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Review

Possible Threats of the Presence of Non-Native Invasive Land Snail Species in Türkiye

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Abstract

Land snails, including some economically important species, are commonly found in forests and mountainous regions of Türkiye. Türkiye's large geomorphological region and four surrounding seas, which offer a variety of malacofauna, help Türkiye achieve an endemism grade of over 65%. Despite extensive research over the past few decades, we still lack a sufficient understanding of the species identification of land snails in Türkiye, particularly in areas like forested mountain regions, and it is assumed that there are more than 1000 taxa in total. This review emphasizes the existence of invasive non-native snail species in Türkiye, such as *Arion ater* (Linnaeus, 1758) and Arion vulgaris Moquin-Tandon, 1855, which have a negative impact on agricultural and horticultural output. In addition to these species, *Theba pisana*, *Cornu aspersum*, *Xeropicta derbentina*, *Xeropicta krynickii, Cernuella virgata* and *Eobania vermiculata* are also found in Türkiye as non-native land snail species. Moreover, it also places a strong emphasis on the necessity of managing invasive species to reduce their detrimental effects on natural ecosystems by giving examples from the world and covers the predictions about the possible threats that the presence of non-native invasive land snail species in Türkiye may pose in the future.

Keywords: *Arion ater*, *Arion vulgaris*, Crop loss, Ecosystems, Environmental problem, Malacofauna

Introduction

Compared to many countries, Türkiye's area is very vast (approximately 1650 km from east to west and about 570 km from south to north) and contains numerous biotopes from extremely damp subtropical to nearly wilderness-like conditions [1]. In addition, Türkiye is surrounded by four seas: the Black Sea, the Mediterranean Sea, the Aegean Sea, and the Sea of Marmara. Thus, Türkiye has a seaboard longer than 8.300 km. These seas differ in terms of parameters such as their biological content, temperature, salinity, and climatic conditions [2]. The fact that Türkiye has such a wide geomorphological area and the overlap of many important biogeographic structures in its region has caused it to have an endemism degree of over 65% [1]. In addition, 44% of the alpine land snail fauna is endemic to Türkiye [3]. It has been reported that 220 species of 74 genus of terrestrial snails detected in Türkiye are endemic to Anatolia [1]. However, land snails, which are endemic to Türkiye, mostly belong to the *Monacha* (Stylommatophora: Hygromiidae) Fitzinger, 1833, *Albinaria* (Stylommatophora: Clausiliidae) Vest, 1867, and *Oxychilus* (Stylommatophora: Oxychilidae) Fitzinger, 1833 genera (20, 13 and 12 species, respectively). For example, *Meijeriella canaliculata* (Stylommatophora: Enidae) Bank, 1985, *Metafruticicola dedegoelensis* (Stylommatophora: Hygromiidae) Hausdorf, Gümüş & Yıldırım, 2004 [4], *Bulgarica denticulata* (Stylommatophora: Clausiliidae) (Olivier, 1801), *Assyriella guttata* (Stylommatophora: Helicidae) (Olivier, 1804) [5] and *Monacha samsunensis* (L. Pfeiffer, 1868) [6] are endemic species of Türkiye. On the other hand, *Helix lucorum* (Stylommatophora: Helicidae) Linnaeus, 1758 is also considered to be a semi-endemic species for the country because it is also found outside the country [7,8].

The first land snails were recorded in Türkiye in 1801. After this record, Türkiye's land snails continued to be researched and a significant number of articles were published, especially in the last fifty years, including approximately one-third of the number of taxa considered valid today [1]. However, it is thought that our knowledge about the species identification of land snails in Türkiye is still insufficient despite many investigations carried out in recent decades. Snail species in some areas, such as most forested mountain areas, are nearly fully unknown. Therefore, although it is assumed that many species have not yet been registered, the total count of species inhabiting Türkiye is estimated to presumably exceed 1000 taxa [1]. On the other hand, although the species belonging to the Achatinidae family are not native species to Türkiye, the term land snail in the Special Hygiene Rules for Animal Foods Regulation of the Ministry of Agriculture and Forestry refers to the terrestrial gastropods of *Helix pomatia* Linnaeus, 1758, *Helix aspersa* Müller, 1774, *Helix lucorum* species and the species belonging to the Achatinidae (Stylommatophora) Swainson, 1840 family [9]. In addition to the presence of plenty of native land snail species of Türkiye, the presence of non-native land snail species has also been reported in the country. For this reason, this paper aims to highlight the record of Non-Native Invasive Land Snail species and possible threats to their existence in Türkiye.

Presence of Non-Native Land Snail Species in Türkiye

Since some synanthropic terrestrial mollusk species hide under various wooden, plastic, and ceramic items, they can spread to new environments because of these items being moved to another place [10]. The Western European snail species *Arion ater* s. l. (Stylommatophora: Arionidae) (Linneaus, 1758) and *A. vulgaris* Moquin-Tandon, 1855, which have recently been reported to exist in Isparta and the Asian and European sides of the Bosphorus in Türkiye [10] are among the most important invasive and harmful mollusk species in terms of their adverse effects on agricultural areas and horticultural production [11].

Biogeographic considerations and a phylogenetic analysis on *Theba pisana* (Stylommatophora: Helicidae) (O. F. Müller, 1774) indicate Morocco as place of origin [12]. This species is found in many regions of Türkiye [13]. *Cornu aspersum* (Stylommatophora: Helicidae) (O. F. Müller, 1774) (the Mediterranean mollusc) is one of the most broadly distributed terrestrial snails on a global scale, North Africa is known as the homeland of this species. Despite it is commonly seen in very humid locations (Istanbul, Antalya, Izmir, Bodrum, etc.), it is not very abundant in regions far from the coastal locations (such as Burdur, Isparta). Furthermore, it is reported in cultivated lands far from the sea in Anatolia [13].

Another land snail, *Xeropicta derbentina* (Stylommatophora: Geomitridae) (Krynicki, 1836), is endemic to the Northern Black Sea steppes, and *X. derbentina* also originally lived in the Eastern Mediterranean, the Caucasus, and Anatolia [14].

Xeropicta krynickii (Krynicki, 1833) is usually recorded in the Eastern Mediterranean. Its main distribution extends between the Black Sea and the Caspian Sea, from Bulgaria and Romania to Iran and Azerbaijan [15]. It is mostly recorded from the Black Sea Region in Türkiye [16].

Cernuella virgata (Stylommatophora: Geomitridae) (Da Costa, 1778) is indigenous to western Europe and Mediterranean. However, this species has also spread rarely in Türkiye. It has been recorded in İstanbul and Marmara Region [17].

Eobania vermiculata (Stylommatophora: Helicidae) (Müller, 1774), is one of the most important examples of the helicid terrestrial snails, achieved a worldwide spread through anthropogenic affairs. It is of Circum-Mediterranean distribution, typical for the coastal areas of Greece, Libya, Spain, Croatia, France, Algeria, Italy, Montenegro, Albania, Tunisia and Morocco. In addition to these countries, it was also recorded in Türkiye [13].

Adverse Effects of Invasive Snail Species on the Environment

An introduced species is a vegetable, animal, or another organism that is not native to a specific region, and which tends to spread to a degree believed to cause damage to the environment, human economy, or human health [18]. Invasive species can be introduced to a new environment through a variety of means, such as accidental or intentional releases by humans, natural dispersal, or through the actions of other animals. Therefore, it is very important to monitor and manage invasive species to minimize their negative effects on native ecosystems [19]. Snails are also among the species introduced in the world in recent years due to their economic importance [20].

Snails have a wide range of feeding habits depending on the species. Some are herbivorous and feed on plants, while others are carnivorous and feed on other invertebrates or even small vertebrates. Some species are scavengers, feeding on decaying organic matter [21]. Snails are hermaphrodites, meaning they possess both male and female reproductive organs [22,23]. They typically reproduce through internal fertilization (self-fertilization) and lay eggs in a variety of habitats such as on the ground, in freshwater, or in marine environments. The eggs hatch

into small, fragile-bodied snails [24,25]. They are important decomposers, breaking down dead plant and animal material and recycling nutrients back into the ecosystem. They are also a food source for numerous animals, including birds, fish, and mammals [26]. Therefore, snails play an important role in many ecosystems [11,27].

Although snails have significant roles in many habitats, some species of snails can be invasive and cause significant ecological and economic damage. For example, the Giant African snail, which is native to Africa, has been introduced in many countries and is considered a pest due to its feeding habits that can damage crops, fruits, and vegetables [28]. Invasive snail species can have a variety of negative impacts on the environment. They can outcompete native snails for resources and habitat, leading to declines in native snail populations. They can also disrupt ecosystem functions and processes, such as decomposition and nutrient cycling. Invasive snails can also cause significant economic damage. For example, some invasive snail species are agricultural pests, feeding on crops, fruits, and vegetables. This can lead to significant crop losses and reduced agricultural productivity [29]. In addition, some invasive snails can act as vectors for disease, spreading diseases that can affect humans and animals. For example, some species of snails can carry bacteria and the parasitic worm responsible for angiostrongyliasis [30] and schistosomiasis, which can cause serious health problems in humans that can be transmitted if handled with bare hands or eaten [31]. In conclusion, invasive snail species can cause significant ecological and economic damage by outcompeting native snails for resources and habitat, disrupting ecosystem functions and processes, and causing damage to crop and infrastructure. Therefore, they can significantly negatively affect the environment, economy, and human health. Thus, it's crucial to monitor and manage invasive snail species to minimize their negative effects on native ecosystems and human activities.

Ecological Impacts of Invasive Snails

Invasive snails can have significant negative ecological impacts, including competition with native species for resources, predation on native species [32,33], disruption of ecosystem processes, the spread of diseases, loss of biodiversity [34-39] and economic impacts [40]. Examples of invasive snail species include the Giant African Snail [41]. Chinese Mystery Snail, European Brown Garden Snail [42] and Golden Apple Snail [43]. These invasive species can have an impact on the population dynamics and distribution of native snails, including alteration of community structure, changes in distribution, and genetic effects [31], which can lead to declines in biodiversity [24].

The Giant African Snail *(Achatina fulica* [Stylommatophora: Achatinidae] [Bowdich, 1822]): This invasive species is native to East Africa and has been introduced to many other parts of the world. It competes with native snails for food and habitat and can also prey on smaller native snails [28,44].

The Chinese Mystery Snail *(Cipangopaludina chinensis* [Architaenioglossa: Viviparidae] [J. E. Gray, 1833]): This invasive species is native to Asia and has been introduced to many parts of North America. It competes with native snails for nutrients and habitat and can also outcompete native snails for space in the substrate (e.g. mud, sand) [25,45,46].

The European Brown Garden Snail *(Helix aspersa):* Scattered throughout Western Europe, Great Britain, and the Mediterranean and Black Sea borders. It was brought to the Atlantic Islands, New Zealand, Australia, South Africa, Mexico, Chile, Argentina, and Australia [25,47,48].

Effects on Population Dynamics and Distribution of Native Snails

Invasive snails can have a significant impact on the population dynamics and distribution of native snails. These impacts can include [30,31]:

Competition for resources: Invasive snails can outcompete native snails for food and habitat, leading to declines in native snail populations [49].

Predation: Some invasive snails, such as the Giant African Snail, can prey on native snails, which can lead to declines in native snail populations [50].

Displacement: Invasive snails can outcompete native snails for space in the substrate (e.g., mud, sand), which can lead to the displacement of native snails from their preferred habitats [51].

Alteration of community structure: Invasive snails can alter the structure of native snail communities by reducing the abundance and diversity of native species [52].

Changes in distribution: Invasive snails can spread to new areas and displace native snails from their natural range, leading to changes in the distribution of native species [53].

Genetic effects: Invasive snails can hybridize with native snails and change the genetic makeup of native snail populations. These impacts can have cascading effects on the ecosystem and can cause declines in biodiversity [54].

Nutrient cycling: Invasive snails can alter nutrient cycling in an ecosystem by consuming large amounts of plant material, which can lead to changes in nutrient availability for other organisms [38].

Decomposition: Invasive snails can alter decomposition processes by consuming large amounts of dead plant material and other organic matter, which can affect the rate and efficiency of decomposition in an ecosystem [55].

Soil formation: Invasive snails can alter soil formation by consuming large amounts of vegetation, which can change the composition of soil and affect the growth of other plants [38].

Disruption of the food web: Invasive snails can disrupt the food web by preying on native snails and other invertebrates, which can lead to declines in native species and altered trophic interactions [40].

Altering ecosystem services: Invasive snails can change ecosystem services such as pollination, seed dispersal, and pest control by reducing the abundance and diversity of native species [51].

Examples of How Invasive Snails Alter Community Structure and Ecosystem Function

In Hawaii, the invasive Giant African Snail *(Achatina fulica)* is known to prey on native snails, which can lead to declines in native snail populations and altered community structure. This can also have ripple effects on other species that rely on native snails as a food source or as part of the ecosystem, leading to declines in biodiversity [42].

Effects on biodiversity and ecosystem services: Invasive snails can lead to a decline in native snail populations and ripple effects on other species that rely on native snails as a food source, resulting in declines in biodiversity. Invasive snails can also alter the structure of native snail communities and ecosystem function through changes in community structure, nutrient cycling [56], decomposition, soil formation, disruption of the food web and altering ecosystem services. These changes can have negative impacts on biodiversity, agriculture, and human health through the spread of diseases. Invasive snails can have negative effects on biodiversity and ecosystem services. These impacts can include:

Loss of native species: Invasive snails can outcompete native snails for resources and prey on native snails, which can lead to declines in native snail populations and loss of biodiversity. This can also have ripple effects on other species that rely on native snails as a food source or as part of the ecosystem [57].

Alteration of community structure: Invasive snails can alter the structure of native snail communities by reducing the abundance and diversity of native species, which can affect the functioning of the ecosystem and lead to declines in biodiversity [58].

Changes in ecosystem services: Invasive snails can change ecosystem services such as pollination, seed dispersal, and pest control by reducing the abundance and diversity of native species. Invasive snails can also change nutrient cycling and decomposition processes, which can affect the functioning of the ecosystem [51].

Economic impacts: Invasive snails can cause damage to crops and gardens, resulting in economic losses for farmers and gardeners [59].

Human health impacts: Some invasive snails can serve as vectors for diseases that can affect humans (i.e., angiostrongyliasis [30] and schistosomiasis [31]).

Overall, invasive snails can have a range of negative impacts on biodiversity and ecosystem services, which can have ripple effects on human well-being and the economy.

Economic Impacts of Invasive Snails

Invasive snails can have a range of economic impacts, which include:

Crop damage: Invasive snails can cause damage to crop by consuming large amounts of plant material, which can result in reduced crop yields and economic losses for farmers [60,61].

Garden damage: Invasive snails can cause damage to gardens by consuming large amounts of plant material, which can result in reduced yields and economic losses for gardeners [56].

Control costs: Invasive snails can be difficult to control, and the costs of control measures can be significant. These costs can include the costs of labor, materials, and equipment for control measures such as snail baiting and manual removal.

Damage to infrastructure: Invasive snails can cause damage to infrastructure such as buildings, roads, and sidewalks by consuming mortar and other building materials [51].

Damage to the tourism industry: Invasive snails can cause damage to natural resources and native ecosystems, which can negatively influence the tourism industry [38].

Reduced exports: Invasive snails can lead to reduced exports of agricultural products and other goods due to quarantine measures and other restrictions [50,61].

Damage to Crop and Agricultural Lands

Invasive snails can cause damage to crops and agricultural lands in several ways:

Crop consumption: Invasive snails can consume large amounts of plant material, which can result in reduced crop yields and economic losses for farmers. This can be particularly damaging to crops such as fruits and vegetables, where even a small amount of damage can greatly reduce the value of the crop [50].

Soil damage: Invasive snails can cause damage to soil by consuming large amounts of vegetation and other organic

matter, which can change the composition of soil and affect the growth of other plants. This can lead to reduced crop yields and decreased productivity of agricultural lands [61].

Spread of diseases: Some invasive snails can serve as vectors for diseases that can affect crops, which can lead to reduced crop yields and economic losses for farmers [50].

Damage to irrigation systems: Invasive snails can cause damage to irrigation systems by consuming the plastic and other materials used in these systems, which can lead to reduced water delivery to crops and decreased crop yields [62].

Damage to farm equipment: Invasive snails can cause damage to farm equipment by consuming the rubber and other materials used in this equipment, which can lead to increased maintenance and repair costs for farmers [44].

Economic Costs of Crop Damage

The economic costs of crop damage caused by invasive snails can be significant. These costs can include:

Reduced crop yields: Invasive snails can consume large amounts of plant material, which can result in reduced crop yields and economic losses for farmers. The cost of this loss can depend on the type of crop, the extent of damage, and the market price of the crop [50].

Increased costs of control and management: Farmers may need to spend additional money on control measures such as snail baiting, manual removal, and other pest management techniques to reduce the damage caused by invasive snails [45].

Damage to irrigation systems: Invasive snails can cause damage to irrigation systems, which can lead to reduced water delivery to crops and decreased crop yields. This can result in additional costs for farmers to repair or replace damaged irrigation systems [46].

Loss of exports: Invasive snails can lead to reduced exports of agricultural products and other goods due to quarantine measures and other restrictions, resulting in economic losses for farmers [38].

Loss of reputation and market value: Damage caused by invasive snails can reduce the quality and market value of crops, which can lead to a loss of reputation and reduced market value for farmers [62].

Reduced productivity: Invasive snails can cause damage to soil and other agricultural lands, which can lead to decreased productivity and reduced crop yields over time [48].

The economic costs of crop damage caused by invasive snails can vary depending on the type of crop, the extent of damage, and the location of the farm. But overall, it can be quite substantial for farmers and the economy [38,46,50,62,63].

Damage to Infrastructure and Buildings

Invasive snails can cause damage to infrastructure and buildings by consuming various materials.

Damage to buildings: Invasive snails can consume mortar and other building materials, which can cause damage to buildings and lead to increased maintenance and repair costs [49].

Damage to roads and sidewalks: Invasive snails can consume asphalt and other materials used in road construction, which can cause damage to roads and sidewalks and lead to increased maintenance and repair costs [44].

Damage to electrical infrastructure: Invasive snails can consume the insulation on electrical wires, which can cause damage to electrical infrastructure and lead to increased maintenance and repair costs [44].

Damage to communication infrastructure: Invasive snails can consume the insulation on telephone and internet cables, which can cause damage to communication infrastructure and lead to increased maintenance and repair costs [44].

Damage to water supply systems: Invasive snails can consume the materials used in water supply systems, which can cause damage to the systems and lead to decreased water delivery and increased maintenance and repair costs [50].

