatao je rezistenciju na aminopeniciline i tetraciklin (28,1%), pri čemu je gen *tetA* bio najčešće detektovan (7,8%). Najviša stopa otpornosti bila je na ciprofloksacin (73.8%) kod sojeva brojlera starih 14 dana, dok je najviša stopa kod izolata brojlera starih između 14 i 28 dana bila je na ampicillin

(65.6%). Stepen otpornosti na tetraciklin bila je 69.0% i 60.6%. Fenotipski profil koji uključuje rezistenciju na beta-laktame, tetraciklin i ciprofloksacin preovladavao je kod 28,8% sojeva iz obe grupe brojlera, pri čemu su najčešće bili detektovani geni *tetA* (26,4%) i *qnrS*. Kod rezistentnih sojeva izolovanih iz prosirke, najviše stope rezistencije zabeležene su prema ampicilinu (100%), zatim tetraciklinu (80%), amoksicilinu/klavulanskoj kiselini (70%) i ciprofloksacinu (60%).

Ključne reči: živina, otpornost, antimikrobni agensi, komensalna *Escherichia coli*

INTRODUCTION

The spread of antimicrobial resistance among bacteria is a significant contemporary public health issue, impacting both animal and human health, as well as the safety of foods of animal origin (EFSA, JIACRA IV, 2024). The selective pressure from the use of antibiotics in poultry farming is one of the essential factors affecting the emergence of resistance in bacteria (Agyare et al., 2018). This process poses risks to various ecological niches in nature beyond the animal population. Additionally, it facilitates the transfer of genetic determinants, contributing to the development of cross-resistance, the emergence of multidrug-resistant strains, and the presence of diverse genetic platforms enabling horizontal gene transfer of resistance traits to chemotherapeutics (Singer and Williams-Nguyen, 2014; Skarzyńska et al., 2020). Several studies advance the hypothesis about a link between the use of antibiotics in animal husbandry, including poultry farming, and the spread of resistance to chemotherapeutics among pathogenic bacterial strains isolated from humans (Silbergeld et al., 2008; Marshall and Levy, 2011; Aworth et al., 2020).

In line with Directive 2003/99/EC of the European Parliament and the Council, EU member states are required to submit annual data on the monitoring of resistance to chemotherapeutic agents in *Salmonella*, *Campylobacter*, methicillin-resistant staphylococci, and indicator *Escherichia coli* isolated from farm animals. From 2021, the requirements for testing resistance in indicator *E. coli* also apply to border control of imported fresh meat, with emphasis on the spread of *Salmonella* and *E. coli* strains producing extended-spectrum beta-lactamases (ESBL), AmpC beta-lactamases and carbapenemases, as some of key indicators of resistance rates in farm animals (ECDC, EFSA, 2020). The prevalence of co-resistance to the third-generation cephalosporins cefotaxime and ciprofloxacin among indicator animal *E. coli* and *Salmonella* spp. isolates is also considered a highly critical issue, given their importance in the therapy

of severe bacterial infections in humans (EFSA, ECDC, 2024). The presumption about inclusion of indicator *E. coli* in monitoring systems and in various scientific studies largely relates to the fact that they are defined as reservoirs of genes in the intestinal microbiome, determining resistance to various chemotherapeutics, which may be transferred between different bacterial species causing infections in humans (EFSA, 2008, 2019).

Conversely, experts from the joint ECDC/EFSA/EMA report for the 2018–2021 period highlighted data showing a clear trend: the prevalence of cephalosporin-resistant indicator *E. coli* isolates producing extended-spectrum beta-lactamases in animals is directly linked to the use of cephalosporins in livestock farming (JIACRA IV, 2024). Their conclusion is similar regarding the use of aminopenicillins and the prevalence of resistant *E. coli* strains from animals. The report also indicates that penicillins (29%) and tetracyclines (23.6%) are the most widely used group of chemotherapeutic agents in animal.

MATERIAL AND METHODS

Materials and bacteriological examinations

Between March 2020 and September 2024, a total of 510 cloacal swab samples were collected from broilers at two poultry farms—one situated in central Bulgaria and the other in the northern region of the country. Twelve poultry litter samples were also collected. Of all samples, 90 were from one-day-old broilers, 140 from 14-day-old broilers and 280 from 28-30-day-old broilers.

For primary isolation of *E. coli* bacteria, swab samples were cultured in McConkey agar (Himedia, Biosciences, India) and incubated at 37 °C for 24 hours. The biochemical identification of *E. coli* was performed using Kligler iron agar (Himedia Biosciences, India), IMViC test (production of indole, methyl red test, Voges-Proskauer test, Simmons citrate agar) produced by Himedia, Biosciences, (India) and *Enterobacteriaceae* identification kit (ENTERO test 24N, Erba Lachema, Czech Republic).

Methods for detection of *E. coli* strains' susceptibility to chemotherapeutics

The susceptibility of *E. coli* isolates to chemotherapeutic drugs was determined through the disk diffusion method and the following disks: ampicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), cefotaxime (10 µg), ceftazidime

(5 µg), gentamicin (10 µg), tetracycline (30 µg) and ciprofloxacin (5 µg) (Hi-media Biosciences, India). The chemotherapeutics' MICs were determined by the E-test (Hi Comb™, Himedia Biosciences, India). The MICs of gentamicin and tetracycline were determined using E-test (Liofilchem, MTS™, Italy). To identify the ESBL-producing *E. coli* strains, an E-test with a combination of ceftazidime/clavulanic acid (0.064-4 µg/mL) and cefotaxime/clavulanic acid (0.016-1 µg/mL) (Hi Comb™, Himedia Biosciences, India) was used. The interpretation of the results was based on the epidemiological cut-off (ECOFFs) values. The reference strain *E. coli* ATCC 25922 was used for control of antimicrobial susceptibility tests.

Genetic methods

The DNeasy Blood Tissue kit (Qiagen, Germany) was used for DNA extraction from pure cultures. The identification of genes conferring resistance to beta-lactams (*bla*_{CTX-M-1}, *bla*_{SHV}), tetracycline (*tetA*, *tetB*) and ciprofloxacin (*qnrS*) was performed with Microbial DNA qPCR assay kits (Qiagen, Germany). The thermal profile of the PCR reaction included denaturation at 95 °C for 10 min, 40 cycles with initial denaturation at 95 °C for 15 sec and amplification/elongation at 60 °C for 2 min. The amplification was performed in a Stratagene Mx3000PqPCR system (Agilent Technologies, USA).

RESULTS

A total of 479 *E. coli* isolates were obtained, including 89 from one-day-old broilers, 126 from 14-day-old broilers, and 264 from broilers aged 28–30-day-old. In addition, 12 samples were collected from poultry litter, from which 10 *E. coli* strains were isolated.

Table 1 presents the results on the sensitivity of the *E. coli* strains to chemotherapeutic agents tested by the disk diffusion method, while Tables 2 and 3 present the MIC values of the chemotherapeutics for isolates obtained from one-day-old broilers and from growing broilers, respectively. Most frequently, commensal *E. coli* bacteria from one-day-old broilers were resistant against ampicillin (49.4%), tetracycline (43.8%), and amoxicillin/clavulanic acid (34.8%). Among all isolates, 7.9% were resistant to the third-generation cephalosporin cefotaxime, and 3.4% showed resistance to ceftazidime. The highest resistance rates among strains from 14-day-old broilers were observed against ciprofloxacin (73.8%), while in isolates from 28–30-day-old broilers, the highest resistance was to ampicillin (65.6%). The prevalence rates of tetracycline-

resistant strains were 69.0% and 60.6%, respectively. The resistance rates to cefotaxime and ceftazidime were 37.3%, 26.9% and 34.1%, 33.0%. The MIC₉₀ of aminopenicillins, cefotaxime, gentamicin and ciprofloxacin in isolates from one-day-old broilers and from older broilers were similar – 16 µg/mL, 1 µg/mL, 2 µg/mL and 0.25 µg/mL, respectively. The respective MIC₉₀ values of ceftazidime were 1 µg/mL and 2 µg/mL, and of tetracycline – 16 µg/mL and 32 µg/mL (Tables 2 and 3).

Table 1. Resistance among commensal *E. coli* strains isolated from broilers (n = 479)

Antimicrobial agents	Isolates from broilers (one-day-old) n=89		Isolates from broilers (14-day-old) n= 126		Isolates from broilers (28-30-day-old) n=264		Total n=479	
	Number (%)	Confidence limits (CL)	Number (%)	Confidence limits (CL)	Number (%)	Confidence limits (CL)	Number (%)	Confidence limits (CL)
Ampicillin	44 (49.4%)	39.1÷60.2	77 (61.1%)	52.5÷69.4	173 (65.5%)	59.8÷71.1	294 (61.4%)	57.0÷65.7
Amoxicillin/ clavulanic acid	31 (34.8%)	25.3÷44.9	64 (50.8%)	42.1÷59.4	154 (58.3%)	52.3÷64.1	249 (52.0%)	47.5÷56.4
Cefotaxime	3 (3.4%)	0.6÷8.1	47 (37.3%)	29.1÷45.8	71 (26.9%)	21.7÷32.4	121 (25.3%)	21.5÷29.2
Ceftazidime	7 (7.9%)	3.2÷14.3	43 (34.1%)	26.2÷43.9	87 (33.0%)	27.5÷38.7	137 (28.6%)	24.7÷32.7
Gentamicin	17 (19.1%)	11.7÷27.8	36 (28.6%)	21.1÷36.7	63 (23.9%)	19.0÷29.2	116 (24.2%)	20.5÷28.1
Tetracycline	39 (43.8%)	33.7÷54.1	87 (69.0%)	60.7÷76.7	160 (60.6%)	54.6÷66.3	286 (59.7%)	55.3÷64.0
Ciprofloxacin	16 (18.0%)	10.7÷26.5	93 (73.8%)	65.8÷81.0	134 (50.5%)	44.5÷56.5	243 (50.7%)	46.2÷55.1