Costs of Control and Management

The costs of control and management efforts for invasive snails can be significant and can include:

Labor costs: Control and management efforts for invasive snails can be labor-intensive, and the costs of labor can be significant. This can include the costs of hiring staff, contractors, or volunteers to carry out control measures [50,64].

Materials and equipment costs: Control and management efforts for invasive snails can require the use of materials and equipment, such as snail bait, pesticides, traps, and protective gear. These costs can vary depending on the type of control measure used [45].

Monitoring and research costs: Control and management efforts for invasive snails often require monitoring and research to assess the effectiveness of control measures and identify new populations. These costs can include the costs of field surveys, laboratory analyses, and data management [51].

Education and outreach costs: Control and management efforts for invasive snails often require education and outreach to inform the public about the risks of invasive

snails and how to prevent the spread. These costs can include the costs of developing educational materials, organizing workshops and presentations, and communicating with stakeholders [51].

Legal costs: Control and management efforts for invasive snails can involve legal measures such as permits, regulations, and enforcement. These costs can include the costs of obtaining permits and compliance with regulations and the costs associated with enforcement actions [45].

Continual costs: Invasive species management is an ongoing effort, and the costs associated with it can be ongoing as well. The population of invasive snails can rebound if the management efforts are not continued [50].

Examples of Control and Management Efforts for Invasive Snails

Physical control: Physical control methods include the manual removal of snails, trapping, and fencing. This method can be effective but can be labor-intensive and costly [52].

Chemical control: Chemical control methods include the use of snail baits, pesticides, and molluscicides. These methods can be effective in reducing snail populations but can also have negative impacts on non-target species and the environment [53].

Biological control: Biological control methods include the release of natural predators or pathogens that target invasive snails. This method can be effective but can also have negative impacts on non-target species and the environment [54].

Cultural control: Cultural control methods include techniques to make the environment less favorable to invasive snails, such as altering the pH of the soil, reducing moisture, and planting repellent plants [38].

Quarantine and regulations: Quarantine and regulations can be used to prevent the introduction and spread of invasive snails by restricting the movement of potentially infested materials and enforcing regulations on the import and export of invasive snails [38].

Public education and outreach: Public education and outreach can help prevent the spread of invasive snails by raising awareness about the risks of invasive snails and how to prevent their spread [51,64].

Invasive snails can have significant economic impacts, including crop and garden damage [28], control costs, damage to infrastructure, harm to the tourism industry and reduced exports. They can cause damage to crop and agricultural lands by consuming large amounts of plant material [38] damaging soil, spreading diseases to crops, and damaging irrigation systems and farm equipment [56]. These impacts can lead to reduced crop yields and decreased productivity of agricultural lands, resulting in economic losses for farmers. The economic costs of crop damage can include reduced crop yields [28], increased costs of control and management, damage to irrigation systems, and loss of exports due to quarantine measures and other restrictions. Invasive snails can also cause damage to infrastructure such as buildings, roads, and sidewalks by consuming mortar and other building materials.

Success and Challenges of Control and Management

Successes and challenges of control and management efforts for invasive snails can vary depending on the invasive species and the ecosystem.

Successes

Physical control: Physical control methods, such as the manual removal of snails and trapping, can be effective in reducing the population of invasive snails in small areas or localized infestations [40].

Chemical control: Chemical control methods, such as the use of snail baits and pesticides, can be effective in reducing the population of invasive snails in large areas [53].

Biological control: Biological control methods, such as the release of natural predators or pathogens that target invasive snails, can be effective in reducing the population of invasive snails over time [54].

Cultural control: Cultural control methods, such as altering the pH of the soil and reducing moisture, can make the environment less favorable to invasive snails and can be effective in preventing the establishment of invasive snails [38].

Quarantine and regulations: Quarantine and regulations can be effective in preventing the introduction and spread of invasive snails by restricting the movement of potentially infested materials and enforcing regulations on the import and export of invasive snails [42].

Challenges

Difficulty in detecting and monitoring invasive snails: Invasive snails can be difficult to detect and monitor, especially in the early stages of an invasion, which can make it difficult to control and manage their populations [51].

Difficulty in controlling and managing large and established populations: Once invasive snails have established large populations, it can be difficult to control and manage their populations effectively [51].

Difficulty in controlling and managing in large and remote areas: Control and management efforts can be difficult and costly in large and remote areas, where access and resources may be limited [51].

Limited knowledge of invasive snails' biology and behavior: Although invasive snails can seriously harm the environment and the economy, managing them successfully can be difficult due to our limited understanding of their biology and behavior.

Control and management efforts for invasive snails can be successful using methods such as physical, and chemical [28], biological control, and quarantine and regulations [38]. These methods can be effective in reducing the population of invasive snails in small areas or localized infestations, large areas, and over time. However, there are also challenges in controlling and managing invasive snails such as difficulty in detecting and monitoring them [57], difficulty in controlling and managing large and established populations, difficulty in controlling and managing in large and remote areas and limited knowledge of their biology and behavior. These challenges can make it difficult to effectively control and manage invasive snail populations.

Importance of Preventing and Managing Invasive Snail Species

Preventing and managing invasive snail species is important for a variety of reasons, including:

Protecting native species and biodiversity: Invasive snails can outcompete, prey on, and alter the habitats of native species, leading to reduced population sizes and distribution of native species and potentially even extinction. Preventing and managing invasive snail species can help protect native species and biodiversity [58,65,66].

Maintaining ecosystem function: Invasive snails can change the composition and dynamics of ecosystems by consuming large amounts of plant material and other organic matter, which can affect the growth of other plants and the functioning of ecosystems. Preventing and managing invasive snails can help maintain the balance and functioning of natural ecosystems [59,67].

Protecting agricultural lands and crops: Invasive snails can cause damage to crop and agricultural lands by consuming large amounts of plant material, causing damage to soil and irrigation systems, and spreading diseases to crops, which can lead to reduced crop yields and decreased productivity of agricultural lands. Preventing and managing invasive snails can help protect agricultural lands and crops [38,58].

Protecting infrastructure and buildings: Invasive snails can cause damage to infrastructure and buildings by consuming various materials, which can lead to increased maintenance and repair costs. Preventing and managing invasive snails can help protect infrastructure and buildings [42].

Saving costs: Control and management efforts for invasive snails can be significant and costly, including labor costs, materials, and equipment costs, monitoring and research costs, education and outreach costs, and legal costs. Preventing the introduction and spread of invasive snails is more cost-effective than trying to manage established populations [59].

Improving the quality of life: Invasive species can have a negative impact on human activities such as agriculture, recreation, and infrastructure. Preventing and managing invasive snail species can improve the quality of life for people who live and work in areas affected by invasive snails [59,66,68].

Future Directions for Research and Management of Invasive Snail Species

Future directions for research and management of invasive snail species can include:

Improving early detection and rapid response methods: Developing more efficient and cost-effective methods for detecting and responding to new invasive snail populations quickly can help prevent the establishment and spread of invasive snails [60,65,66].

Conducting more research on the impacts of invasive snails: More research is needed to understand the full range of impacts that invasive snails have on native species, ecosystems, and human activities, such as agriculture and infrastructure [54].

Developing and testing new control and management methods: New control and management methods, such as biological control methods that use natural predators or pathogens to target invasive snails, may be developed and tested to improve the effectiveness and sustainability of control and management efforts [54].

Incorporating citizen science and public engagement: Involving the public in monitoring, reporting and controlling invasive snails through citizen science programs and outreach can increase the speed and effectiveness of management efforts [61].

Enhancing international cooperation: Invasive snails can be spread through international trade and travel; therefore, international cooperation is needed to develop

and implement effective prevention and management measures [61].

Evaluating the long-term effectiveness of management actions: Long-term monitoring and evaluation of management actions are necessary to assess the effectiveness of management efforts over time and to identify any potential unintended consequences.

Improving risk assessment: A more robust risk assessment process for identifying invasive snails that are likely to establish in new regions and cause harm, will help to prioritize management efforts and resources.

In summary, invasive snail species can have a wide range of negative impacts on the environment, including on native species, ecosystems, and human activities such as agriculture and infrastructure. Preventing and managing invasive snail species is important to protect native species and biodiversity, maintain ecosystem function, protect agricultural lands and crops, protect infrastructure and buildings, and save costs. Future directions for research and management of invasive snail species can include improving early detection and rapid response methods and conducting more research on the impacts of invasive snails [62]. Also, developing more effective control and management strategies, such as biological control and habitat management, can help reduce the negative impacts of invasive snails [64].

Conclusion and Recommendations

Adverse Effects of Invasive Snail Species on the Environment

Invasive snail species can have a wide range of adverse effects on the environment. These include competition with native snails, which can lead to reduced population sizes and distribution of native species, predation on native species, alteration of community structure and ecosystem function, damage to crops and agricultural lands, damage to infrastructure and buildings, and costs of control and management efforts. On the other hand, invasive snails can consume large amounts of plant material and other organic matter, which can affect the growth of other plants and the functioning of ecosystems. Furthermore, they can cause damage to crop and agricultural lands by consuming large amounts of plant material, causing damage to soil and irrigation systems, and spreading diseases to crops, which can lead to reduced crop yields and decreased productivity of agricultural lands, and they can cause damage to infrastructure and buildings by consuming various materials, which can lead to increased maintenance and repair costs. Control and management efforts for invasive snails can be significant and can include labor costs, materials, and equipment costs, monitoring and research costs, education and outreach costs, and legal costs. Overall, invasive snails can have a wide range of negative impacts on the environment, including on native species, ecosystems, and human activities such as agriculture and infrastructure.

Possible Threats of the Presence of Non-native Invasive Land Snail Species in Türkiye

Land snails are accepted in the aquatic products group in Türkiye. Importation of foreign live fishery products to Türkiye is stated in the Fisheries Regulation, "For the fisheries to be imported, it is obligatory to submit a Health Certificate and a Certificate of Origin, issued by the official institutions of the seller country, stating that they are free from infectious diseases and are found to be healthy. In the importation of broodstocks, these documents must also be approved by the Consulates" [69]. In addition, concerning "non-native species", in Article 13 of the "Regulation on the Protection of Wetlands" of the Ministry of Forestry and Water Affairs [70] it is stated that "non-native species cannot be stocked in areas protected by the Ramsar Convention and wetlands of national importance, for whatever purpose, without scientific research and without the approval of the Ministry" and "Removal of non-native species from the area, which have been stocked in the past and which have been found to have a serious negative impact on the wetland ecosystem as a result of scientific research, and if this is not possible, control of their populations is provided by the relevant Administrations under the coordination of the Ministry". However, despite these laws, the existence of non-native invasive land snail species has been reported in Türkiye.

Many non-native and invasive creatures can reach new geographic regions by many means of transport [66]. One of the most important ways of transporting these creatures to other environments is the worldwide ornamental animal trade, which increases its impact with the speed of global trade [22,25,72,73]. The exotic pet trade has increased rapidly in recent years, especially as interest in live animals kept for hobby purposes has increased, and there has been a simultaneous increase in the number of invasive species dispersed in this way [73]. The easy and fast access provided by the trade of pets over the internet has also accelerated this increase. One of the most striking examples of this situation is the sale of members of the African Giant Snail (Achatinidae family), which are known for their invasive and intermediate host properties for some zoonotic parasites [74], offered for sale in web shops in Türkiye. It is expected that these family members, whose invasive characteristics, and damage in the new habitats they enter, are imported to Türkiye, together with all freshwater snails, are indirectly possible according to the regulations [75] and will disperse within the country through various trade channels in the coming years. However, it is predicted that this distribution will create undesirable negativities soon

in terms of both ecological and health risks. For example, the negative impact of *A. vulgaris* on agriculture products has been reported in Düzköy, Trabzon city. It has been stated that *A. vulgaris* is particularly damaging to crops such as potatoes, beans, and cabbage. Therefore, residents, who have been trying to fight the snail species that have been affecting the district for about 4 years, are asking for help from the authorities.

Preventing the introduction and distribution of invasive snail species in Türkiye is essential to protect the country's biodiversity and ecosystems. Invasive snails can cause a wide range of negative impacts on the environment, including competition with native snails, which can lead to reduced population sizes and distribution of native species, predation on native species, alteration of community structure and ecosystem function, damage to crops and agricultural lands, damage to infrastructure and buildings, and costs of control and management efforts in Türkiye. It is therefore important for the Government and public to be aware of the potential risks and report any sightings to the authorities, and for the agricultural industry and travelers to be cautious and not to transfer and release any invasive snails into the wild. The presence of any non-native snail species in any location should be reported. Cooperation of the government, researchers, industry, and the public is vital to prevent the transfer, introduction, and spread of invasive snail species in Türkiye.

In Türkiye, the public, administrations, and especially entrepreneurs who want to import foreign species need to be conscious of the protection of natural genes and resources and the potential dangers and negative effects of foreign species stocking. In addition, it is of great importance to enact more effective import-controlling laws and to implement existing laws. If this is not successful, the quality of Türkiye's resources and the structure of our natural genetic resources will have deteriorated because of the introduction of foreign species into Türkiye's natural environment to gain economic income. Therefore, the importation and sale of invasive non-native snail species must be banned by the government for any purpose. For example, in many countries, including giant African snails, invasive snail species are prohibited from importing, bringing in by any means, and purchasing online, as exotic snails are likely to escape and spread, causing environmental problems. Moreover, more research should be done on the biology, ecology, artificial production, feeding, and rearing of economically important native snail species of Türkiye.

Highlight Keypoints

- The presence of non-native invasive snail species in Türkiye could lead to a decline in the country's unique biodiversity. These invaders might outcompete or prey upon native snails found exclusively in Turkish ecosystems, potentially disrupting the delicate balance of species in various regions across Türkiye.
- Given Türkiye's diverse agricultural practices and varied environmental landscapes, invasive snail species could cause disruptions in these systems. These invaders might negatively affect the intricate harmony within Turkish agricultural practices and environmental ecosystems, potentially leading to economic losses and environmental imbalances in different parts of the country.
- Invasive snails pose a threat to native snails by competing for resources, leading to reduced populations and altered distributions of native species. This disruption can shift the balance in ecosystems, impacting their structure and function.
- Invasive species cause significant harm to agriculture by consuming large quantities of plant material. This not only damages crop directly but also affects soil quality, irrigation systems, and spreads diseases among crops, ultimately decreasing yields and productivity.
- Invasive snails can wreak havoc on infrastructure and buildings by consuming various materials, resulting in increased maintenance and repair costs. Their activities pose threats to the integrity of structures and the need for continuous repairs.
- Managing invasive snail populations comes with considerable expenses, including labour, materials, equipment, monitoring, research, education, and legal costs. Controlling their spread and mitigating their impact demands substantial financial and resource investments.

Declarations

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

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Research Article

Examination of the Vertebral Heart Scale and Anatomical Structure of Different Dog Breeds by Computed Tomography

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Abstract

This study aims to reveal the statistical difference by measuring the vertebral heart scale and anatomical structures of the heart in different dog breeds. The vertebral heart scale (VHS) has recently been described as a method for measuring the heart silhouettes of dogs and cats. Vertebral heart scale (VHS) may vary in dog and cat breeds due to age, weight, etc. Twenty-one dogs (10 males and 11 females) of different ages and weights were used. Among the dog breeds used are the French Bulldog, Russian Poodle, Rottweiler, Jack Russell, Golden Retriever, King Charles, Pekingese, Belgian Shepherd, Husky, Chihuahua, Cocker, Terrier, Bulldog, Bouvier and Flanders breeds. Multislice detectors were scanned at 80 kV, 200 MA, 639 mGY, and 0.625 mm slice thickness. The resulting images were saved in Digital Imaging and Communications in Medicine (DICOM) format. The obtained sections were measured in 3D-Slicer software. Thoracic height (TH) and cranial vena cava (CVC) measurement parameters were statistically significant with weight ($P<0.05$). Vertebral heart scale (VHS) was not statistically significant with any parameter in the correlation analysis ($P>0.05$). At the correlation table of animal weight with the measurements, it was seen that it had a very significant positive correlation with other measurement parameters except vertebral heart score (VHS) ($P<0.01$). Vertebral heart scale was determined as 9.09±1.37 vertebrae in females and 9.50±0.52 vertebrae in males. The study aims to contribute to veterinary anatomy, surgery and internal medicine.

Keywords: Computed tomography, Dog, Vertebral heart scale

Introduction

Dogs are beings that have been in constant interaction with humans since the past. Today, dogs live in our homes like a member of the family. Apart from taking part in our lives as companions, dogs are also involved in many areas such as search and rescue work [1].

Computed tomography (CT) is the processing of cross-sectional images with high levels of ionizing radiation using X-rays and computers [2]. Computed tomography provides three-dimensional information about the structure, shape, position, and relationships of internal organs [3]. Axial, sagittal, and coronal sections obtained from CT and MR allow better evaluation of these structures. Moreover, the application of the morphometric method to these radiological images brings a new perspective to this analysis [4-6]. Since it is viewed from a single position in 2D (2D) imaging methods, it causes technical errors such as superposition and minimization. This increases the value of 3D (3D) works [7-9]. With the effect of developing technology in recent years, there are many studies in the field of anatomy with radiological, 3D modeling and different modeling techniques [10-13].

Early diagnosis of heart diseases in dogs is important for increasing the quality of life of the patient and prolonging its life. In the diagnosis of heart diseases, besides computed tomography, electrocardiography and echocardiography, biomarkers have been used recently [14,15]. Thoracic radiographs play an important role in the diagnosis of heart disease and also include prognostic information.

In particular, it contributes greatly to the subjective assessment of cardiomegaly. The wide variation in thoracic conformation among dog breeds leads to significant differences in the appearance of the heart silhouette [16,17]. Vertebral heart scale (VHS) has been described as a method to measure heart silhouettes of dogs and cats [18]. The development of the method started with the positive correlation between heart weight and body length in cats. The measurements have proven to aid in the diagnosis of heart disease in dogs [19]. It has been suggested that the vertebral heart scale may be a useful aid in cardiac assessment for inexperienced observers who may be prone to false positive interpretations in dogs, particularly when examining radiographs of puppies, brachycephalic breeds, or obese dogs [20]. The normal VHS values between 9.7 vertebrae(v), 8.7v and 10.7v in dogs are generally considered physiological [21]. Although the VHS value is high in some breeds, these values are normal for the breeds and each breed should be evaluated on its scale [19].

This study aims to reveal the statistical difference for the vertebral heart scale in dogs of different breeds and to determine whether there is a significant difference between the reference intervals of the breeds. It is also to examine the anatomical structures in the heart of dog breeds using computerized tomography images.