Table 2. MIC (µg/mL) of tested antimicrobial drugs in commensal *E. coli* isolates from one-day-old broiler (n = 89)

Antimicrobial agents	MIC ₉₀	0.06	0.125	0.250	0.5	1	2	4	8	16	32	64	128
Ampicillin	16						2	10	33*	35	7	2	
Amoxicillin/ clavulanic acid	16					1	5	24	28*	21	4	5	1
Cefotaxime	1				4	82	3*						
Ceftazidime	1					82	5	2*					
Gentamicin	2				19	43	10*	17					
Tetracycline	16							47	3*	16	21	1	1
Ciprofloxacin	0.25	7	17	49	16*								

Legend: MIC thresholds are marked with asterisks

Table 3. MIC (µg/mL) of tested antimicrobial drugs in commensal *E. coli* isolates from 14-30-day-old broilers (n = 390)

Antimicrobial agents	MIC ₉₀	0.06	0.125	0.250	0.5	1	2	4	8	16	32	64	128	256
Ampicillin	16						4	25	111*	189	37	19	5	
Amoxicillin/ clavulanic acid	16						7	11	154*	41	154	14	7	2
Cefotaxime	1				15	257	114*	4						
Ceftazidime	2				19	241	18	112*						
Gentamicin	2				19	93	179*	99						
Tetracycline	32							23	120*	157	37	50	1	2
Ciprofloxacin	0.25	3	21	139	215*	12								

Legend: MIC thresholds are marked with asterisks

Table 4 presents the phenotypic resistance profiles of *E. coli* strains isolated from both one-day-old and growing broilers, along with selected genetic determinants associated with resistance to beta-lactams, tetracycline, and ciprofloxacin. The predominant phenotype profile among resistant isolates from one-day-old broilers included aminopenicillins and tetracycline (28.1%), with the *tetA* gene (7.8%) being predominant. The phenotype profile including resistance to beta-lactams, tetracycline and ciprofloxacin was dominant among of strains isolated from growing broilers (28.8%), with the highest prevalence of the *tetA* (26.4%) and *qnrS* (15.1%) genes. The prevalence rates of the *bla*_{CTX-M-1} and the *tetB* genes were 1.8% and 1.3%, respectively. The *bla*_{SHV} gene was not detected in any of the isolates resistant to beta-lactams from broilers of different age categories and from poultry litter.

Table 4. Phenotype resistance profiles and genes of antimicrobial resistance in commensal *E. coli* isolates from broiler chickens

Phenotype profiles	Genes of antimicrobial resistance				
	<i>bla</i> _{CTX-M-1}	<i>bla</i> _{SHV}	<i>tetA</i>	<i>tetB</i>	<i>qnrS</i>
One-day-old broilers (n = 89)					
CIP (n = 3)	-	-	-	-	-
G (n = 6)	-	-	-	-	-
Amp, G CIP (n = 5)	-	-	-	-	-
Amp, T, CIP (n = 5)	-	-	2 (2.2%)	-	1 (1.1%)
Amp, AMC, T (n = 25)	-	-	5 (5.6%)	-	-
Amp, AMC,CAZ, T (n = 3)	-	-	-	-	-
Amp, AMC, CTX, G, T (n = 3)	-	-	-	-	-

Phenotype profiles	Genes of antimicrobial resistance				
	<i>bla</i> _{CTX-M-1}	<i>bla</i> _{SHV}	<i>tetA</i>	<i>tetB</i>	<i>qnrS</i>
Amp, G, T, CIP (n=3)	-	-	-	-	2 (2.2%)
14-30-day- old broilers (n = 390)					
T (n = 24)	-	-	6 (1.5%)	-	-
CIP (n = 6)	-	-	-	-	-
T, G (n = 67)	-	-	49 (12.6%)	-	-
AMP, G, T, CIP (n = 3)	-	-			
AMP, CAZ, G,T (n = 19)	-	-	3 (0.8%)	-	-
AMP, CTX, CAZ, G, T (n = 10)	2 (0.5%)	-	4 (1.0%)	3 (0.8%)	-
AMP, AMC, CTX,CIP (n = 105)	15 (3.8%)	-	-	-	73 (18.7%)
AMP, AMC, CTX, CAZ, T, CIP (n = 113)	7 (1.8%)	-	103 (26.4%)	5 (1.3%)	59 (15.1%)
Total (n = 479)	24 (5.0%)	-	172 (36.0%)	8 (1.7%)	135 (28.2%)

Legend: AMP-ampicillin, AMC-amoxicillin/clavulanic acid, CTX- cefotaxime, CAZ- ceftazidime, G- gentamicin, T- tetracycline, CIP- ciprofloxacin

The results about the susceptibility of the poultry litter isolates to the studied antibiotics and their phenotypic profiles are presented in Tables 5 and 6. In the resistant poultry litter strains, the highest rates were against ampicillin (100%), tetracycline (80%), amoxicillin/clavulanic acid (70%) and ciprofloxacin (60%). The most common phenotype profile of resistance included beta-lactams, tetracycline and ciprofloxacin (40%), with the *qnrS* gene being observed in 40% of the strains and the *tetA* gene in 50% of the resistant *E. coli* bacteria. The MIC₉₀ values for *E. coli* isolates from poultry litter were 16 µg/

mL for aminopenicillins and tetracycline, 2 µg/mL for gentamicin and third-generation cephalosporins, and 0.25 µg/mL for ciprofloxacin.

Table 5. Resistance among commensal *Escherichia coli* strains isolated from poultry litter (n = 10)

Antimicrobial agents	Isolates from poultry litter (n = 10) Number (%)	Confidence limits (CL)	MIC ₉₀
Ampicillin	10 (100%)	90.7÷100	16
Amoxicillin/clavulanic acid	7 (70.0%)	40.7÷92.8	16
Cefotaxime	2 (20.0%)	2.3÷48.7	1
Ceftazidime	4 (40.0%)	13.4÷70.2	2
Gentamicin	6 (60.0%)	44.1÷86.5	2
Tetracycline	8 (80.0%)	51.2÷97.6	16
Ciprofloxacin	6 (60.0%)	44.1÷86.5	0.25

Table 6. Phenotype resistance profiles and genes of antimicrobial resistance in commensal *E. coli* isolates from poultry litter

Phenotype profiles	Genes of antimicrobial resistance				
	<i>bla</i> _{CTX-M-1}	<i>bla</i> _{SHV}	<i>tetA</i>	<i>tetB</i>	<i>qnrS</i>
Poultry litter (n=10)					
AMP, G, T (n=2)	-	-	2 (20%)	-	-
AMP, AMC, T, CIP (n=4)	-	-	2 (20%)	-	3 (30%)
AMP, AMC, CAZ, G, T (n=1)	1 (10%)	-	1 (10%)	-	-
AMP, CAZ, G, T (n=1)	1 (10%)	-	-	1 (10%)	-
AMP, AMC, CTX, CAZ, G CIP (n=2)	2 (20%)	-	-	-	1 (10%)
Total (n=10)	4 (40.0%)	-	5 (50.0%)	1 (10%)	4 (40%)

Legend: AMP-ampicillin, AMC-amoxicillin/clavulanic acid, CTX-cefotaxime, CAZ- ceftazidime, G- gentamicin, T- tetracycline, CIP- ciprofloxacin

DISCUSSION

In the present study, 28.1% of the resistant *E. coli* strains from one-day-old chickens exhibited a phenotypic resistance profile that included both ampicillin and tetracycline. Overall, resistance to ampicillin was observed in 49.4% of the isolates, while 43.8% were resistant to tetracycline. According to Nilsson et al. (2014) and Projahn et al. (2018), the emergence of resistance in *E. coli* bacteria isolated from day-old broilers may be a consequence of vertical transfer of genetic factors during incubation and transport. In 28.8% of the resistant *E. coli* isolates from 14-30-day-old broilers, the phenotype profile included ampicillin, tetracycline and ciprofloxacin, with the highest resistance rate observed against ciprofloxacin (73.8%) in strains from 14-day-old broilers, and against ampicillin (65.6%) in those isolated from 28-30-day-old broilers. Chuppava et al. (2019) and Luiken et al. (2020), hypothesized that the environment in poultry farms, e.g. poultry litter and dust, played a major role in the spread of resistant *E. coli* in growing broilers. In their experimental study Montoro-Dasi et al. (2021) also reported a wider spread of penicillin- and ciprofloxacin-resistant *E. coli* isolates from growing broilers. The authors affirmed that the presence of strains resistant against third-generation cephalosporins was mainly based on environmental horizontal gene transfer and defined this circumstance as critical for the breeding of broiler birds. On the other hand, WHO experts (2017) placed an emphasis on the speculative opinion about a possible risk associated with the transmission of environmental genetic factors determining resistance to fluorinated quinolones in commensal intestinal bacteria, and the thesis that the risk was mainly related to the selective pressure from their use in poultry farming. In the Netherlands, Hesp et al. (2019) conducted a comparative analysis of resistance profiles in commensal *E. coli* strains isolated from broilers before and after 2009. They noted that despite the implementation of restrictive policies on the use of fluoroquinolones in poultry farming after 2009, resistance rates to ciprofloxacin in *E. coli* remained high. The authors attributed this finding on the fact that the observed resistance against ciprofloxacin was not plasmid-determined, unlike the genetic background of resistance against third-generation cephalosporins. In our study, alongside the observed high resistance rate to ciprofloxacin (73.8%) in *E. coli* strains from growing broilers, the plasmid-mediated *qnrS* gene was detected in 34% of the resistant strains and in 40% of the isolates from poultry litter.