Material and Methods

Ethical Statement

The required ethics committee report for the study was obtained from Animal Experiments Local Ethics Committee of Istanbul University-Cerrahpaşa (Approval No: İÜC-HADYEK/29.11.2023-848033) and the Istanbul University-Cerrahpaşa, Faculty of Veterinary Medicine Ethics Committee (Report Number: 2022/38). An "Informed Consent Form" was obtained from the animal owners before to conducting tomography scans

Animals

Twenty-one dogs, which were 11 females and 10 males, were used in the study. The age ranges are between 1 and 16 years old. The weight range is between 4 and 43 kg. The dog breeds used were French Bulldog, Russian Fino, Rottweiler, Jack Russell, Golden Retriever, King Charles, Pekingese, Belgian Shepherd, Husky, Chihuahua, Cocker, Terrier, Bulldog, Bouvier, and Flanders breeds. All of the images were obtained from the archive images of Istanbul University-Cerrahpaşa Veterinary Faculty Animal Hospital. Samples were obtained from mature animals. Dogs with any evidence of pathological lesions, deformation, or other damage were excluded from this study.

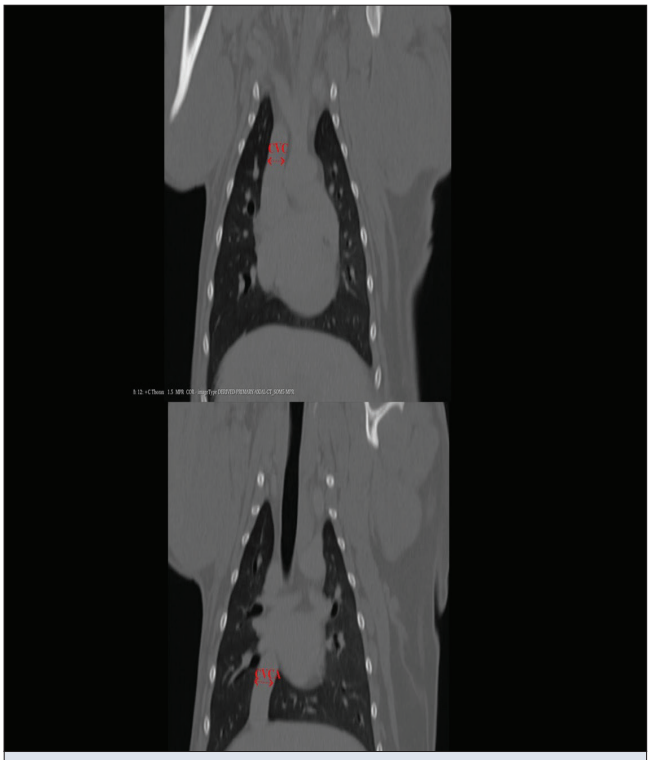

Fig 1. Measurement points of anatomical structures on the heart (CVC and CVCA)

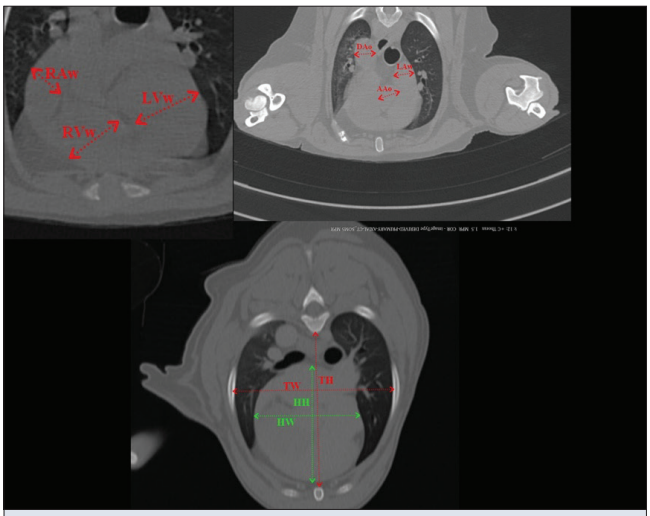

Fig 2. Measurement points of anatomical structures on the heart (TH,TW,HH,HW, Ao,AAo,and DAo)

Computed Tomography Images

Computed tomography images were scanned with 64-detector multislice 80 kV, 200 MA, 639 mGY, 0.625 mm slice thickness. The resulting images were saved in DICOM format. The obtained images were measured in 3D-Slicer software. The measurement points, specified in *Table 1*, were used from the sources specified [22-24]. Measurement points taken on the heart are shown in *Fig. 1*, *Fig. 2* and *Fig. 3*.

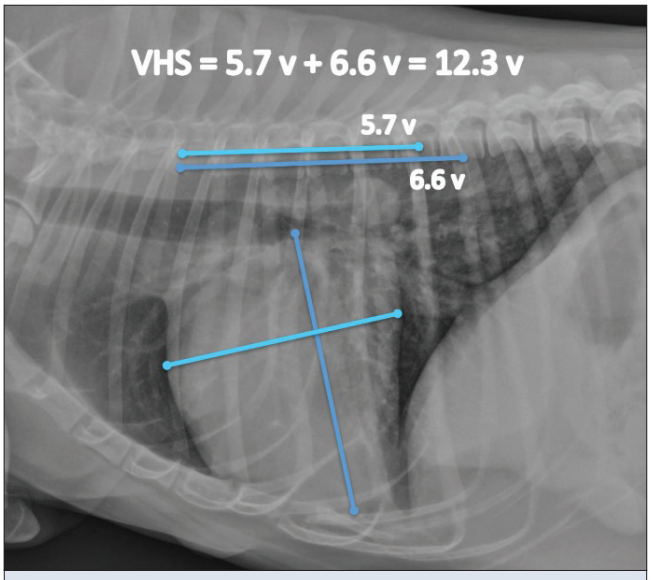

Fig 3. Vertebral heart scale measurement points

Statistical Analysis

Statistical analyses were performed in the SPSS 22.0 software program. Descriptive statistics of measurement values were used as mean, standard deviation and one-way ANOVA depending on more than one variable. The Mann-Whitney U test was used for gender comparisons according to measurements. Spearman correlation coefficient was calculated to determine the relationship between the measurements provided that they are different.

Results

The anatomical structures of the heart and VHS were measured using axial, sagittal and coronal images of the dogs participating in the study. The statistical comparison of the measurements made in our study with the measurements of age, gender and weight on the anatomical structures of the heart is given in *Table 1*. It

Table 1. *Descriptive statistics of measurements of anatomical structures of the heart in dogs by sex, age, and weight*

Parameters	Gender	N	Mean	Std. Deviation	P Value for Age	P Value for Weight	P Value for Gender
TH	Male	10	96.33	16.00	NS	*	NS
	Female	11	93.79	18.78			
TW	Male	10	102.57	25.67	NS	NS	NS
	Female	11	104.77	25.02			
HH	Male	10	63.72	11.63	NS	NS	NS
	Female	11	61.11	9.72			
HW	Male	10	71.87	16.21	NS	NS	NS
	Female	11	59.76	21.56			
Ao	Male	10	17.29	6.10	NS	NS	NS
	Female	11	15.33	5.77			
AAo	Male	10	18.91	6.35	NS	NS	NS
	Female	11	15.72	5.82			
DAo	Male	10	14.96	4.41	NS	NS	NS
	Female	11	13.72	3.84			
LVw	Male	10	32.35	8.23	NS	NS	NS
	Female	11	29.80	10.62			
LAw	Male	10	20.17	6.29	NS	NS	NS
	Female	11	17.41	5.91			
CVC	Male	10	13.36	3.38	NS	*	NS
	Female	11	11.05	3.28			
CVCA	Male	10	13.41	3.27	NS	NS	NS
	Female	11	10.90	3.50			
RVw	Male	10	22.49	6.05	NS	NS	NS
	Female	11	19.97	6.31			
RAw	Male	10	13.95	4.87	NS	NS	NS
	Female	11	13.24	4.75			
VHS	Male	10	9.50	0.52	NS	NS	NS
	Female	11	9.09	1.37			

TH: *Thoracic high,* ***TW:*** *Thoracic width,* ***HH:*** *Hearth high,* ***HW****: Hearth width,* ***Ao:*** *Aorta,* ***AAo:*** *Ascendens aorta,* ***DAo:*** *Descendens aorta,* ***LVw:*** *Left ventricular width,* ***LAw:*** *Left atrium width,* ***CVC:*** *Cranial vena cava,* ***CVCA:*** *Caudal vena cava,* ***RVw:*** *Right ventricular width,* ***RAw:*** *Right atrium width,* ***VHS:*** *Vertebral high scale*

Table 2. *Correlation between measurements of anatomical structures of the heart in dogs by age and weight (Green: P<0.01; Yellow: P<0.05; Red: P>0.05)*

Items	Age	Weight	TH	TW	HH	HW	Ao	AAo	DAO	LV	LA	CVC	CVCA	RV	RA	VHS
Age	1															
Weight	0.279	1														
TH	0.28	.721(**)	1													
TW	.462(*)	.738(**)	.649(**)	1												
HH	0.261	.675(**)	.686(**)	.858(**)	1											
HW	.455(*)	.820(**)	.568(**)	.697(**)	.687(**)	1										
Ao	0.389	.819(**)	.751(**)	.770(**)	.732(**)	.804(**)	1									
AAo	0.229	.879(**)	.769(**)	.700(**)	.649(**)	.799(**)	.901(**)	1								
DAO	0.218	.870(**)	.609(**)	.656(**)	.617(**)	.813(**)	.822(**)	.873(**)	1							
LV	0.368	.755(**)	.466(*)	.665(**)	.551(**)	.795(**)	.869(**)	.781(**)	.839(**)	1						
LA	0.255	.825(**)	.571(**)	.688(**)	.562(**)	.739(**)	.922(**)	.886(**)	.862(**)	.939(**)	1					
CVC	0.300	.900(**)	.488(*)	.648(**)	.631(**)	.899(**)	.791(**)	.817(**)	.881(**)	.862(**)	.849(**)	1				
CVCA	0.315	.825(**)	0.401	.605(**)	.587(**)	.875(**)	.791(**)	.778(**)	.883(**)	.871(**)	.834(**)	.958(**)	1			
RV	0.367	.854(**)	.517(*)	.714(**)	.599(**)	.813(**)	.796(**)	.731(**)	.843(**)	.878(**)	.843(**)	.875(**)	.845(**)	1		
RA	0.344	.804(**)	0.23	.658(**)	.473(*)	.770(**)	.668(**)	.674(**)	.806(**)	.856(**)	.799(**)	.887(**)	.860(**)	.895(**)	1	
VHS	-0.418	-0.25	-0.328	-0.183	-0.068	-0.206	-0.408	-0.289	-0.296	-0.285	-0.32	-0.189	-0.225	-0,143	-0,131	1

was determined that age and gender were not statistically significant in the measurements (P>0.05). Anatomical structures of the heart were found to be statistically significant with the weight of dogs only with thoracic height (TH) and cranial vena cava (CVC) (P<0.05). Vertebral heart scale (VHS) males are larger than females. Only thoracic width (TW) females were found to be larger than males in the measurements obtained. The correlation of measurements of age, weight and anatomical structures is given in *Table 2*. There is a significant correlation between age, thoracic width and heart width (P<0.05). It was found to be statistically insignificant with other parameters (P>0.05). Vertebral heart scale was found to be statistically insignificant with any measurement parameter (P>0.05).

Discussion

In this study, fifteen different breeds of dogs of different weights, whose CT taken in our hospital, were evaluated to determine the values of VHS and heart anatomical structures.

Vertebral heart scale has become an important marker in the diagnosis of heart diseases in cats and dogs. In clinical practice, breed-related differences in VHS value have been observed between dog breeds and accordingly weight differences. This study was conducted to determine whether the weight, age, sex and breed factor differed on the results by compiling the tomography images of the dogs brought to our hospital with different complaints.

Cardiomegaly, which is the most common heart disease, is formed in dogs with valvular diseases, dilated cardiomyopathy, congestive heart failure and congenital heart diseases [25]. The type of heart disease will affect the accuracy of a diagnosis based on measurements of the vertebral heart scale. Heart diseases can impose different loads on the heart depending on their pathophysiology and the heart's response varies according to the load [26]. Volume-loading diseases such as mitral regurgitation cause eccentric hypertrophy or enlargement of the heart chambers with a corresponding increase in the external dimensions of the heart. Diseases that exert pressure loads, such as aortic stenosis, tend to cause concentric hypertrophy, i.e. thickening of the myocardium occupying the ventricular lumen without any significant change in external dimensions. The observed inter-racial differences in the diagnostic accuracy of the vertebral heart scale are at least partly due to pathophysiological differences between the conditions to which the breeds are predisposed [19]. As Lamb et al.[19] noted, vertebral heart scale values greater than 10.4v for the Yorkshire terrier and 11.1v for the Cavalier King Charles Spaniel should provide about 80 percent accuracy in diagnosing heart disease. With these data obtained from breed-based research, the veterinarian's diagnosis of heart diseases is accelerated.

Considering that dogs being male or female may show differences in VHS measurements, the gender was also included in our study as a statistical criteria and differences were determined according to gender. The same researchers also indicated that female dogs have a lower VHS value than male dogs. According to Buchanan and Bücheler [20], there was no difference in the VHS value in dogs between males and females. The fact that the thoracic width determined in our study is greater in females and the VHS value is higher in male dogs supports the effect of gender on sizing.

Sagoglu [26] calculated the VHS value as 11v and above in dogs with mitral valve disease, dilated cardiomyopathy, congestive heart failure, congenital heart disease and developed cardiomegaly. Buchanan and Bücheler [20] reported in their study that healthy dogs have a VHS value of 10.5 and below.

The margin of error that may arise in heart score measurements using X-ray images is important during diagnosis. It is seen that many different results have been obtained in the researches. Regarding the direction of X-rays, some researchers noted that the heart silhouette appears larger than the left view because the heart is on the left side in Whippets [27]. It is also showed that a greater view on heart radiographs taken from the right view in Beagles [21]. Different notifications are also seen in studies conducted in rodents [28-30].

In the present study, VHS values were determined with many dog breeds. The values determined in dogs are equivalent to the studies performed and give results equivalent to the values obtained in healthy dogs. The VHS value increased with the size of the dog, but it was observed that the heart scale increased in dogs with heart disease, regardless of size. Mean values were calculated in our study. However, it has been detected that the Pekingese dog has a VHS of 12v. This indicates that this small-sized dog has a higher VHS value than the larger-sized dogs and it shows as cardiomegaly in a this Pekingese dog.

There is a difference between male and female VHSs. Males appeared to have a greater VHS value than females. In the data obtained from the right and left radiographs, it was observed that there was no difference between the directions of the VHS value.

Wagner et al.[23] in their study on healthy cats, they reported that chest width was higher than chest height, and heart height was higher than heart width. They also found that the parameters in the measurements of males were higher than females. In this study, thoracic width was found to be greater than thoracic height in dogs. However, it was observed that the heart width was greater than the heart height in dogs. It was determined that thoracic width was higher in females than in males.

In the measurement of the anatomical structures of the heart in dogs in this study, the measurements made from different dog breeds were similar to other studies. There were small differences on average depending on the weight of the breed type of the dogs. In studies conducted on different dog breeds, the aortic root was measured as 12.8±0.31 mm and the left atrium as 15.9±0.38 mm [26], on English Bulldogs, the left atrium as 47.57±5.26 mm and the right atrium as 31.0±96.05 mm [15], in another research the average length of the left atrium was measured as 22.56 mm and the average diameter of the right atrium was 18.00 mm [31]. Changes in these values also support the importance of breed and weight differences between dogs in the diagnosis of cardiac problems.

In conclusion, this study aimed to reveal the heart anatomical measurements and vertebral heart scales taken from different breed of dogs. This study would be a guide to the researches conducted by examining CT images of dogs of the same breed and to precise their VHS and heart anatomical structure reference values.

Declarations

Availability of Data and Materials: The authors declare that data supporting the study findings are also available to the corresponding author.

Funding Support: None.

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

Ethical Approval: The ethics committee report for the study was obtained from Animal Experiments Local Ethics Committee of Istanbul University-Cerrahpaşa (Approval No: İÜC-HADYEK/ 29.11.2023-848033) and the Istanbul University-Cerrahpaşa, Faculty of Veterinary Medicine Ethics Committee (Report Number: 2022/38).

Author Contributions: In the author's contribution to this study, BCG and EO collected CT. BCG, DOE, YA manuscript design. BCG and ÇPY Completed the article hypothesis and writing phase.

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Research Article

Estrous Cycle Length in the Algerian Arbia Goat: Exfoliative Vaginal Cytology and Serum Progesterone Levels

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Abstract

This study aimed to estimate the estrous cycle length of the Algerian Arbia goat in Northern Algeria. For this, eighteen (18) Arbia goats, aged between 2 and 6 years, were used in our work that took place in the experimental farm of the Saad Dahlab University (Blida, Algeria). Blood samples were taken from each goat twice a week (at a 2 or 3-day interval) for 3 months. The serum progesterone concentration was determined by Radio-Immuno-Assay. Smears of the vaginal mucosa were taken at the same time as the blood samples. The predominance of superficial cells on the smear of the vaginal mucosa as well as a serum progesterone level less than 1 ng/mL expressed the return to estrous which was considered the beginning of a new cycle. A negative correlation was observed between the percentage of superficial cells (SC) and serum progesterone (P4) levels in all goats. Our results showed a significant difference ($P<0.05$) between the means of different cycle lengths obtained among the females. In addition, normal cycles had an average of 20.11±1.85 days (17-25 days) representing 59.6% of cycles. Besides, a large number of short cycles (<17 days) with an average of 14.41±1.51 days were found representing 25.5% of recorded cycles. The number of long cycles (>25 days; with an average of 32.14±5.58 days), represented 14.9% of recorded cycles. Following these results, it can be concluded that the local goat in Northern Algeria had different types of cycles (normal, short, and long) with a large percentage of normal cycles.

Keywords: *Arbia goat*, *Cycle length, Serum progesterone, Vaginal cytology smears*

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Introduction

Small ruminant production is one of the most important sources of meat in Algeria and plays a vital role in the country's food security [1]. Furthermore, the reproductive performance of ruminants is among the major concerns of breeders and their technical supervisors [2]. Knowledge of the reproductive physiology of the estrous cycle is important for animal management and to determine the reproductive and productive potential of animals [3]. The latter plays a key role in farm economics, not only in determining animal performance but also in decisions concerning selection and culling [2].

Perfect knowledge of the characteristics of the sexual cycle and its different stages is of decisive value in the success of breeding. In females with normal cycles, morphological, endocrine and secretory changes that occur in the ovaries and tubules usually represent the stage of the cycle. These changes have been associated with sexual steroid hormone levels. In the absence of infection, circulating levels of progesterone and estradiol 17β are the main determinants of the model of vaginal cytology [4,5].