In comparison to our findings, Racewicz et al. (2022) reported higher resistance rates in *E. coli* strains from broilers in Poland, with 100% resistance to ampicillin and 92% to ciprofloxacin. The authors underlined the high resistance against ampicillin (100%) and doxycycline (100%) in strains isolated

from poultry litter. However, the genetic profile of the beta-lactam-resistant strains showed a low prevalence of ESBL genes, with the *bla*_{CTX-M} gene detected in only one *E. coli* strain (4%). Their results were comparable regarding the prevalence of the *qnrS* gene in ciprofloxacin-resistant *E. coli* bacteria. Regarding doxycycline-resistant *E. coli*, the *tetA* and *tetB* genes were detected in 36% of strains from broilers and in 37.5% of strains isolated from poultry litter. The presence of the *tetA* gene predominated in 75% of the strains from poultry litter and in 52% of isolates from broilers. In this regard, our results also showed a wider spread of the *tetA* gene compared to that of the *tetB* gene in *E. coli* isolates from both broilers (36%; 1.7%) and poultry litter (50%; 10%).

Over the past years, multidrug-resistant commensal *E. coli* producers of ESBLs have been recognized as a specific and important indicator of the spread of resistance to chemotherapeutics in various public health sectors. The plasmid-mediated genes conferring resistance to extended-spectrum beta-lactams are often linked to co-selection mechanisms that facilitate the transfer of resistance to other classes of chemotherapeutic agents. This, combined with selective pressure from drug use, poses a significant risk to various public health sectors (Schink et al., 2013; Poirel et al., 2018). In this context, a collective mechanism involving plasmid-encoded efflux pump genes—contributing to resistance against fluoroquinolones and other antibiotic classes—may also be involved. Efflux pump mechanisms also play a key role in the development of resistance to tetracyclines. Since tetracyclines are among the most commonly used antimicrobials in livestock and poultry farming, the associated risks are amplified by the strong selective pressure resulting from their widespread use.

CONCLUSION

As previously mentioned, this study demonstrated the prevalence of multidrug-resistant phenotypic profiles including resistance to beta-lactams, tetracycline, and ciprofloxacin in *E. coli* isolates from both growing broilers and poultry litter. Strains isolated from one-day-old broilers predominantly showed resistance to aminopenicillins and tetracycline. Concerning the diversity of genetic mechanisms and factors influencing resistant *E. coli* as a specific reservoir within the intestinal microbiome of broiler chickens, the major trends observed in this four-year study align with predicted patterns of resistance spread in poultry reported in other European countries. On the other hand, despite the restrictive antibiotic policies implemented at the two studied farms, the possibility remains that factors beyond selective pressure in poultry farming contribute to the spread of such resistant strains across different public health sectors.

Author's Contribution:

VU contributed to the conceptualization and design of the study, participated in the investigation, methodology, supervision, and was involved in writing, reviewing, and editing the manuscript. RS participated in the investigation, contributed to methodology and resources, and was involved in drafting the manuscript. DD contributed to the investigation, methodology, and provision of resources. All authors read and approved the final manuscript.

Competing interest

The authors declare no conflict of interest related to the present study.

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BLOOD-BRAIN BARRIER OF CAPILLARIES OF THE BRAINSTEM OF DOMESTIC CHICKEN

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Abstract

The aim of the study was to establish the structural organization of the blood-brain barrier of the brainstem capillaries of domestic chicken. The material used for the study was fragments of the brainstem tissue of the White Loman cross chicken. Their fixation and further processing to produce ultrathin sections were carried out in accordance with generally accepted methods. The obtained sections were examined using an electron microscope Jem-1011. The manuscript was written using the terminology of the International Histological Nomenclature. It was established that the brainstem blood-brain barrier of domestic chicken is formed by four structural elements: endothelial cells, basal membrane, pericytes, and processes of glial cells in astrocytes. The endotheliocytes that form the lumen of the capillaries are in contact using tight junctions, preventing the possibility of large molecular compounds to penetrate the blood-brain barrier. If there are pericytes accompanying the capillary, they are surrounded by cleavages of their basal membrane. The latter form a single layer along the capillaries, surrounding their wall from the outside. These structures ensure the selective transport of substances from the bloodstream to the nervous tissue.

Key words: neurology, brain, histohematic barriers, blood-brain barrier, homeostasis

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KRVNO-MNOŽDANA BARIJERA KAPILARA MOŽDANOG STABLA DOMAĆE KOKOŠKE

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Kratak sadržaj

Cilj ove studije je da se utvrdi struktura krvno-moždane barijere kapilara u moždanom stablu kod domaćih kokošaka. Za studiju su korišćeni fragmenti tkiva moždanog stable kokošaka hibridne rase “beli Loman”. Njihovo fiksiranje i dalja obrada kako bi se dobili ultra tanki preseći obavljano je u skladu sa opšteprihvaćenim metodama. Dobijeni uzorci pregledani su pomoću elektronskog mikroskopa “Jem-1011”. U radu je korišćena Internacionalna histološka nomenklatura. Ustanovljeno je da se krvno-moždana barijera moždanog stabla kod domaćih kokošaka sastoji iz četiri strukturalna elementa: endotelnih ćelija, bazalne membrane, pericita i produžetaka glijalnih ćelija u astrocitima. Endotelne ćelije koje formiraju lumen kapilara su povezane putem “*tight junctions*” veza, sprečavajući prolazak jedinjenja velike molekulske mase kroz krvno-moždanu barijeru. Ako periciti okružuju kapilare, oni su odvojeni pukotinom bazalne membrane. Površina bazalne membrane koja je okrenuta ka nervnom tkivu u kontaktu je sa izraštajima astrocita. Poslednje spomenuti formira jedan sloj duž kapilara, okružujući njihov zid sa spoljne strane. Ove strukture obezbeđuju selektivni transport supstanci iz krvotoka do nervnog tkiva.