It is well known that the female reproductive tract is a target for sex steroid hormones. The endometrium and the vaginal epithelium are especially influenced by sex hormones which determine their development and function [6]. The serum levels of estradiol 17β (E2) and progesterone (P4) directly influence the cytology pattern of the vagina. Examination of vaginal cells is a useful indicator of the estrous cycle in different species [7,8].

Vaginal cytology changes during the estrous cycle have been studied in sheep [9], goats [6], bovine [10] and rodents [11]. The morphology of exfoliated cells has been found to be very useful to determine the physiological and pathological status of the female as well as a tool for hormonal bioassay in several animal species [5,12]. Many researchers have studied the variations which occur in the vaginal mucosa at different phases of estrus cycle by using vaginal smears [13,14]. These variations occur under the influence of steroid hormones [15]. Exfoliated cells are a normal occurrence during the estrous cycle due to the increase of estrogen. As the stages of the cycle advance to estrus, mostly cornified epithelial cells are present [14]. The relative proportion of different types of vaginal epithelial cells can be used as a marker of the endocrine environment [15,16].

In Algeria, as far as the authors know, there is little or no work done on the nature of reproductive cyclicity in local goats and the characterization of sexual cycle parameters. These data are imperfectly known and are still unclear. Thus, the purpose of the present work was to study the reproductive cyclicity by determining the estrous cycle length of the Algerian Arbia goat.

Material and Methods

Ethical Statement

All the animal studies were conducted with the utmost regard for animal welfare, and all animal rights issues were appropriately observed. No animal suffered during the course of the work. All the experiments were carried out according to the guidelines of the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research (Agreement Number 45/DGLPAG/DVA.SDA.14).

Study Area

This current study took place in the experimental farm of the Saad Dahlab University of Blida 1, in Blida region, located between latitude 36°28' North and longitude 2°49' East. It is bordered in the North by Tipaza and Algiers, East by Boumerdes and Bouira, South by Medea and West by Ain Defla *(Fig. 1)*. It has a Mediterranean climate characterized by cold and rainywinters and hot and dry summers.

Animals

Eighteen Arbia goats, aged between 2 to 6 years, with an average live weight of 31.28 kg were used in our experiment.

To ensure that the females were not pregnant, an ultrasound examination was performed for each goat. Throughout the study period, the goats were separated from the males (buck) to avoid any unwanted protrusions.

Before the start of the study, the entire herd used for the experiment underwent internal and external antiparasitic treatment using Ivermectin (Ivomec 1%®, Merial). The study took place during September, October, and November.

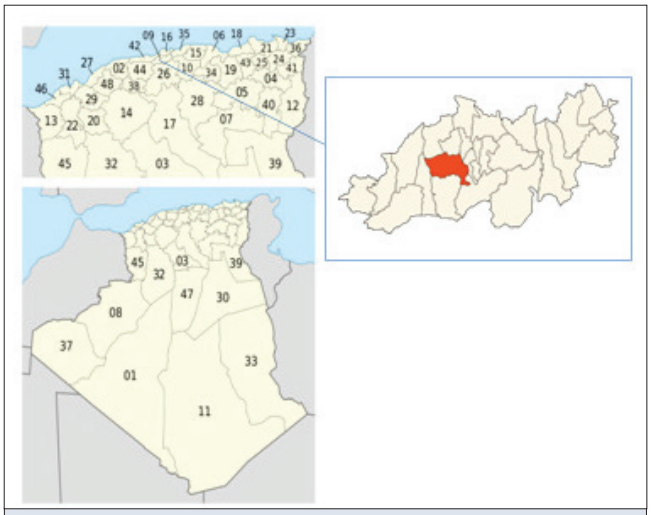

Fig 1. Study area localization

Diet

The entire herd received an identical diet throughout the experiment period. The animals received a daily feed ration consisting of oat hay, and a concentrate based on crushed barley, corn and bran distributed at a rate of 500 g/day/animal (In addition to the contribution acquired by free grazing in the meadow of the experimental station). Water was supplied *ad-libitum*.

Vaginal Smear and Cytology

Vaginal smears were collected from each female twice a week (at 2 or 3-day interval) for 3 months. We studied 312 smears in total. The smear collection procedure involved using a vaginoscope for parting the vulva lips and inserting a 10 cm long cotton-tipped sterile swab into the vagina to a depth of about 5-7 cm. The swab inside the vagina was rotated through 2-3 revolutions against the vaginal wall. It was withdrawn and rolled on a clean glass slide to form two parallel tracks of smear material on the glass surface. The smear was immediately fixed with absolute methanol, air-dried and stained with the Giemsa stain. The vaginal cells were classified under a light microscope into three basic cell types with different diameters; superficial squamous cells with light cytoplasm, intermediate squamous cells and parabasal cells [14]. Each cell type was counted and then expressed as a percentage of the total [15]. The predominance of superficial cells on the smear of the vaginal mucosa means the return to heat which was considered the beginning of a new cycle [17].

Hormonal Assay

In order to measure serum progesterone, blood samples (5 mL) were taken from the jugular vein of all females

twice a week (at a 2 or 3-day interval) for 3 months (in the same time as vaginal cytology samples). Following centrifugation at 3000 rpm for 20 min, serum was harvested and stored at -20°C until assayed. Progesterone concentration was determined by the RIA technique using an Immunotech kit (RIA Progesterone from IMMUNOTECH SAS France). The analysis was carried out at the Nuclear Research Center in Draria, Algiers.

Estrous cycle length was defined as the number of days between the onsets of two consecutive estrous periods [18]. Serum progesterone levels below 1 ng/mL associated with the predominance of superficial epithelial cells on vaginal smears indicated the beginning of a new cycle [19]. We were therefore able to determine the different lengths of estrous cycles by the detection of estrous, the perception at the same time of a large percentage of superficial cells on vaginal smears and the decrease of serum progesterone (<1 ng/mL).

Twenty-four samples were taken from each goat during the 3 months of the study (vaginal smears and blood samples), corresponding to 864 samples analyzed (432 smears and 432 serums).

Statistical Analysis

The differences between the percentage of epithelial cell types as well as the cycle length recorded after analysis of vaginal cytology and the determination of serum progesterone levels were calculated by the ANOVA test (statistical software Past3).

Results

According to our results *(Table 1)*, in 75.7% of all vaginal smears, intermediate epithelial cells predominated, followed by the superficial epithelial cells with 20% of smears, and a rate of 4.3% for the parabasal epithelial cells that were rarely encountered (P<0.05) *(Table 2)*.

Table 1. *Percentage of recorded epithelial cells*

Sample	Percentage of Superficiel Cells (%)																	
	goat 1	**goat 2**	**goat 3**	**goat 4**	**goat 5**	**goat 6**	**goat 7**	**goat 8**	**goat 9**	**goat 10**	**goat 11**	**goat 12**	**goat 13**	**goat 14**	**goat 15**	**goat 16**	**goat 17**	**goat 18**
1	2	9	30	30	9	9	11	0	0	20	2	22	12	10	1	29	76	2
2	7	12	50	40	3	52	4	1	0	80	4	0	16	64	5	87	23	13
3	12	65	7	10	50	76	10	3	80	5	7	3	75	30	23	38	2	73
4	21	43	0	1	64	33	32	2	33	3	0	9	26	12	68	18	7	33
5	26	5	2	0	13	15	99	0	11	2	80	20	23	0	12	2	0	5
6	73	8	90	0	38	5	65	5	67	9	76	39	0	2	4	6	28	2
7	25	12	20	12	17	0	8	10	32	18	11	18	20	5	0	12	64	12
8	2	15	10	34	14	0	2	55	11	74	2	8	65	88	0	0	23	69
9	5	43	1	80	71	0	1	92	39	20	4	30	25	20	15	24	10	21
10	11	16	3	0	77	10	5	42	34	2	34	11	4	15	56	77	75	11
11	12	6	45	10	22	8	7	22	56	12	42	22	5	9	40	12	9	5
12	28	7	70	5	17	22	11	30	25	65	76	11	10	12	11	5	2	3
13	29	8	0	0	78	32	15	27	19	7	10	50	22	25	10	2	16	6
14	56	52	0	0	49	49	55	0	4	5	8	22	78	90	82	17	22	56
15	0	62	2	22	5	38	78	4	25	12	2	5	33	33	24	22	11	11
16	15	5	1	0	10	15	5	2	65	0	56	10	12	20	2	92	73	20
17	32	18	0	8	85	21	13	0	45	6	31	65	5	12	1	53	20	8
18	33	19	78	23	20	9	5	4	32	66	78	21	8	6	21	25	8	4
19	46	12	23	0	15	78	0	0	19	1	23	54	0	23	90	19	11	5
20	3	86	3	11	12	14	0	0	20	9	3	32	0	62	28	3	4	3
21	33	82	4	42	10	8	4	2	5	32	15	77	10	27	17	1	9	0
22	4	5	12	90	79	6	60	0	10	30	3	81	15	5	6	67	94	1
23	63	3	30	67	9	0	88	1	72	28	5	17	24	10	11	18	6	9
24	10	11	88	30	71	10	76	2	12	90	2	32	17	2	65	2	0	2

Table 2. *Percentage of epithelial cell type encountered on analyzed smears*

Cell Type	Parabasal	Superficial	Intermediate
Smear rate where cell type predominates	4.3%	20%	75.7%
P-value	<0.05		

Table 3. *Progesterone levels*

Sample	Progesterone Levels (ng/mL)																	
	goat 1	goat 2	goat 3	goat 4	goat 5	goat 6	goat 7	goat 8	goat 9	goat 10	goat 11	goat 12	goat 13	goat 14	goat 15	goat 16	goat 17	goat 18
1	1.8	1.6	1.1	0.8	1.5	1.6	0.9	1.8	1.1	1.3	0.9	1.2	1.8	1.9	1.7	2	0.2	1.8
2	1.9	1.6	0.5	0.5	1.3	1.1	1	1.6	1.4	0.4	0.6	1.7	2	0.2	0.9	0	0.9	1.1
3	1.3	0.6	1.6	1.2	0.7	0.6	1.1	1.5	0.6	1.2	0.8	1.3	0.3	1.6	1.5	1.3	1.9	0.4
4	1.2	1	1.9	1.4	0.8	1.5	1	1.5	1.5	1.4	0.9	1.5	1.1	1.3	0.2	1.8	1.3	1.9
5	0.9	0.8	1.5	1.3	1.3	1.2	0.6	1.7	1.5	1.3	0.7	0.6	1.3	1	1.5	1.2	2.4	2.1
6	0.3	0.8	0.6	1.2	1.1	1.7	1	1.4	0.3	1.1	0.8	1.5	1.9	1.9	1.3	1.4	1.5	1.9
7	0.8	0.9	1.3	1.1	1.2	1.3	1.9	1.6	1.6	1.3	1.4	1.3	1.1	1.7	1.9	1.1	0.6	0.9
8	2	1.2	1.4	1.3	0.9	1.6	1.2	1.8	1	0.5	1.3	1.9	0.6	0.7	1.2	2.3	0.8	0.3
9	1.2	0.5	1.2	0.7	0.6	1.7	1	0.1	0.9	1.7	1.2	1.2	1.1	0.9	1.7	1.8	1.1	1.8
10	0.8	0.7	1.6	1.6	1	1.7	1.3	0.9	1	1.8	0.6	1.4	1.5	1	0.6	0.4	0.3	1.9
11	1.1	1	1.4	1.2	1	3	1.1	1	0.5	1.1	0.7	0.8	1.9	1.4	0.8	1.9	2	1.5
12	1.7	1.2	0.8	0.8	0.6	1.6	1.5	1.2	1.3	0.7	0.6	1.5	1.7	2.1	1.1	1	1.5	1.5
13	2.3	1.1	6.2	1.2	0.5	1.3	1.2	1.1	1.7	1.3	1.5	0.4	1.2	1.1	1.2	1.2	1.3	1.2
14	0.1	0.8	1.3	1.5	0.7	1.2	1.7	1.8	5.9	1.4	1.2	1.2	0.7	0.5	0.3	1.3	1.1	0.7
15	1.6	0.6	1.5	1.2	0.9	0.8	0.5	4.8	3.3	1.5	1.1	1.3	1.3	1.5	1	0.9	1.6	1.1
16	0.9	1.2	1.2	1.1	1.5	3	1.5	1.4	0.2	1.2	0.5	1.2	1.8	3	1.8	0.2	0.4	1.5
17	1.8	1	1.4	0.8	0.1	1.5	1.2	1.4	0.9	0.9	0.5	0.5	2.1	1.9	2.2	1.4	1.7	1.2
18	1.8	0.9	0.1	1.5	1.2	1.2	1	1.5	1.6	0.5	0.9	1.1	1.5	1.6	1.3	1.6	1.1	1.7
19	1.6	1.3	1.2	1.3	1.1	0.4	2	1.2	1.6	1.8	1.3	1.4	2	1.1	0.2	1.2	1.9	1.8
20	0.4	0.1	1.2	1.2	1	1.6	1	1.2	1.3	1.5	1.2	1.2	1.8	0.3	1.1	1	1.3	1.2
21	1.4	0.5	1.4	0.8	1.2	1.2	1.3	1.5	0.8	0.7	1	0.8	0.9	1.8	1.4	1.1	1.5	1.3
22	1.3	1.4	1.2	0.8	0.2	1.4	1	1.5	1	1.1	1.2	1.2	1.3	2	1.5	0.3	0.1	2.3
23	0.3	1.2	1.1	0.9	1.3	1.3	0.7	2.2	0.4	1.3	1.4	1.1	1.5	1.8	0.3	2.4	1.8	2.2
24	1.2	1.3	0.7	1.1	0.3	1	0.8	1.1	1.4	0.8	1.4	1.2	0.7	0.9	0.9	1.2	2.1	1.3

In this study, variations in progesterone levels ranging from 0.1 ng/mL to 6.2 ng/mL were found *(Table 3)*. A negative correlation was observed between the percentage of superficial cells (SC) and serum progesterone (P4) levels in all goats (when the percentage of the superficial cells increased, the serum progesterone level decreased) *(Fig. 2)*.

Throughout the study, we recorded for all the goats, 47 cycles of different types (lengths) which are presented in *Table 4*.

The average cycle length observed was; 20.11±1.85 days for normal cycles, 14.41±1.51 days for short cycles, and 32.14±5.58 days for long cycles *(Table 5)*. There was significant difference in the estrous cycle lengths among the different goats (P<0.05).

Fig. 3 shows the frequency of the different cycle lengths revealed throughout the study. It was noted that the majority of cycles were between 14 and 24 days with some cycles less than 14 days and some cycles more than 25 days.

Data in *Table 3* indicate that the frequency of normal cycles (from 17 to 25 days) was the most important which reached a rate of 59.6% (28 cycles of 47cycles recorded) followed by short cycles (<17 days) with a rate of 25.5%

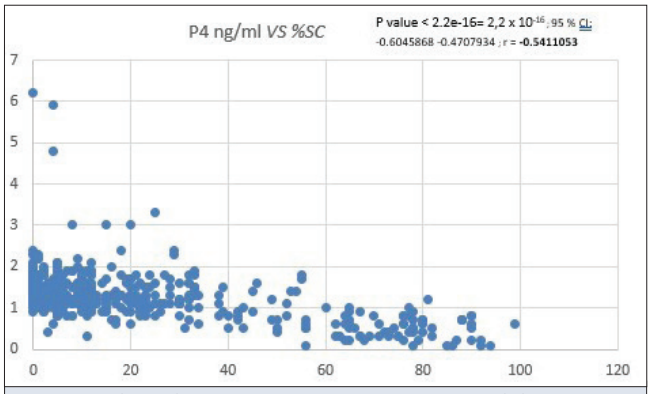

Fig 2. Correlation between serum progesterone rates and the percentage of superficial cells

Table 4. *Number and type of cycle recorded in all females throughout the study*

Goat Number	Number and Duration of Cycle		
	Short (< to 17d)	Normal (17 to 25d)	Long (> to 25d)
1	-	-	(31d), (35d)
2	-	(21d), (18d), (21d)	-
3	(14d), (14d)	(17d), (21d)	-
4	(16d)	-	(42d)
5	(15d)	(21d)	-
6	-	(18d)	(26d)
7	-	-	(28d), (35d)
8	-	-	-
9	(11d)	(17d), (19d), (24d)	-
10	(15d)	(21d), (21d), (21d)	-
11	(15d)	(24d)	-
12	(15d), (15d)	-	-
13	(16d)	(21d)	-
14	-	(20d), (19d), (20d)	-
15	(15d)	(18d), (21d), (19d)	-
16	-	(20d), (22d)	(28d)
17	(12d)	(21d), (19d), (22d)	-
18	-	(17d), (20d)	-
Average +/- SD	14.41±1.51d	20.11±1.85d	32.14±5.58d
P value	0.02623		

d: day

(12 cycles of 47 cycles recorded) and finally the lowest rate was that of long cycles (>25 days) with a rate of 14.9% (7 cycles of 47 cycles recorded).

Table 5. *Frequency and percentage of different cycle lengths*

Parameter	Cycle Length (days)			
	Short (< 17d)	Normal (17d - 25d)	Long (> 25d)	Total
Number of cycle	12	28	7	47
Percentage of cycles	25.5	59.6	14.9	100
P-value	0.005			

d: day

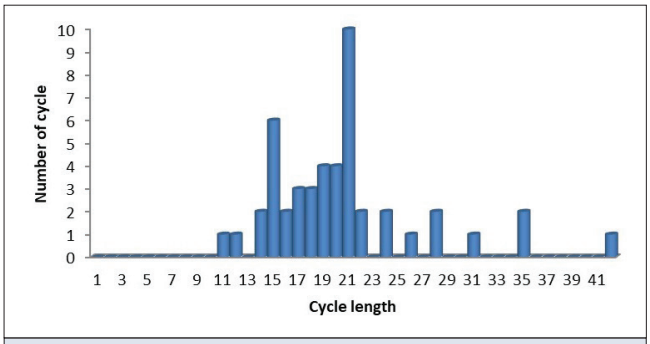

Fig 3. Frequency of recorded cycle lengths

A significant difference (P<0.05) was recorded between the percentages of cycle lengths found.

Discussion

The predominance of intermediate cells in the majority of analyzed smears seems to be related to the luteal phase with a high rate of serum P4 (which is the longest phase of the estrous cycle). On the other hand, smears dominated by superficial epithelial cells corresponded to samples with a decrease in P4 levels, which indicates the estrous phase as reported previously [7]. Sitaresmi [17]found that intermediate cells dominated the majority of the smears, especially on the metestrus and diestrus. Ola et al.[6] noted that intermediate and parabasal cells were more commonly encountered in the smears from days corresponding to the luteal phase under progesterone control. These results are in agreement with those reported by Zarkaoui and soukouti [20] in Damascus does, which have shown that the follicular phase had an average length of 3.1±0.6 days (range: 2-5 days), with a mean progesterone level of 0.68±0.79 nmol L^{-1} (range: 0.00-2.81 nmol L^{-1}).

These cyclical relationships between the exfoliated cells and the ovarian steroid hormones have been strongly established for small ruminants and for other species [5,6,8,21].

In the current study, data showed that the average normal estrous cycle length in the Algerian Arbia goat was 20.1±1.85 days (17-25 days). In addition to the normal cycles, the females presented a great percentage of short

cycles (25.5%) with an average of 14.41±1.51 days. Long cycles were found with a percentage of 14.9% and an average of 32.14±5.58 days. These results are consistent with the study conducted by Yahia et al.[22]. These latter concluded that the average length of normal cycles for Algerian local goats was 19.23 days. In the same context, Charallah [23] confirmed that the normal estrous cycle length was 20 days in the Bedouin goat, with the existence of other types (short and long cycles). Derquaoui and El Khaldi [24] noticed that the average duration of the estrous cycle in D'man goats was20.96±2.84 days for normal cycles and 10.5±3.45 days for short cycles. In local Moor goats in Tunisia, the average duration of normal cycles was 21.1±1.5 days [25]. Corteel [25] found that the goat showed only 6-8estrous cycles during each year and the frequent duration was 21 days.

On the contrary, longer or shorter cycles (less or more than 21 days) were observed by several authors. Lahirigoyen [27] mentioned that the average duration of short cycles was 6 days and those of long cycles ranged between 30 and 44 days. The west African dwarf goats exhibited medium (regarded as normal) cycle lengths of between 19-22 days [28,29]. In fact, 86%, 32%, of cycles were short, respectively, in the Nubian goat [30], and in Creole [31]. A study with Alpine goats during the breeding season recorded 77% of normal cycles (17-25 days) with an average duration of 20.7 days, 14% were short cycles (8 days on average) and 9% were long cycles (39 days on average) [32], which is in agreement with our results. It would seem that the relatively high incidence of short cycles was a characteristic of the goat species [24,32]. The origin and etiology of short cycles in small ruminants are not fully elucidated, but they can be explained by the fact that the corpus luteum of short cycles is of bad quality and that its secretory function is limited as a consequence [31]. This fact is strongly influenced by food level [33].

In addition, the periods found in this work are similar to those reported for a certain tropical alpine breed in different countries. Greyling [34] showed that the normal estrous cycle length was 20.7±0.7 days.The normal cycle duration of 19.7±1.5 was revealed in the Matou goat in China by Moaeen-ud-Din et al.[35]. The normal estrous cycle length recordedin our study (20.11±1.85 days), is very close to those reported in Red Sokoto goats in Nigeria [36] and in Criollo goats in Chile [37] (21.3 and 20.7, respectively).

The present study showed a negative correlation between the percentage of superficial cells (SC) and serum progesterone (P4) levels in all goats, and the average normal estrus cycle length in Algerian Arbia goats was 20.11±1.85 days with a percentage of 59.6%. To this type of cycles, were added short and long cycles with different rates. In addition, it has been suggested that a vaginal cytology exam is highly indicative of the effect of progesterone in the goat reproductive tract. It was possible through this work, for the first time, to characterize the estrous cycle length of the Algerian Arbia goat and to determine the estrus phase by researching the high rate of superficial epithelial cells and the low level of serum progesterone. These findings are important and useful in assessing the physiology of the estrous cycle of the Algerian Arbia goat as a primary parameter of reproduction.

Declarations

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

Funding Support: There is no funding source.

Ethical Statement: All the experiments were carried out according to the guidelines of the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research (Agreement Number 45/DGLPAG/DVA.SDA.14).

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

Author Contributions: YA, NH, KS, KH, NM: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing - Original Draft, Writing - Review & Editing.

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Research Article

Temperature Humidity Index: Influence on Milk Yield and Milk Composition of Multiparous West African Dwarf Does

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Abstract

This study examined the influence of ambient temperature (AT), relative humidity (RH) and temperature-humidity index (THI) on milk yield (MY) and milk compositions (MC) of West African Dwarf (WAD) goats using a total of thirty-six (36) lactating does. The goats were milked twice daily for twelve (12) weeks during which, AT and RH were monitored. The AT and RH data were used to generate THI. The milk collected at every milking was quantified and also analysed for MC, The AT, RH and THI during each milking period were categorized into low, medium and high ranges, under which the corresponding MY and MC were fixed. The data generated were subjected to Analysis of Variance of a Completely Randomized Design. The result revealed that the highest MY (350.28 mL), recorded during low AT range, was not significantly different from 329.43 mL obtained during medium AT, while high RH range (70-99%) facilitates higher MY (364.30 mL) compare to low and medium ranges. AT exerted no significant difference on all the MCs except protein. Milk fat was highest (7.34%) at low THI, while the lactose (11.30%) at high THI range was significantly higher compared to other ranges. A relatively higher MY (324.35 mL) and milk density (1034.94 kg/m^3) was obtained within medium THI range (75.6-85.6). It is concluded that, AT and RH exhibit a synergistic effect on MY and some of the MCs.It is recommended that thermo-comfort THI for dairy WAD goats is between 75.6 and 85.6.

Keywords: Ambient temperature, Milk composition, Milk yield, Relative humidity, and Temperature- humidity index

Introduction

Agriculture contributes to climatic change and as well, being negatively affected by it. The nature and the extent of animal agriculture practised in any region of the globe are commonly influenced by the interactions among numerous physical, biological and socioeconomic factors [1,2]. As agriculture tends towards increased commercialization, impact on climate change becomes more apposite [3]. Thus, improving animal agriculture needs to consider the interactions between animals and the environment. Complexities between livestock sector and climatic change were identified to be significant and generally overlooked, particularly in Africa where livestock plays a crucial role in poverty alleviation and rural development [4].

Small ruminants play a predominant role in the economy of million people and have provided meat, milk, skin and many other indirect benefits for centuries [5]. The demand for livestock (including small ruminants) products and consequential increase in production has been largely driven by the rapid growth of human population, increases in income and urbanization [6]. Therefore, climatic change becomes a serious long- term challenge faced by small ruminants' owners worldwide. The influence of climatic change in terms of sudden temperature change has caused significant losses in animal production [7] and therefore generates the need for detailed studies on the adaptive processes of breeds, especially goats, to better understand the adaptation parameters and processes of these animals.

Change in relative humidity and ambient temperature constitute meaningful effects on animal behaviour as a result of neuron endocrine response which consequently influences production and health status of animals [8.9]. Thus, temperature change is a major threat to the viability and sustainability of milk production in goats as well as relative humidity [10]. Livestock generally express their full potential when environmental conditions are favourable.

Earlier researchers demonstrated that air temperature and relative humidity are the major natural physical environmental factors affecting livestock. Changes in these factors are recognized as a potential hazard in livestock growth and production [4] and thus, seasonal variations in these factors are therefore undeniable physiological stressors which affect the animal's biological systems.

High-producing animals tend to have reduced productivity when subjected to greater influence by climatic factors, particularly those raised under tropical conditions, due to high air temperature and relative humidity [11]. Some authors also asserted that high temperatures may reduce feed intake, lower milk production, leading to energy deficits that may lower fertility, fitness and longevity in animal [12]. Research findings by [13,14], indicated that effective environmental temperature above 30°C activates the stress response system in lactating goats and in response goats reduces intake of feed which is directly associated with negative energy balance, which is largely responsible for the decline in milk synthesis. However, the findings by [15] concluded that goat breeds differ in capability to tolerate heat. Evaluation of goat milk yield and composition with respect to ambient temperature and relative humidity on West African Dwarf does is practically non-existent in literature. Therefore, this study was designed to investigate the influence of ambient temperature, relative humidity and relative-humidity index on the milk yield and milk composition of West African Dwarf does.

Materials and Methods

Ethical Approval

All the handling of the goats conforms to the guidelines of Ethical Review Committee of University of Ilorin, Nigeria. Ethical approval to undertake the research was given by the Ethical Review Committee of University of Ilorin, Nigeria. The protocol identification Code and Ethical Approval Number assigned to the research are UERC/AGR/174 and UERC/ASN/2020/2039 respectively.

Experimental Site

This research was carried out at the Small Ruminant Unit of the Teaching and Research Farm, University of Ilorin, Nigeria. The ambient temperature of the site ranges from 19.00°C to 42°C while its relative humidity ranges from 21-83% depending on the period of the year.

Animals and Management

This research was conducted using thirty-six (n=36) apparently healthy lactating West African Dwarf (WAD) does. The does were free of mastitis and other physiological disorder. All the does were fed *ad-libitum* with the same ration *(Table 1)*, compounded to satisfy the nutrient requirement of lactating does [16]. The experiment lasted for a period of twelve weeks. The does were managed within housing units that permit ample exercise and limited degree of freedom which was strictly in line with guideline of University of Ilorin ethical review committee. The milking of each of the does commenced at five-day post-paturm and for a period of twelve weeks. The records of milk yield, ambient temperature and relative humidity were taken at every milking period.

Table 1. *Composition of diets fed to experimental goats*

Feed Ingredient	Percentage (%)
Wheat Bran	25
Palm Kernel Cake	15
Groundnut Cake	5
Rice Bran	5
Corn Bran	10.5
Maize	20
Cassava Peel	19
Salt	0.5
Estimated TDN	59.09
Estimated CP	12.23

Experimental Design and Data Collection

This experiment was laid out in a Completely Randomized Design (CRD). The does were milked twice daily (morning: 07.00 am - 09.00 am; evening: 05.00 am - 07.00 am) for a period of twelve weeks. Hand stripping was done in order to stimulate milk let down before the use of the milking machine, which has a calibrated cylinder for measuring the milk yield of each doe. Milk samples from the does were taken to the laboratory for milk composition analysis using a milk analyzer (Milch analyser, Ultrascan 3100, count: 1288, SN: 28214 PC151). A digital thermo-hygrometer placed within the housing unitswas used to monitor the ambient temperature (AT) and relative humidity (RH) twice per day during every milking process. The recorded morning and evening AT and RH values were averaged to obtain daily day AT and RH during the period of the experiment. These values were categorized as low, medium and high. The AT ranges for each of the categories are: low (17.5-25.6); medium (25.7-33.8) and; High (33.9-42.0) while the ranges for the RH are: low (10-39%); medium (40-69%); and high (70-99%). The sample sizes being the total number of animals whose data collection period fell within the stated categories of AT and RH throughout the period of the experiment were: Low (n=31), Medium (n=27), and High (n=26) for AT, while that of RH are; Low (n=27), Medium (n=28), and High (n=29). Collected data on AT and RH were used to calculate temperature-humidity index (THI) as reported [17].

THI = 0.8×AT+RH%× (AT-14.4) + 46.4

where, AT = air temperature in °C; RH = relative humidity in %.

The THI values were also categorized as: low (65.5-75.50; medium (75.6-85.6) and; high (85.7-95.7). The sample sizes being the number of animals observed within each THI categories were: Low (n=28), Medium (n=26) and High (n=30).

Statistical Analysis

The three categories of each of AT, RH and THI (low, medium and high) were treated as independent variables while the corresponding values of milk yield and milk composition were considered as dependent variable. The data obtained were tested for normal distribution using DATAtab statistic calculator. The parameter whose data that did not follow normal distribution were transformed prior analysis of variance using Minitab Statistical package Version 17. Least Significant Difference Test was used for post hoc.

Results

The influence of ambient temperature on daily milk yield (DMY) and milk composition of WAD does is presented in *Table 2*. A significant difference (P<0.05) was observed in the DMY of the multiparous WAD does at different ranges of ambient temperature (AT). The DMY when AT was medium (350.28 mL/day) as well as low (329.43 mL/day) were not significantly different from each other but were significantly higher (P<0.05) than 210.56 mL/day obtained when AT was within high range. All the milk compositions evaluated were not affected by ambient temperature except milk protein. The average milk protein of the does during the days within high AT (4.72%) was significantly higher (P<0.05) compare to when AT was either within low or medium range. The milk produced during the days with medium and low AT had comparable milk protein of 3.83 and 3.85% respectively.

The influence of relative humidity (RH) on the milk yield and milk composition of WAD does is presented in *Table 3*. The DMY of the multiparous WAD does was significantly (P<0.05) influenced by RH with the highest yield (364.30 mL/day) being produced when the RH of the day was high. The yield during moderate RH (332.18 mL/day) was also significantly higher than 257.48 mL/day produced during the day with low RH range. The milk fat obtained when the RH was on the high side was also significantly lower compare to when RH was low or medium. All other milk compositions were not affected by RH. The SNF, density, FP, protein, lactose and salt content of the milk showed no significant difference (P>0.05) at the different levels of relative humidity.

The influence of temperature-humidity index (THI) on milk yield and milk composition of lactating WAD goats is presented in *Table 4*. Significant differences (P<0.05) were observed in the milk yield of the does and, in the fat, density and lactose content of the milk at the three THI categories (low, medium and high). The highest milk yield (324.35 mL) and milk density (1034.94 kg/m^3) were obtained when the THI range was medium. These values were significantly higher than the corresponding values obtained at low and high THI (MY: 246.30 mL vs 205.00 mL; Density: 1032.38 vs1030.10). The milk fat and milk density during the days that exhibited medium and high THI were not significantly different from each other. A significantly higher milk fat, and lower milk density was however recorded during the day with low THI. The milk lactose recorded during high THI days (11.30%) was significantly higher than 5.66 and 6.07% recorded for low and moderate THI days respectively. No significant difference (P>0.05) was observed between classes of THI with respect to SNF, FP, protein and salt content of the milk.

Table 2. *Influence of ambient temperature on average milk yield and milk composition of West African Dwarf does*

Milk Parameters	Ambient Temperature			SEM	P-Value
	Low (n =31)	Medium (n =27)	High (n =26)		
DMY (mL)	350.28^a	329.43^a	210.56^b	22.45	0.001
Fat (%)	7.09	6.95	7.76	0.85	0.114
SNF (%)	11.03	10.94	10.65	0.19	0.955
Density (kg/m^3)	1033.19	1034.30	1034.15	1.15	0.167
FP (°C)	-0.68	-0.50	-0.73	0.29	0.692
Protein (%)	3.83^b	3.86^b	4.72^a	0.54	0.018
Lactose (%)	5.68	6.05	7.05	0.39	0.131
Salt (%)	0.86	0.90	0.87	0.06	0.442

^{a,b} *Means with different superscript in the same row are significantly P<0.05 different from one another. DMY: Daily Milk Yield; SNF: Solid Non-Fat; FP: Freezing point*

Table 3. *Influence of relative humidity on milk yield and milk composition of lactating West African Dwarf goats*

Milk Parameters	Relative Humidity			SEM	P-Value
	Low (n = 27)	Medium (n=28)	High (n=29)		
DMY (mL)	257.48[c]	332.18[b]	364.30[a]	25.55	0.000
Fat (%)	7.34[a]	7.33[a]	6.17[b]	0.98	0.000
SNF (%)	10.46	11.57	10.38	1.34	0.300
Density (kg/m^3)	1034.17	1033.42	1034.44	0.79	0.228
FP (°C)	-0.72	-0.69	-0.36	0.39	0.345
Protein (%)	4.32	3.87	3.82	0.57	0.146
Lactose (%)	6.46	5.77	6.16	0.72	0.397
Salt (%)	0.87	0.87	0.90	0.06	0.561

[a,b,c] *Means with different superscript in the same row are significantly P<0.05 different from one another. DMY: Daily Milk Yield; SNF: Solid Non-Fat; Fp: Freezing point*

Table 4. *Influence of Temperature-humidity index on milk yield and milk composition of West African Dwarf does*

Milk Parameters	Temperature-Humidity Index			SEM	P-Value
	Low (n = 28)	Medium (n=26)	High (n=30)		
DMY (mL)	246.30[b]	324.35[a]	205.00[c]	23.45	0.000
Fat (%)	7.55[a]	6.78[b]	7.13[b]	0.38	0.009
SNF (%)	11.41	10.62	10.77	0.82	0.590
Density (kg/m^3)	1032.38[b]	1034.94[a]	1030.01[c]	2.36	0.000
FP (°C)	-0.68	-0.52	-0.726	0.32	0.772
Protein (%)	3.80	4.05	4.00	0.27	0.482
Lactose (%)	5.66[b]	6.07[b]	11.30[a]	3.23	0.000
Salt (%)	0.86	0.89	0.90	0.08	0.373

[a,b,c] *Means with different superscript in the same row are significantly P<0.05 different from one another. DMY: Daily Milk Yield; SNF: Solid Non-Fat; FP: Freezing point*

Discussion

Variation in adaptability to fluctuations in weather and other environmental factors is one of the primary factor responsible for global distribution of species and breeds of livestock [18-20]. Despite being reported to be adaptable to a vast weather condition, some specific breeds of goat are strictly predominant in few regions than others. Thus, definition of thermo-comfort zone using temperature range subsists. However, discrepancies exist in the reported range, as some researchers [21,22] concluded that goats in the hot climate are liable to perform excellently between (12-24°C) while other researchers [23] indicated between 20 and 30°C. The current result, however, disagrees with the thermo-comfort temperature specified for goat by these authors, with the comparable yield (350.28 mL and 329.43 mL) obtained at low (17.5-25.6) and medium (25.7-33.8) ambient temperature ranges focused in the current study. This may suggest breed variation in adaptability to AT, which was also reported by some authors [24,25]. A marked reduction in the milk yield at AT of between 33.9 and 42.0°C suggests inhibition of production capacity and thermo-regulatory compensation for energy balance [26]. The milk yield obtained during low and medium AT in the current study are of close range with 3339.20mls reported for white WAD goat [27].

The present result also corroborates with the report in which the production and quality of goat's milk were indicated to be directly related to climatic variations and the combined action of these factors [23]. The significant effect of AT on protein, RH on fat and THI on fat, density and lactose reveals that synergy exist between AT and RH. However, the present study contradicts [28] who reported that stressful environmental conditions may not alter the composition of goat milk if they are well adapted to their environment. The trends of THI, RH and AT impacts on milk fat, protein and other compositions in the current study may explain the complexities in the thermo-regulatory mechanisms in goat and thus reaffirm the statement that environmental effects on milk composition is not conclusive [26]. A similar increase in milk protein but not other milk compositions, as observed in the

current study, was also reported [26] in goat during period of high AT while some previous studies [10,29,30] reported a contrary result. Reduction in milk yield at the expense of maintaining milk fat at high AT and, increased milk protein at low RH, as well as high lactose when THI was high substantiates the influence of these environmental factors on metabolic changes and hormonal reactions [24]. In contrary to some authors [31] who noted that goats are more comfortable in environment where average RH is 65%, the highest milk in the current study was recorded when RH was between 70-99%. This might suggest breed variation in adaptability to weather factors [15].