Ključne reči: neurologija, mozak, histohematske barijere, krvno-moždana barijera, homeostaza

INTRODUCTION

Histohematic barriers ensure the constancy of the composition of tissue fluid by regulating metabolic processes between blood and tissues (Drozdova

and Kundryukova, 2010; Gareev et al., 2023; Prusakova et al., 2024; Baltazar-Lara et al., 2022; Prusakova et al., 2023). In each organ, its structure is distinguished by its specific features (Akmalova et al., 2018). The main element of any histohematic barrier is the hemocapillary (Prusakov et al., 2019; Strizhikov et al., 2021). The pathogenesis of many diseases (Baryshev et al., 2022; Kuznetsov et al., 2022; Alekhin et al., 2023; Galitovskaya, 2018; Ponamarev et al., 2023), both in humans and animals, is based on structural changes in histohematic barriers (Yashin and Prusakov, 2021; Prusakov et al., 2020; Liu et al., 2023). Therefore, examining the characteristics of their organization is not only of theoretical interest but also critically important for practical veterinary medicine (Prusakov et al., 2019).

The study of the chicken blood-brain barrier involves routine histological examinations with hematoxylin and eosin staining. However, in the study, its ultrastructural organization was examined using electron microscopy. It enables the validation of hypotheses regarding the existence of specific intercellular junctions that are crucial for regulating barrier permeability. These data open new perspectives for understanding the mechanisms of protection of the central nervous system in birds and can be used to develop new methods for the treatment of diseases associated with impaired blood-brain barrier function. The data obtained from such studies are valuable and relevant, as they enhance our understanding of how substances are transported across histohematic barriers under both normal and pathological conditions.

The exchange of substances between the blood and the nervous tissue of the brain occurs through the blood-brain barrier. This barrier acts as a highly selective filter for various types of substances (Dyatlova et al., 2022).

Compared to mammals, the function of the blood-brain barrier in birds remains poorly understood. It is often assumed that their functions are alike due to the structural similarity between the avian and mammalian blood-brain barriers (Dunton et al., 2021).

Given the above, the goal was to establish the structural organization of the blood-brain barrier in the brainstem capillaries of the domestic chicken.

MATERIAL AND METHODS

The study material consisted of brainstem tissue fragments, each no larger than 2.0 mm³, collected from five adult White Loman chickens that showed no signs of nervous system disease during their lifetime. At least five samples of brain stem tissue were collected from each animal. They were fixed in a 2.0% glutaraldehyde solution in cacodylate buffer (pH 7.2–7.4) for 2 hours. Then,

after washing in three portions of the same buffer, they were postfixed in a 1.0% osmium tetroxide solution (prepared in cacodylate buffer, pH 7.2–7.4) for 1 hour. After that, the samples were dehydrated in ascending alcohols and absolute acetone. Then they were poured into Epon-812 in accordance with the generally accepted method (Anwar Ul-Hamid, 2018). Ultrathin sections were obtained on an ultramicrotome (LKB-III, Sweden), contrasted with a 2.0% aqueous solution of uranyl acetate and a solution of lead citrate (Reynolds, 1963). The resulting ultrathin sections were photographed using a Jem-1011 electron microscope (JEOL, Japan) at magnifications of 2500–3000. The manuscript was prepared using terminology from the International Histological Nomenclature (Semchenko et al., 1999). The experiment was approved by the ethical committee Saint Petersburg State University of Veterinary Medicine.

RESULTS AND DISCUSSION

It was established that the blood-brain barrier in the brainstem of the domestic chicken functions as a boundary structure separating nervous tissue from the circulatory system. Four structural elements are clearly distinguishable in their composition: endothelial cells, basal membrane, pericytes, and processes of glial cells in astrocytes. (Figure 1)