Defining heat stress using THI in goat has been discussed by several authors [32,33]. There is, however, a clear demarcation of discomfort levels across breeds. This is equally noticed in the current study with WAD goat producing significantly higher milk yield at THI higher than 79 which was reported [34] to be a dangerous level for Saanen goats managed in Brazil. The result in the current study aligns with the report by some authors [35] which indicated in good performance in Anglo-Nubian goat at THI of 83.00 denoting better adaptability of these breeds to tropical regions. A relatively high milk yield between the THI of 75.6 and 85.6 in the present study portrays a disagreement with the earlier findings [36] which noted 80-85 THI as a dangerous level. However, a marked reduction when THI is between 85.7 and 95.7 may be an indication that the WAD goats are heat-stressed within this range [29]. The effect of heat stress on milk fat content is contentious. Some authors reported a negatively correlated effects [37,38] while [39] established no relationship. The present result, however, agrees with [40], who found lower values of milk fat content when the temperature-humidity index was higher than 75. Similarly, earlier findings [39,40] found milk lactose content to be non-significantly between dairy animals maintained at temperature-humidity index <75 and those maintained at temperature-humidity index >75 but, the current study is not in line with these two reports as the lactose content recorded for high (85.7-95.7) temperature-humidity index was significantly different ($P<0.05$) from the lactose content recorded for low (65.5-75.5) and moderate (75.6-85.6) temperature-humidity index. Alteration in milk protein reported by these authors as temperature humidity index changes was also not observed in the current study. All contradictions might be as a result in difference in species, breed, nutrition and other environmental factors. The response of the goats in relation THI result in the current study aligns with the report that there is higher tendency of better performance at THI of between 65-72 [30].

It is concluded that changes ambient temperature, relative humidity and temperature-humidity influence milk yield in a non-uniform trend while only THI affects more milk compositions than AT and RH. It is also concluded that WAD goat for dairy purpose perform optimally at AT of 17.5 to 33.8°C, RH of 70-99%, and THI of between75.6 and 85.6.

Declarations

Availability of Data and Materials: The data that supports the findings of this study are available on request from the corresponding author (A. T. Yusuff).

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Research Article

Effects of Dietary Supplementation with *Clostridium butyricum* on Rumen Fermentation, Rumen Microbiota and Feces in Beef Cattle

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Abstract

This study investigated how *Clostridium butyricum* affected rumen fermentation and the microbial communities of rumen and feces in beef cattle. Twenty beef cattle were divided into two groups: the control group (CK) and the *C. butyricum* group (CB, fed 2.5 x 10^8 CFU/kg of dry matter intake per day). The results showed that *C. butyricum* increased rumen pH, ammonia-N concentration, and microbial crude protein (MCP) concentration (P<0.05). Ruminal propionate and butyrate concentration increased, while the ruminal acetate to propionate ratio decreased (P <0.05). For rumen microbiota, observed species, Chao 1, and ACE indices were higher (P<0.05) with supplemented *C. butyricum*. At the phyla level, the *C. butyricum* enhanced the proportion of Firmicutes and decreased Bacteroidota (P<0.01). *Christensenellaceae* R-7 group, *Methanobrevibacter, Oscillospiraceae* NK4A214 group, *Desulfovibrio, Streptococcus,* and *C. butyricum* were increased (P<0.05) at the genus and species levels in the CB group. The proportion of *Prevotella*, *Christensenellaceae* R-7 group, *Blautia*, and *Megasphaera elsdenii* increased, while *Escherichia coli* decreased (P<0.05) in feces. *E. coli and Salmonella* populations were significantly reduced (P<0.01). These results indicated that diets supplemented with *C. butyricum* could improve rumen fermentation by increasing the diversity and altering the microbial community structure of the rumen. Additionally, the supplemented *C. butyricum* changed the fecal microbiota and decreased the harmful bacteria population in beef cattle.

Keywords: *Clostridium butyricum*, Fecal *E. coli*, Fecal microbiota, Rumen fermentation, Rumen microbiota, Microbial population

Introduction

Ruminants have many microorganisms in their gastrointestinal tracts. They are crucial for animal health processes, such as digesting nutrients and mediating animal immune and physiological responses [1]. The rumen microorganisms enable ruminants to use energy stored in plant material through complex interactions [2]. Changing the rumen microbial composition can affect the energy-harvesting ability, health, and rumen function of ruminants [3]. A typical and stable microbial community is an essential guarantee of ruminant health, playing vital roles in promoting the development of gastrointestinal morphology and structure, maintaining normal immune function, resistance to exogenous pathogenic factors, and so on. The microbial composition in the gastrointestinal tracts is fundamental because it can affect production performance and animal health [4]. Understanding the relationships between microbial communities in the gastrointestinal tracts and the ruminant animal has been shown to provide essential animal benefits. It has been reported that dietary-supplemented probiotics can improve productive performance by altering gastrointestinal bacterial communities in ruminants [5].

Clostridium butyrium, a strictly anaerobic bacterium, is a gram-positive bacteria that can form endospores. Additionally, *C. butyrium* can tolerate complex environments in the gastrointestinal tracts of ruminants compared with *Lactobacillus* and *Bifidobacterium* [6]. Therefore, *C. butyricum* belongs to typical intestinal microorganisms and is used in feed additives. Furthermore, extensive

research studies have confirmed that *C. butyricum* could enhance intestinal health and function in weaned piglets [7] and chickens [8]. Additionally, diets supplemented with *C. butyricum* can improve the short-chain fatty acids (SCFAs) content, the microbial diversity of gastrointestinal tracts, and production performance in Pekin ducks [9]. Additionally, *C. butyricum* produces lipoteichoic acid, SCFAs, hydrogen, and bacteriocin, helping to enhance the anti-oxidant and anti-bacterial functions of the intestines of animals [10]. Probiotic effect of *C. butyricum* has been demonstrated in monogastric animals, but few studies have been revealed in ruminants. Ruminants have a complex digestive system, and diet digestion occurs initially in the rumen. Rumen microorganisms break down diet components such as carbohydrates, plant fiber, and proteins, producing short-chain fatty acids. Thus, we hypothesized that dietary supplements with *C. butyricum* would affect rumen microbiota and rumen fermentation function of beef cattle. In addition, rumen microorganisms have attracted considerable attention in ruminant nutrition [5], but limited attention has been focused on the hindgut microorganisms. Therefore, this research examined how *C. butyricum* affected rumen fermentation and the microbial communities of rumen and feces.

Material and Methods

Ethical Approval

The Institutional Animal Care and Use Committee of Northwest A&F University (NWAFAC1008) approved this animal study.

Animals, Experimental Design, and Feeding Management

This animal experiment was conducted at a beef cattle breeding base in Guangdong VTR Bio-Tech Co., Ltd. (Zhuhai, China). Twenty beef steers (500±34 kg) were divided into two groups according to body weight, with 10 beef cattle in an open-sided house. Beef steers in the control group (CK) were fed a basal diet, and the experimental group (CB) was fed a basal diet with 2.5 x 10^8 CFU/kg *C. butyricum* of dry matter intake, respectively. *C. butyricum* was deposited in the Guangdong Microbial Culture Collection Center (GDMCC) and provided by Guangdong VTR Bio-Tech Co., Ltd. The deposition number was GDMCC NO: 61311. The experimental period lasted 40 days. The basal diet was designed to meet the requirements for the growth of beef cattle based on the Feeding Standard of Beef Cattle (NY/Y 815-2004). Beef cattle were fed twice daily at 7:30 and 14:30 and allowed free access to water.

Collection of Samples

On the last days of the trial (day 40), a flexible oral stomach tube (the Laboratory of the Chinese University of Agriculture, Beijing, China) was used to collect rumen samples at 3, 6, and 9 h after feeding [11]. The first 50 mL of rumen samples were discarded to minimize contamination with saliva. Rumen samples from each beef cattle were homogenized and filtered using four layers of gauze to obtain rumen liquids [12]. The rumen liquid was immediately used to determine pH and stored at -20°C freezer for determining rumen fermentation parameters. Approximately 2 mL of rumen samples were placed in a sterile frozen tube, immediately frozen in liquid nitrogen, and stored at -80°C freezer for microbial community analysis. On the same day, rectal fecal samples were collected 4 h after feeding, placed in a sterile frozen tube, and stored at -80°C freezer for further analysis of the fecal community [13]. Approximately 20 g of fresh fecal samples were used to determine the microbial population.

Measurements of Rumen Fermentation Parameters

The pH value was obtained via a PHS-3C pH meter (INESA Scientific Instruments, Shanghai, China) after the collection of the rumen fluid. Then, the sample was centrifuged at 10.000 x g for 20 min, and an aliquot (3 mL) of supernatants was used to determine the contents of ammonia-N (NH_3-N), microbial crude protein (MCP), acetic acid, propionic acid, and butyric acid. First, the MCP content was measured using the spectrophotometric method [14]. Next, NH_3-N concentration was determined using the phenol/hypochlorite method [15]. Finally, the concentrations of acetic, propionic, and butyric acids were determined using gas chromatography (Agilent GC 8860, Agilent Company, US) [16]. Briefly, an aliquot (0.1 mL) of supernatants was added to 0.8 mL 25% (w/v) metaphosphoric acid. Then, the supernatant sample was injected into a silica column of GC after centrifuging at 12.000 × g for 20 min.

Microbial Community Analysis

Rumen and fecal samples were used to determine bacterial flora in the digestive tract and investigate microbial community changes after dietary supplementation with *C. butyricum*. First, total DNA was extracted from the rumen and fecal samples using the sodium dodecyl sulfate (SDS) method [17]. Subsequently, the integrity and concentration of the DNA were verified using 0.7% agarose gel electrophoresis. The distinct V3-V4 regions of 16S rRNA were sequenced on a sequencing platform (Novaseq6000, Novogene Technology Company, China). The raw sequence data were obtained after sequencing and stored as fastq format [11]. The sequence data were filtered to remove barcodes and primers. Then, the sequence data were spliced using Fast Length Adjustment of Short Reads (FLASH; Version 1.2.7, *http://ccb.jhu.edu/software/FLASH/)* according to Quantitative Insights Into Microbial Ecology (QIIME; Version 1.9.1, *http://*

qiime.org/scripts/split_libraries_fastq.html) process [18]. After quality filtering, the effective tags were assembled to obtain operational Taxonomic Units (OTUs) at a 97% similarity level by the clustering method of the UPARSE-OTU algorithm (Version 7.0.1001; *http://www.drive5.com/uparse/)* and relative abundance information. Based on the OTU results, alpha diversity analysis was obtained using QIIME [19]. Online repositories *(https://www.ncbi.nlm.nih.gov/PRJNA852290)* contained the datasets.

Microbial Population Analysis

The populations of *Escherichia coli*, *C. butyricum*, and *Salmonella* were measured using the spread-plate method. Briefly, samples (20 g) were homogenized in sterile water (180 mL) and shaken at room temperature for 20 min. The colonies were counted after inoculating serial dilutions on agar plates and spreading them evenly [19]. *E. coli* was incubated on MacConkey agar at 37°C for 24 h. *C. butyricum* was incubated using a reinforced medium for *Clostridia* agar at 37°C under anaerobic conditions for 18 h. *Salmonella* was incubated using Selenite Cystine Broth agar for 24 h.

Statistical Analysis

A one-way analysis of variance (ANOVA) was used to analyze all data based on a completely randomized using SPSS Statistics (version 22.0; IBM Corp., Armonk, N. Y., USA) general linear model procedure. Replications were considered experimental units. A P-value <0.05 was defined as significant, and a P-value <0.01 as extremely significant.

Results

Rumen Fermentation Parameters

The results of ruminal pH, ammonia-N, MCP, acetate, propionate, and butyrate concentrations are shown in *Table 1*. There was a tendency to an increase in rumen pH (P=0.078) by adding *C. butyricum*. The contents of ammonia-N and MCP in the CB group were higher (P<0.05) than those of the CK group. Propionate and butyrate concentrations increased (P<0.05) by adding *C. butyricum*. At the same time, the acetate-to-propionate ratio decreased (P<0.05). The acetate concentrations did not differ in the two groups.

Rumen Microbiota

These OTUs of the rumen sample that are shared and unique among the two groups are shown in *Fig. 1*. An evaluation of the distribution of OTUs was conducted using the Venn and Flower diagrams. In total, 2521 OTUs were clustered. Of the 1.952 common OTUs, 202 and 367 were unique to the CK and CB groups. The population of OTUs was more enhanced than in the CK group (2.319 vs. 2.154). Alpha diversity can reflect the species richness and diversity of the microbial community. The alpha diversity indices of the rumen samples in the two groups are given in *Table 2*. The observed species, Chao 1, and ACE indices were increased (P<0.05) with added *C. butyricum*. There were no effects on the Shannon, Simpson, and PD-whole-tree indices in the two experimental groups with added *C. butyricum*. Our results indicated that dietary added *C. butyricum* positively affected rumen microbial structure.

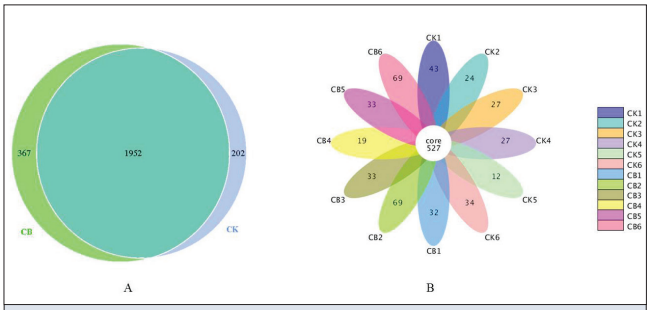

Fig 1. Venn analysis of operational taxonnmic units (OTUs) of rumen sample. (A), Each circle represented a group. The common OTUs were showed in the overlapping part, while the numbers in the non-overlapping part represent unique OTUs in each group. (B), Each petal represented a sample, while different colors represented different samples. The numbers of common OTUs were showed in the overlapping part. CK, the control group; CB, the *C. butyricum* group

Table 1. *Ruminal fermentation parameters of beef cattles with C.butyricum supplementation*

Item	Treatment		SEM	P-value
	CK	CB		
pH	6.58	6.65	0.021	0.078
Ammonia-N (mg/100 mL)	8.01^{b}	8.18^{a}	0.043	0.034
MCP (mg/mL)	6.11^{b}	6.22^{a}	0.190	0.001
Acetate (mmol/L)	54.36	56.89	1.432	0.401
Propionate (mmol/L)	15.14^{b}	19.89^{a}	1.117	0.025
Butyrate (mmol/L)	9.68^{b}	13.32^{a}	0.955	0.050
Acetate/propionate	3.73^{a}	2.88^{b}	0.208	0.033

CK, the control group; CB, the C. butyricum group; SEM, standard error of the mean; MCP, microbial crude protein

Table 2. *OTUs number of alpha diversity indices of microbial community of rumen*

Items	Observed-Species	Shannon	Simpson	Chao1	ACE	Goods-Coverage	PD-Whole-Tree
CK	1287[b]	7.915	0.986	1396.8[b]	1404.1[b]	0.995	94.919
CB	1403[a]	7.892	0.982	1512.2[a]	1516.4[a]	0.995	99.476
SEM	29.543	0.0997	0.002	28.029	27.373	0.0002	2.283
P	0.044	0.916	0.373	0.031	0.032	0.664	0.342

CK, the control group; CB, the C. butyricum group. SEM, standard error of the mean

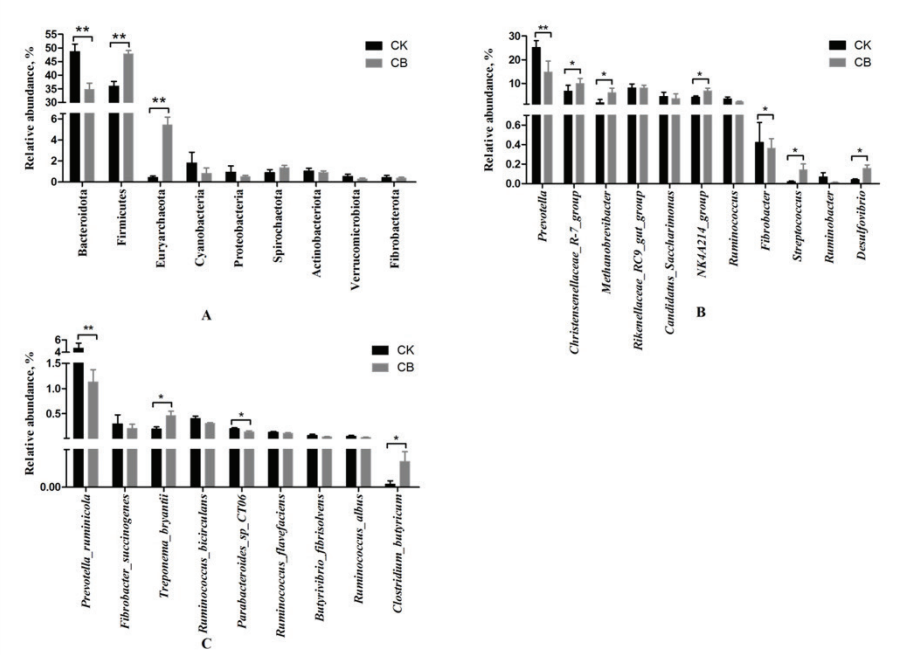

Fig 2. The relative abundances of rumen microbiota composition at the phylum level (A), genus (B), and the species level (C). CK, the control group; CB, the *C. butyricum* group

The proportion of ruminal microbiota is shown in *Fig. 2*. At the phyla level, a diet supplemented with *C. butyricum* had an enhanced proportion of Firmicutes (36.0 vs. 47.9; P<0.001), Euryarchaeota (0.43 vs. 5.44; P<0.001), and decreased the proportion of Bacteroidota (48.7 vs. 34.9; P=0.003). In addition, dietary supplemented with *C. butyricum* enhanced the proportion of *Christensenellaceae* R-7 group (4.08 vs. 9.94; P=0.026), *Methanobrevibacter* (0.48 vs. 3.88; P=0.019), *Oscillospiraceae* NK4A214 group (4.18 vs. 6.92; P=0.043), *Desulfovibrio* (0.04 vs. 0.16; P=0.010), *Streptococcus* (0.02 vs. 0.15; P=0.042), and reduced the proportion of *Prevotella* (27.2 vs. 11.2; P<0.001), and *Fibrobacter* (0.21 vs. 0.42; P=0.038) at the genus level. At the species level, dietary supplemented with *C. butyricum* enhanced the proportion of *Treponema bryanti* (0.20 vs. 0.46; P=0.022), *C. butyricum* (0.000 vs. 0.003; P=0.022), and decreased *Prevotella ruminicola* (4.67 vs. 1.13; P=0.002), *Parabacteroides* sp._CT06 (0.20 vs. 0.14; P=0.039). On the other hand, *Ruminococcus bicirculans* tended to decrease with *C. butyricum* supplementation (0.40 vs. 0.31; P=0.093).

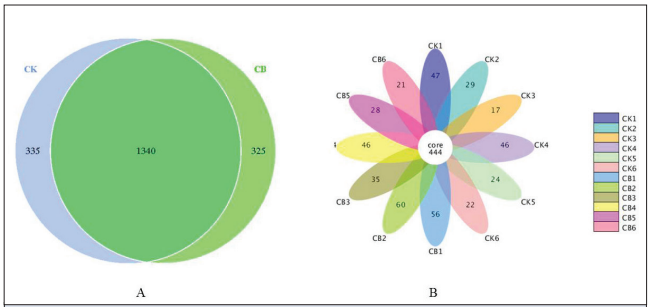

Fig 3. Venn analysis of operational taxonomic units (OTUs) of fecal sample. (A), Each circle represented a group. The common OTUs were showed in the overlapping part, while the numbers in the non-overlapping part represent unique OTUs in each group. (B), Each petal represented a sample, while different colors represented different samples. The numbers of common OTUs were showed in the overlapping part. CK, the control group; CB, the *C. butyricum* group

Fecal Microbiota

The shared and unique OTUs of the fecal samples among the two groups are illustrated in *Fig. 3*. An evaluation of the distribution of OTUs was carried out using the Venn and Flower diagrams. In total, 2000 OTUs were clustered.

Table 3. OTUs number of alpha diversity indices of microbial community in feces							
Items	**Observed-Species**	**Shannon**	**Simpson**	**Chao1**	**ACE**	**Goods-Coverage**	**PD-Whole-Tree**
CK	1011[b]	7.683[b]	0.989	1093.06	1091	0.997	71.103
CB	1083[a]	7.897[a]	0.989	1137.85	1145	0.997	79.374
SEM	16.249	0.053	0.0005	15.429	15.95	0.0001	2.310
P	0.016	0.035	0.640	0.155	0.088	1.000	0.070
CK, the control group; CB, the C. butyricum group. SEM, standard error of the mean							

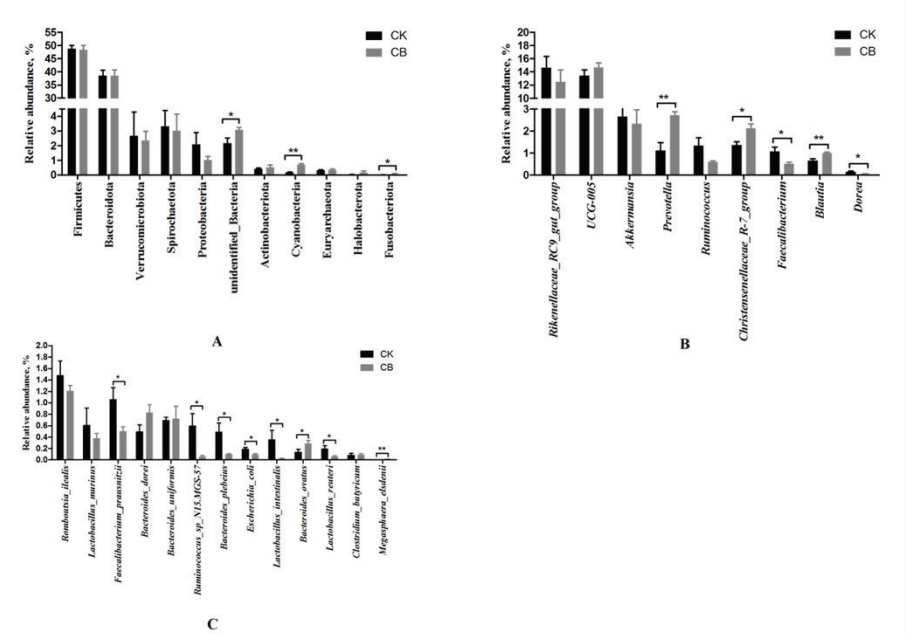

Fig 4. The relative abundances of fecal microbiota composition at the phylum level (A), genus (B), and the species level (C). CK, the control group; CB, the *C. butyricum* group

Table 4. Effects of C. butyricum on the microbial population of rumen and fecal sample				
Items	**Rumen *C. butyricum***	**Feces**		
		C. butyricum	***E. coli***	***Salmonella***
CK	1.22[b]	3.18	4.84[a]	2.96[a]
CB	5.54[a]	2.98	3.76[b]	1.74[b]
SEM	0.653	0.102	0.185	0.210
P	<0.001	0.332	<0.001	<0.001
Bacterial number is expressed as Log_{10} colony forming units per fecal contents. CK, the control group; CB, the C. butyricum group. SEM, standard error of the mean				

Of the 1340 common OTUs, 335 and 325 were unique to the CK and CB groups. The alpha diversity indices of the fecal samples in the two groups are given in *Table 3*. The observed species and Shannon indices were higher (P<0.05) than the CK group. In addition, ACE and PD-whole-tree indices increased with added *C. butyricum*. However, the two groups did not differ in Simpson, Chao 1, or goods-coverage indices.

The microbial relative proportions of the fecal sample are shown in *Fig. 4*. At the phyla level, dietary supplemented with *C. butyricum* led to an enhanced relative proportion of Cyanobacteria (0.14 vs. 0.69; P<0.001), Unidentified Bacteria (2.16 vs. 3.07; P=0.046), and Fusobacteriota (0.006 vs. 0.079; P=0.023). At the genus level, dietary supplemented with *C. butyricum* enhanced the proportion of *Prevotella* (1.11 vs. 2.72; P=0.002), *Christensenellaceae* R-7 group (1.36 vs. 2.12; P=0.014), *Blautia* (0.65 vs. 0.99; P=0.007) and reduced the proportion of *Faecalibacterium* (1.07 vs. 0.50; P=0.031), *Dorea* (0.14 vs. 0.05; P=0.050). *Ruminococcus* decreased in the CB group compared to the

CK group (1.33 vs. 0.59; P=0.070). At the species level, dietary added *C. butyricum* enhanced the proportion of *M. elsdenii* (0.002 vs. 0.013; P=0.003) and decreased the proportion of *Faecalibacterium prausnitzii* (1.06 vs. 0.50; P=0.033), *Ruminococcus* sp._N15.MGS-57 (0.60 vs. 0.06; P=0.029), *Bacteroides plebeius* (0.49 vs. 0.10; P=0.034), *Lactobacillus reuteri* (0.20 vs. 0.06; P=0.031), *Lactobacillus intestinalis* (0.42 vs. 0.02; P=0.033) and *E. coli* (0.19 vs. 0.10; P=0.005).

Microbial Rumen Population and Fecal Samples

The microbial rumen population and fecal samples are shown in *Table 4*. The *C. butyricum* rumen population in the CB group increased (P<0.001), while the *E. coli* and *Salmonella* feces populations decreased significantly (P<0.001). There were no observed effects on the *C. butyricum* population in feces with dietary *C. butyricum* supplementation.

Discussion

Ruminal fermentation parameter includes a range of indicators and can reflect the function and health status of the rumen. Ruminal pH is mainly related to the dietary digestion rate, especially the degradation rate of concentrate grains. Therefore, it is a critical fermentation parameter for the rumen environment and function [20]. It has been reported that added probiotics resulted in a high ruminal pH in cows [15]. Cai et al. reported that dietary supplemented with *C. butyricum* increased ruminal pH in goats [16]. Our results showed that ruminal pH increased with added *C. butyricum*, potentially associated with the rising proportion of bacteria that utilize lactic acid [15]. The result indicated that added *C. butyricum* could stabilize the ruminal pH as other probiotics.

Furthermore, the appropriate ammonia-N concentration can provide a nitrogen source for microorganisms and promote the synthesis of MCP [21]. The principal fermentation products of rumen are acetate, propionate, and butyrate, which account for as much as 70% of the overall metabolizable energy provision in ruminants [22]. A previous study reported that added *C. butyricum* enhanced ruminal NH_3-N concentration but did not affect the MCP concentration in goats [16]. In our research, adding *C. butyricum* improved the concentration of NH_3-N, propionate, and butyrate but decreased the acetate-to-propionate ratio in beef cattle. Our results indicated that adding *C. butyricum* enhanced ruminal protein and energy supply and positively affected rumen fermentation. Ruminal fermentation parameters can reflect the situation of the dietary digestion in the rumen and are closely related to ruminal microorganisms [2]. Therefore, we analyzed the ruminal microbial flora to find out why *C. butyricum* affected rumen fermentation.

Rumen microbiota is a vital factor in immune function and the efficiency of nutrient digestion. There is a large number of microorganisms in the gastrointestinal tracts of ruminants. The degradation of nutrients by ruminal microorganisms produces acetate, propionate, butyrate, and MCP, which supply energy and protein for ruminants. Therefore, determining the function of rumen microbiota is essential for understanding their role in animal metabolism [4]. In the research, adding *C. butyricum* enhanced the number of OTUs and the observed species, Chao 1, and ACE indices. The results indicated that *C. butyricum* positively affected microbial diversity, consistent with previous research [23].

Bacteroidota and Firmicutes were the most abundant bacteria in the rumen [24]. Many microorganisms belonging to Firmicutes can degrade fiber from dietary compounds and produce SCFAs [24]. The Firmicutes to Bacteroidota (F/B) ratio is a valuable indicator of the ability to absorb and store energy [25]. Our results indicated that added *C. butyricum* enhanced the proportion of Firmicutes but reduced the proportion of Bacteroidota. The F/B ratio was enhanced, which indicated that added *C. butyricum* improved the energy absorption capacity of the rumen microbiota. At the genus level, *Christensenellaceae* R-7 group and *Oscillospiraceae* NK4A214 group were the top two species belonging to Firmicutes. Our results showed that supplemented with *C. butyricum* could enhance the proportion of *Christensenellaceae* R-7 group and *Oscillospiraceae* NK4A214 group, thereby enhancing the relative proportion of *Firmicutes*. Changing the rumen microbial composition can affect the energy-harvesting ability and rumen function of ruminants [14]. The results indicated that added *C. butyricum* changed the ruminal microbial flora structure, affecting the rumen fermentation in beef cattle.

The effects on the fermentation parameters were closely related to the rumen flora structure [14]. Specifically, *Butyrivibrio fibrisolvens*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Fibrobacter succinogenes* belong to cellulolytic bacteria and primarily produce acetate by degrading plant fiber [12]. Our results indicated that supplementation with *C. butyricum* did not affect the proportion of dominant cellulolytic bacteria including *F. succinogenes, B. fibrisolvens*, *R. albus*, and *R. flavefaciens*. Therefore, the acetate concentration showed no difference between the two groups. *Streptococcus* and *Ruminobacter amylophilus* are amylolytic bacteria that produce propionate [25]. *R. bromii* can degrade resistant starch and xylan, while *Ruminococcus* degrades complex deoxy sugars, such as fucose and rhamnose [11]. Our study indicated that the proportion of *Streptococcus* increased. This result was probably why the propionate content in the CB group was higher. *Provotella*, the most abundant

bacterial genus [26], can produce SCFAs by metabolizing dietary fiber [12]. Our study showed that the proportion of *Provotella* decreased with added *C. butyricum*. The low ruminal pH can promote the growth of *Prevotella* [26]. Therefore, the reduced proportion of *Provotella* in the CB group was probably due to the higher rumen pH. The increased rumen pH might be linked to *Desulfovibrio*, a lactate-utilizing bacteria. The proportion of *Desulfovibrio* increased with added *C. butyricum*. The *C. butyricum* also stimulated the development of lactate-fermenting bacteria via outcompeting lactate-producing bacteria for using sugar [25], thereby inhibiting lactate accumulation and increasing ruminal pH.

Methanobrevibacter, an essential member of methanogenic archaea, could produce methane by using H_2 as a substrate to reduce CO_2 [27]. In the study, the proportion of *Methanobrevibacter* was increased, potentially associated with the rising proportion of fiber-degrading bacteria, including carbohydrate-fermenting and H_2-producing bacteria [27]. In addition, *C. butyricum* positively affected production performance and ruminal nutrition digestibility [16]. Therefore, the increased proportion of *Methanobrevibacter* did not cause adverse effects on beef cattle.

Rumen microorganisms have attracted considerable attention, but limited attention has been paid to the hindgut microorganisms of ruminants. Diverse gut microorganisms are prominent in host metabolism, nutrient digestion, growth performance, and overall animal health [17]. Previous studies have reported that diets supplemented with *C. butyricum* could affect the intestinal microbiota by increasing bacterial abundance and diversity [28]. The observed species, Chao 1, and ACE indices increased with *C. butyricum* supplementation. These results showed that added *C. butyricum* enhanced the diversity of fecal microbial communities. The diversity of the intestinal microbiota serves as the foundation for nutrient digestion, intestinal functions, and promoting intestinal immune system development in animals [29]. Zeng et al.[8] found that Firmicutes, Bacteroidota, and Proteobacteria are the dominant phyla in the feces of ruminants. Added *C. butyricum* enhanced the Firmicutes proportion but reduced the Proteobacteria proportion [23]. However, our results showed that the addition of *C. butyricum* improved the abundance of Cyanobacteria and Fusobacteriota without affecting the proportion of Firmicutes, Bacteroidota, and Proteobacteria in feces. This lack of impact on impact on the latter phyla may be attributed to the presence of rumen microbial communities in ruminants.

This research revealed that by adding *C. butyricum* to the diets of the analyzed ruminants, the proportions of *Prevotella* and *M. elsdenii* increased, while the relative proportion of *E. coli* decreased. *Provotella* can decompose hemicellulose and is essential for utilizing non-fibrous polysaccharides [6]. Such an addition improves the production of acetates, propionates, and butyrates with the degradation of starch, xylan, and proteins [30]. The proportion of *Provotella* is predominant in animal feces [31]. *M. elsdenii* is an essential bacteria that converts lactate to acetate, propionate, butyrate, and valerate [32]. A previous study reported that *C. butyricum* could enhance the concentrations of SCFAs in feces [32]. The SCFAs could promote beneficial bacteria proliferation and inhibit the harmful bacteria *E. coli*, possibly due to the reduced pH [33]. Adding *C. butyricum* to diets reduced the proportion of *E. coli* and regulated the intestinal microbial structure by enhancing the amino acid metabolism and recombining proteins related to microbiota [34].

Broilers fed with *C. butyricum* reduced the *E. coli* and enhanced *Bifidobacterium* and *Lactobacillus* populations [34]. Zhang et al.[32] observed that *C. butyricum* could benefit the gut ecosystem by increasing the *Lactobacillus* population and reducing the counts of *C. perfringens*. The microbial composition in the gastrointestinal tracts of ruminants is fundamental because it can affect production performance and animal health. The results indicated that added *C. butyricum* reduced the harmful bacteria *E. coli* and *Salmonella* population compared with the CK group. This result may be attributed to the ability of *C. butyricum* to produce various beneficial materials and compete with pathogens for nutrition and attachment sites, thereby inhibiting the growth of harmful bacteria [35]. In this experiment, beef cattle fed *C. butyricum* could decrease the harmful bacteria counts and were beneficial to the gastrointestinal tract and animal health.

Declarations

Availability of Data and Material: The corresponding author can provide the datasets of this research upon reasonable request.

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Competing Interests: There is no conflict of interest between the manuscript's material and any financial organization.

Ethics Statement: The Institutional Animal Care and Use Committee of Northwest A&F University (NWAFAC1008) approved this animal study.

Author Contributions: Conceptualization: J. He, X. Xie, Z. Wu; Data curation: J. He, L. Yu, L. Li; Formal analysis: J. He, G. Zhao; Methodology: G. Zhao, D. Wang; Software: D. Wang, L. Yu; Validation: D. Wang; Investigation: X. Xie; Writing - original draft: J. He; Writing - review & editing: J. He, X. Xie.

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Research Article

Effect of Dietary Inclusion of Alfalfa Polysaccharide on Performance and Immune System Efficacy of Broiler Chickens

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Abstract

This study was conducted to investigate the effect of Alfalfa polysaccharide (APS) on the growth performance and immune responses of broilers. A total of 400 1-day-old Avian broiler chicks were randomly divided into four treatment groups and administered intranasal with different levels of APS (0, 2.5, 5.0 or 10.0 mg/dose) on day 1 and day 14. Growth performance, phagocytic activity, T lymphocyte transformation, and serum antibody titers against the Newcastle disease virus (NDV) vaccine were examined. During the study period of 42 days, no significant differences were observed in food intake among treatment groups (P>0.05). The APS administered groups displayed a low FCR, especially the 2.5 mg groups (P<0.05). Broilers with 2.5 mg APS displayed a much higher phagocytic activity than the control group (P<0.05). At the same time, APS enhanced the T lymphocyte transformation in response to phytohaemagglutinin (PHA). The antibody titer to NDV was not influenced by APS on day 14, while it improved on day 28 and day 42, especially in the 2.5 mg group (P<0.05). And the above data suggest that APS could improve innate and cellular immune responses in broilers.

Keywords: Alfalfa polysaccharide, Broilers, Diseases, Growth, Immunity

Introduction

Polysaccharides are biomolecules existing in higher plants, animal cell membranes, and microbial cell walls [1]. They are important components of all living organisms, serving as both energy source for cell metabolism and structural element of the cell. They are involved in several physiological and pathological processes of the body [2]. Studies have proved that polysaccharides have anti-inflammatory and anti-infective properties, prevent gastrointestinal inflammation, promote the development of immune organs, and enhance the body's immune system [3,4]. However, although polysaccharides have a wide range of functions, they are difficult to produce synthetically on a large scale due to their complex structure, difficult synthesis process, and high production costs. Based on that, naturally extracted plant polysaccharides appear particularly convenient.