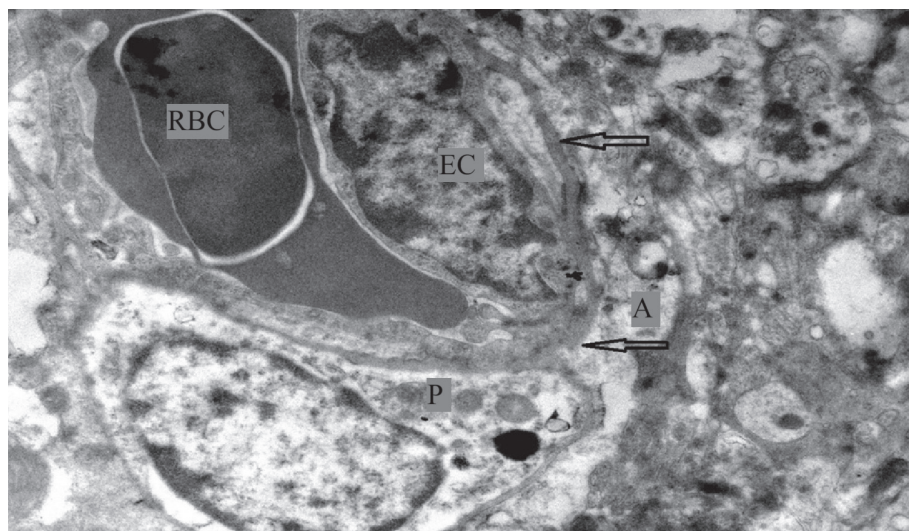


Figure 1. Ultrastructure of the blood-brain barrier of a bird's brainstem capillary. Electron microphotography (magnification $\times 15000$): EC—endothelial cell; RBC—red blood cell in the lumen of a blood vessel; A – astrocyte stalk; P—pericyte; $\uparrow$ – basal membrane

Endothelial cells form a continuous lining of the capillaries that traverse the brainstem's nervous tissue, connecting to one another via tight junctions. These junctions effectively prevent the spontaneous passage of large-molecular compounds across the blood-brain barrier. (Figure 2)

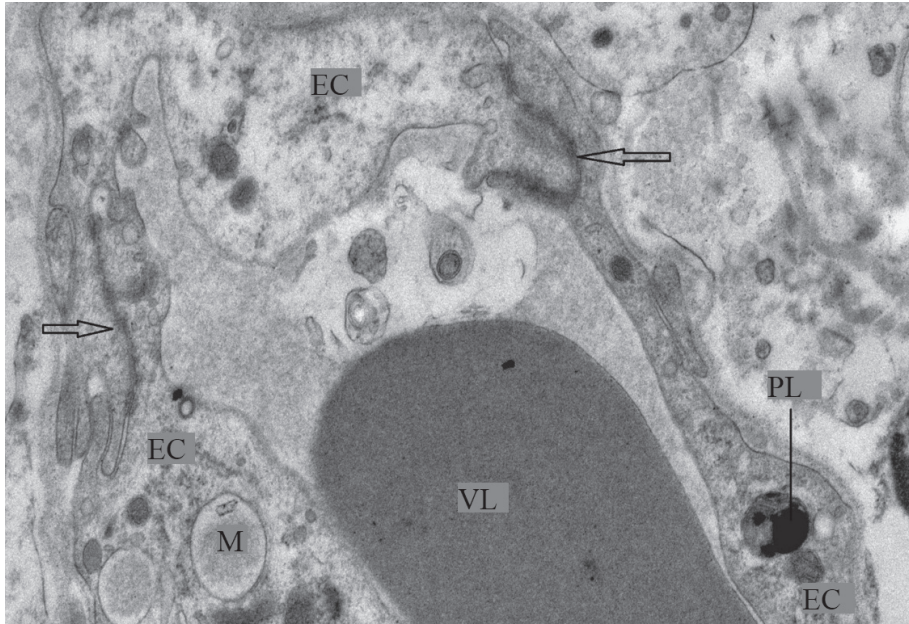


Figure 2. Ultrastructure of an endotheliocyte in a bird's brainstem capillary. Electron microphotography (magnification $\times 15000$): EC - endothelial cell; VL – vessel lumen; M – mitochondria; PL – phagolysosome; $\uparrow$ —basal membrane

Each endotheliocyte contains one nucleus, oval or elongated. The nuclear matrix in the central part of the nucleus is light, and heterochromatin in the form of electron-dense lumps is concentrated along the inner layer of the karyolemma. The nuclei occupy a greater volume of cells, while the cytoplasm surrounds them in the form of a thin rim. Despite its small volume, the cytoplasm of endothelial cells is rich in organelles, primarily various electron-dense ribosomes and polyribosomes. Additionally, it contains occasional short cisternae of rough endoplasmic reticulum, as well as isolated lysosomes and phagolysosomes. There are also mitochondria that have an electron-bright mitochondrial matrix. Owing to their low electron density, the mitochondria display sparse but distinguishable dark cristae - folds of the inner mitochondrial membrane. Occasionally, channels of the lamellar Golgi complex are also observed.

The electron diffraction patterns studied did not show any signs of active pinocytosis. This was evidenced by a small number of pinocytotic vesicles in the cytoplasm of endothelial cells.

In accordance with the location of the endothelial cells of the brainstem capillaries, two surfaces are distinguished: luminal and basal. The luminal surface faces the capillary lumen. Depending on the functional state of the cell, its topography can change. Thus, with increased activity, it can form many outgrowths and protrusions. The basal surface contacts the basal membrane.

The basal membrane is a non-cellular structure formed by a layer of fibrillar material. Its thickness varies between 40 and 100 nm. With the side facing the nervous tissue, the basal membrane contacts either the processes of astrocytic glia cells or the accompanying cells, pericytes (Figures 1 and 3). In areas where pericytes are absent, the blood-brain barrier's thinnest sections are observed, along with noticeable loosening. However, where pericytes are present, the basal membrane splits, forming duplications that entirely encase their bodies.

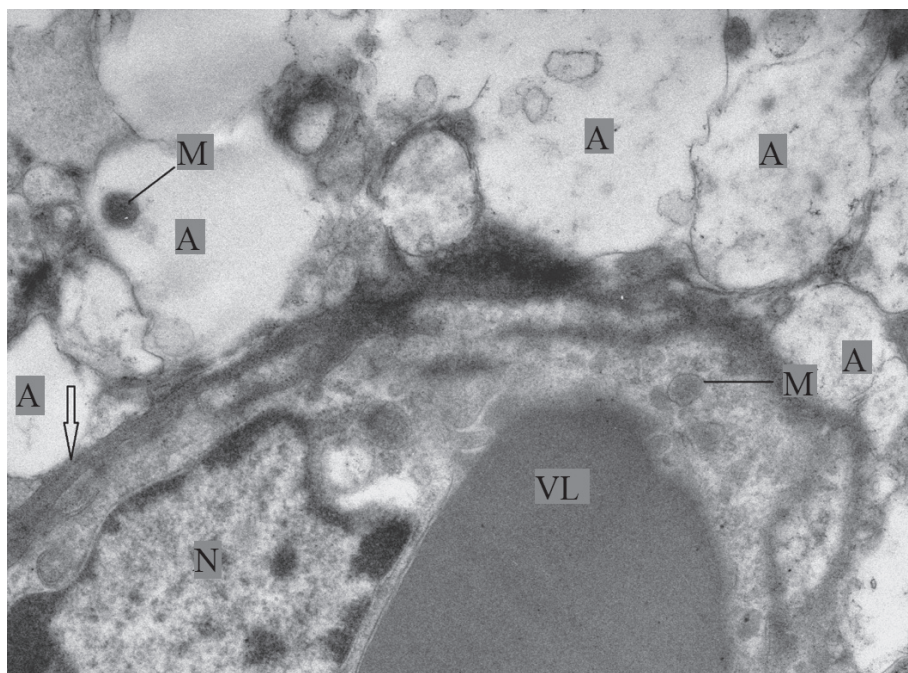


Figure 3. Ultrafine links between the avian brainstem capillary endotheliocyte and astrocytes. Electron micrography (magnification $\times 15000$): A – astrocyte stalks; N – endothelial cell nucleus; VL – vessel lumen; M – mitochondria; $\uparrow$ – basal membrane.

Pericytes protrude the cells enveloped in a basal membrane. Their cytoplasm is poor in organelles. It contains single mitochondria with a light matrix and small electron-dense lysosomes. Additionally, a few channels of the granular endoplasmic reticulum, small heterogeneous granules, and phagolysosomes are also present. Pericyte nuclei have a rounded-elongated shape with an electron-light matrix. In the phase of increased functional load, changes in pericytes, in the shape and size of the nuclei are noted. Thus, they increase in size, and their nuclear membrane becomes convoluted. In this case, large, dark heterogeneous phagolysosomes are revealed in the cytoplasm of these cells.

Pericyte-free areas of the basal membrane, as well as the surrounding basal membrane, are in contact with the processes of astrocytes. The ends of the latter are expanded, and thin microfilaments and a small number of mitochondria are detected in their cytoplasm. Due to the processes of astrocytes along the capillaries, a single layer is formed, surrounding their wall from the outside. (Figure 3)

CONCLUSION

Thus, the blood-brain barrier of the brainstem in the domestic chicken is composed of four structural elements: endothelial cells, the basal membrane, pericytes, and the processes of glial cells in astrocytes. Endothelial cells forming the lumen of capillaries contact by means of tight contacts, preventing the possibility of penetration of large-molecular compounds through the hematoencephalic barrier. If there are pericytes accompanying the capillary, they are surrounded by splits in their basal membrane. The surface of the basal membrane facing the nervous tissue is in contact with the processes of astrocytes. Along the length of the capillaries, a single layer of astrocyte processes surrounds the capillary wall externally. These structures provide selective transport of substances from the bloodstream to the nervous tissue.

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Author's Contribution:

AP – Research design, collecting samples, literature review, results analysis;
VP – Writing of the manuscript, translation, literature review, results analysis.

Competing interest

The authors declare that they have no competing interests.

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Williams R.B. 2015. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. *Avian Pathology*, 34: 159-180. <https://doi.org/10.1080/03079450500112195>

Bailey M.A., Macklin K.S., Krehling J.T. 2013. Use of a multiplex PCR for the detection of toxin-encoding genes netB and tpeL in strains of *Clostridium perfringens*. *ISRN Veterinary Science*, Article ID 865702, 1-4. <https://doi.org/10.1155/2013/865702>

Books:

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Statistica (Data Analysis Software System). 2006. v.7.1., StatSoft, Inc., USA (statsoft.com).

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