Plants polysaccharides can be extracted from a wide range of plant-based sources and have low cost, low toxicity, and lesser side effects, thus overcoming the shortcoming of synthetic polysaccharides. This has attracted the attention of many scholars [3]. So far, a variety of polysaccharides have been isolated from plants. Research has suggested that plant polysaccharides have obvious effects on growth promotion, macrophage phagocytosis, cellular immunity, and humoral immunity of livestock and poultry in a certain dose range [5]. The effectiveness of plant polysaccharides as an immuno-enhancer is influenced by various factors, such as their components and structure, the dosage used, the route of administration, and the species of animal being treated. It has been found that a variety of plant polysaccharides with immune activity, such as Astragalus polysaccharide, can significantly enhance the phagocytic function of macrophages, increase the spleen weight of mice, and have bactericidal, antiviral and anti-infective effects [6,7]; another polysaccharide lentinan can promote the oxidative stress around lung cancer cell tumor [8].

Alfalfa is an important source of legume feed for livestock. It is called the king of forage. It has the effects of clearing heat, detoxicating, and curing jaundice, urinary calculus, and other diseases [9,10]. The alfalfa polysaccharide (APS) is extracted from the stems and leaves of alfalfa. The

extraction process is simple and feasible, and the cost is low. It is suitable for large-scale production and feed supplementation [11]. However, the effect of additive APS in the diet of broiler remains unclear.

In the present research, different doses of APS were administered to 1-day- old broilers to study its effect on growth performance and immune function, including macrophage phagocytosis activity, peripheral lymphocyte transformation and antibody titer. These findings can provide a theoretical reference for the application of APS as an immune-enhancer in the poultry industry.

Material and Methods

Ethical Statement

Experimentation with animals was approved by the Experimental Animal Management Methods of Xinxiang Medical University (Approval number: 201206078) and followed Henan Authority's Experimental Animal Regulations.

Alfalfa Polysaccharide

The APS used in this study was purchased from Shanghai Moqi Biology Co.Ltd. (Shanghai, China). The purity of APS was 95%. The molecular weight of APS was determined by LC-MS and ranged from 8 to 160.000 daltons.

Experimental Animals and Management

A total of 400 1-day-old Avian broiler female chicks (Dayong Broiler Breeding Corp., Henan, China) were randomly assigned into four groups, with 10 replicate pens per group and 10 birds per pen for a 42-day feeding trial. Different dosage of APS (0, 2.5 mg, 5 mg, 10 mg) was dissolved in 50 μL PBS and administered intranasally to broilers on day 1 and day 14.

Maize-soybean-based basal diets without antibiotics were formulated to meet the nutrient requirements as recommended by the National Research Council (1994) during the starter (days 1-21) and finisher (days 22-42) periods. The ingredient compositions of the basal diet are shown in *Table 1*. Body weight gain (BWG) was recorded on day 1 and day 42, and feed intake (FI) was measured over the period of 42 days to calculate the feed conversion ratio (FCR).

All the birds were housed in electrically heated cages and had free access to clean water and feed. Under the lighting program, the broilers were provided light for 23 h for the first 2 weeks, and 20 h afterwards. The birds were kept at a temperature of 33-34°C for the first week. Then, the temperature was decreased by 2-3°C per week till 24°C was attained.

Macrophages Phagocytic Activity Assay

Phagocytic ability was determined by carbon clearance, as proposed by previous study elsewhere [12]. Briefly, two broilers from each cage on day 14 and 28 were randomly selected and injected colloidal carbon (drawing ink, Pelikan; 1 mL/kg BW) intravenously. Two (T1) and ten (T2) minutes after the injection, 200 μL blood was collected from the wing and mixed with 2 mL sodium citrate, which was then centrifuged at 530 g for 5 min. The supernatant was used to measure the optical density (OD) at 600 nm. The phagocytic index was calculated as follows: phagocytic index = body weight/(liver weight + spleen weight) × K1/3. The carbon clearance (K) was calculated as follows: K = (lgOD1-OD2)/T2-T1. OD1 and OD2 were the optical density at 600 nm of the supernatant at T1 and T2, respectively.

***Table 1**. Composition of basal diet*

Composition	0-3 w	4-6 w
Corn (%)	52.71	59.01
Soybean (%)	40.00	33.80
Soybean oil (%)	3.00	3.00
Calcium hydrophosphate (%)	1.90	1.25
mountain flour (%)	1.10	1.80
Salt (%)	0.37	0.37
Trace element premix (%)[a]	0.50	0.50
Multi-vitamin (%)[b]	0.20	0.20
methionine (%)	0.19	0.07
lysine (%)	0.05	0.03
Choline chloride (CC) (50%)	0.20	0.16
Metabolic energy (MJ/Kg)[c]	12.71	12.78
Crude protein (%)[d]	21.37	18.99
Calcium (%)	1.08	1.03
Available phosphorus (%)	0.62	0.55
Lysine (%)	1.25	1.10
methionine (%)	0.54	0.39

[a] *Provided the following per kilogram of diet: Vit. A (β-carotene), 1500 IU; Vit. D, 1250 IU; Vit. E (dl-α-tocoperol), 15 IU; Vit. K, 2.2 mg; Vit. B_1, 1.5 mg; Vit. B_2, 8.0 mg; Vit. B_6, 2.5 mg; Vit. B_{12}, 0.011 mg; niacin, 44.00 mg; D-pantothenic acid, 11.00 mg; folic acid, 0.9 mg; D-biotin, 0.11 mg; choline, 550 mg*
[b] *Fe, 80.00 mg; Cu, 8.00 mg; I, 0.35 mg; Se, 0.15 mg; Zn, 80.00 mg*
[c,d] *measured value*

T Lymphocyte Transformation Rate

A sterile collection of 5 mL blood was performed from chicken hearts on days 14, 28, and 42. Heparin (1 mL) was used for anticoagulation. The blood samples were allowed to stand for 1 h and then 6 mL of lymphocyte separation solution was added (the ratio of blood and separation solution is 1:1). Next, the sample was centrifuged for 20 min at 3000 rpm to absorb the middle cloudy white blood cell layer, washed three times with Hank's solution, and centrifuged again for 10 min at 2000 rpm. The living

cells (trypan blue staining) were resuspended and the number of cells was adjusted to 3 $\times 10^6$ per mL using RPMI1640 culture medium (Gibco, Los Angeles, CA, USA). Thereafter, a certain dose of phytohemagglutinin (PHA) (sigma, St. Louis, MO, USA) was added with a final concentration of 10 μg/mL. After giving a slight shaking, the cells were cultured in a 5% CO_2 incubator at 37°C for 72 h (Thermo, Waltham, MA, USA).

Then, the cells were centrifuged at 1000 rpm for 10 min, and the supernatant was discarded. The precipitate cell (1 mL) was placed on a clean slide and fixed with methanol. After washing with PBS and drying, cells were stained with Giemsa for no less than 3 h. Finally, the number of transformed cells in 200 lymphocytes was observed and counted under the microscope.

Newcastle Disease Virus Antibody Titer

The birds were administrated the Newcastle disease virus (NDV) vaccine (La Sota) at day 1 (intranasal) and day 14 (intramuscular). And on days 14, 28, and 42, 10 broilers were randomly selected from each group. Blood was collected from these chickens and serum was separated by centrifugation. The haemagglutination inhibition (HI) assay was performed using 96-well V-shaped haemagglutination platelets. The titer of antibodies was measured by log2.

Statistical Analysis

Data were reported as means and analyzed by one-way analysis of variance (ANOVA) using SPSS 17.0. The significance of differences among different groups was evaluated by least significant difference (LSD) post-hoc multiple comparisons test.

Results

The effects of APS treatment on growth performance were evaluated through FI, BWG, and FCR and are shown in *Table 2*. No significant differences were found in the FI of broilers among different treatment groups (P>0.05). Compared to the control group, the APS caused positive effects on BWG and FCR of other groups (P<0.05). A quadratic decrease in FCR was observed in groups supplemented with APS. The highest ADG and lowest FCR values were observed in the 2.5 mg APS group. These data suggest that APS improves the broilers' growth performance.

To explore the effect of APS on phagocytic ability, India ink was injected into the peripheral vein of broilers and the carbon clearance level was determined on days 14 and days 28 *(Table 3)*. It was demonstrated that chicks administered with APS possessed a faster carbon clearance rate, which revealed a higher phagocytosis activity than that of the control group. The highest phagocytosis index (9.48) on day 14 was observed in the group administered with 2.5 mg APS (P<0.05). The same tendency was also observed in the 2.5 mg group on day 28; the highest phagocytosis index was 11.13 (P<0.05). On day 42, 2.5 mg and 5 mg APS administered groups showed an improvement in the phagocytosis index (P<0.001), and the highest index was 14.77. These data suggest that APS can improve the phagocytosis ability of peritoneal macrophages in broilers.

To further explore the effect of APS on cellular immunity, the lymphocyte transformation rate after PHA stimulation was measured. The T lymphocyte transformation rate at different ages of broilers in each group is displayed in *Table 4*. We found no significant difference in the T

***Table 2**. Effect of APS administration on growth performance of broilers*

Items	APS Dosage (mg)				P-value		
	0	2.5	5	10	ANOVA	Linear	Quadratic
FI	82.25±1.049	82.28±0.748	82.23±0.940	82.33±1.017	1.000	0.964	0.969
BWG	43.18±1.083 [a]	49.02±1.070 [b]	45.93±0.920 [a]	45.96±1.092 [a,b]	0.008	0.274	0.011
FCR	1.90±0.023 [a]	1.68±0.025 [b]	1.79±0.025 [c]	1.79±0.027 [c]	0.000	0.069	0.000

Means that do not share similar letter in row are significantly different, P≤0.05.

***Table 3.** Phagocytic index of macrophages in peripheral blood of broilers*

Items	APS Dosage (mg)				P-value		
	0	2.5	5	10	ANOVA	Linear	Quadratic
14d	6. 76±0.434 [a]	9.48±0.166 [b]	6.82±0.387 [a,c]	6.76±0.825 [a,c]	0.002	0.259	0.013
28d	8.18±0.848 [a]	11.13±0.819 [b]	9.29±0.582 [a,b]	8.45±0.442 [a,c]	0.029	0.744	0.013
42d	10.08±0.249 [a]	14.77±0.329 [b]	11.10±0.271 [c]	10.83±0.335 [a]	0.000	0.749	0.000

Means that do not share similar letter in row are significantly different, P≤0.05

Table 4. *Lymphocyte transformation rate of broilers*

Items	APS Dosage (mg)				P-value		
	0	2.5	5	10	ANOVA	Linear	Quadratic
14d	9.14±0.283 [a]	11.64±0.180 [b]	10.90±0.396 [b]	8.90±0.106 [a,c]	0.000	0.236	0.000
28d	12.92±0.595 [a]	15.91±1.301 [b]	14.1±0.415 [a,b]	13.83±0.351 [a,b]	0.075	0.791	0.045
42d	17.9±0.465 [a]	22.81±0.378 [b]	20.38±0.404 [c]	20.31±0.182 [c]	0.000	0.010	0.000

Means that do not share similar letter in row are significantly different, P≤0.05

Table 5. *Antibody titers to Newcastle disease virus vaccine (log2)*

Items	APS Dosage (mg)				P-value		
	0	2.5	5	10	ANOVA	Linear	Quadratic
14d	5.5±0.141	5.7±0.106	5.5±0.115	5.3±0.082	0.135	0.130	0.093
28d	7.5±0.124 [a]	8.0±0.124 [b]	7.7±0.118 [a,b]	7.7±0.141 [a,b]	0.078	0.604	0.063
42d	7.5±0.141 [a]	8.2±0.129 [b]	7.7±0.103 [a,c]	8.0±0.124 [b,c]	0.004	0.089	0.126

Means that do not share similar letter in row are significantly different, P≤0.05

lymphocyte transformation rate among the 5 mg and 10 mg APS administered groups on day 14 and 28 (P>0.05). However, on day 42, all of the APS administered groups (2.5 mg, 5 mg, 10 mg) showed marked improvement in T lymphocyte transformation in the peripheral blood (P<0.001) compared to the control group. Between the three APS administered groups, the 2.5 mg group showed significant improvement in the blood T lymphocyte conversion rate during the study period (P<0.05). These data suggest that APS can improve the peritoneal T lymphocyte transformation in broilers.

We measured the antibody titer to Newcastle disease virus (NDV) vaccine in the serum. As show in *Table 5*, there was no significant difference among the groups in the NDV antibody titer on day 14 (P>0.05). However, on day 28 and day 42, groups with APS dose of 5 mg and 10 mg showed increased NDV antibody titer at a certain extent (P>0.05) compared with the control group. Moreover, the 2.5 mg group showed a significant increase in NDV titer of broilers (P<0.05). These data suggest that APS can improve the humoral immunity in broilers at a certain extent.

Discussions

The biological activity of polysaccharides as an immune-enhancer is not only related to their molecular weight, solubility, viscosity, and chemical structure, but also closely related to the route of administration, dosage, animal species, and other factors [13,14]. In this research, the water-soluble APS extracted from the stems and leaves of alfalfa during pregnancy was non-starch polysaccharide, which was given to broilers through nasal drip after autoclaving. Experimental results suggest that water-soluble APS given intranasal can improve the growth performance and immune ability of broilers.

Macrophages are very important to the immune system of the body. They play the functions of monitoring, defense, antigen presentation, and regulation and elimination of apoptotic cells in the body, and are the first line of defense of the body's immune system [15,16]. The enhancement of phagocytic rate or phagocytic ability is one of the most significant characteristics of activated macrophages, which helps to improve the body's defense and anti-infection ability. In this research, our data demonstrate that APS could increase the phagocytic activity of macrophages. Our results are consistent with previous studies that suggested that bioactive polysaccharides could enhance the phagocytic activity of macrophages [17,18].

T lymphocyte proliferation is a direct indicator reflecting the characteristics of cellular immune function. Generally, lymphocytes can be divided and proliferate after being stimulated by corresponding antigen substances and produce antibodies or lymphoid factors against related antigens. Therefore, lymphocytes are the most important immune effector cells in animals [19,20]. The results of this study show that different dosages of APS could promote the proliferation of T lymphocytes in chicken peripheral blood, indicating that APS has immune potentiation. However, this effect was found to be more significant in the low dosage group (2.5 mg) than in the medium (5 mg) and high (10 mg) dosage groups, indicating that APS can promote the proliferation of peripheral blood T lymphocytes but its effect depends on the dosage and that the appropriate dose is an important factor affecting its immune enhancement effect. These data were further verified by other plant polysaccharides with suitable dosage on the proliferation activities of murine spleen lymphocytes [21].

The titer of Newcastle disease HI antibody in broiler serum was determined by erythrocyte agglutination test at different growth stages. It can be seen from the results

of the titer of the HI antibody in the serum on day 14 that the enhancement effect on the humoral immune function of broilers is not obvious. On day 28 and day 42, the 2.5 mg group showed enhanced humoral immune function. There is a certain difference between the results of NDV HI antibody across the three stages. Part of the difference may be due to the interference of the maternal antibody of NDV in the younger chicken, resulting in the insignificant increase of the HI titer of NDV by APS [22].

In the present research, chicks administered with different doses of APS demonstrated a positive effect on their growth performance, phagocytic ability, T lymphocyte proliferation, and NDV antibody titer. However, when different doses of APS were given to broilers through a nasal drip, they did not display dose dependence for the above indicators. This may be attributed to the complex structure of APS. As they are complex biological macromolecule material, polysaccharides could interact with each other to create a network structure and absorb water molecules when the concentration is increased, which could affect their biological activity [23].

In conclusion, intranasally administered APS at a dose of 2.5 mg had positive effects on broiler performance. It stimulated phytohemagglutinin-induced T-lymphocyte transformation and increased phagocytic activity without adversely affecting Newcastle disease virus antibody production. The immunomodulatory effects of APS on poultry production are likely to enhance the response the birds' diseases resistance ability.

Declarations

Availability of Data and Materials: The data and materials set during the current study are available from the corresponding author (S. Zhang) on reasonable request.

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Competing of Interest: The authors declare no competing financial interest.

Ethical Statement: Experimentation with animals was approved by the Experimental Animal Management Methods of Xinxiang Medical University (Approval number: 201206078) and followed Henan Authority's Experimental Animal Regulations.

Author's Contribution: Y. Dong and S. Zhang designed this study. Y. Dong and L. Wang executed the experiment and analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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Research Article

Immunohistochemical Localization of Leptin and Ghrelin in Kidney Tissue of Capsaicin Administered Diabetic and Non-diabetic Rats

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Abstract

In this study, we investigated the effect of Capsaicin (CAP) on leptin and ghrelin expression in diabetic kidneys by immunohistochemical method. The subjects were separated into 5 groups: control, sham, diabetes, CAP, and D+CAP. Streptozotocin (45 mg/kg, single dose) was administered to each rat in the diabetes and D+CAP groups intraperitoneally. Rats with blood glucose levels of 200 mg/dL or higher were considered diabetic. Capsaicin (1 mg/kg) was administered subcutaneously to the rats in the CAP and D+CAP groups every day for 2 weeks. H&E, PAS, and Crossman's Triple staining were performed to examine the histology of the kidney. Leptin and ghrelin in the kidney were examined by immunohistochemical method. Histological examination revealed normal kidney tissue in the control, sham, and CAP groups. The diabetes group showed the glomerulus with scattered and narrowing of the Bowman's capsule. While leptin immunoreactivity was most intense in the diabetes group, it was observed the weakest in the CAP and D+CAP groups. While strong ghrelin immunoreactivity was seen in the control and sham groups, mild immunoreactivity was seen in the CAP group, and weak immunoreactivity was seen in the diabetes and D+CAP groups. In this study, we concluded that the application of CAP could prevent loss of appetite by reducing the leptin expression during diabetes, but could not protect ghrelin from adverse effects of diabetes.

Keywords: Capsaicin, Diabetes, Ghrelin, Immunohistochemical, Leptin

Introduction

Diabetes Mellitus (DM) is a metabolic disease characterized by insulin secretion disorder or increased insulin resistance. A diabetic patient has an increased blood glucose level because of both of these conditions [1]. DM is asymptomatic or characterized by clinical symptoms. These clinical symptoms, which include weight loss, polyuria, polydipsia, polyphagia, and weakness, affect many organs and systems [2].

Capsaicin (CAP) is a chemical compound of chili and regulates the spiciness of hot chili pepper, usually termed a hot pepper *(Capsicum annuum L.)* [3]. *Capsicum annuum* L. contains protein, ascorbic acid, thiamine, red carotenoids, iron, copper, manganese, magnesium, zing, oxalates, phosphorus, flavonoids, calcium, water and vitamins, alongside capsaicin [4,5]. CAP consists of a long hydrophobic carbon tail containing a polar amide group (C18H27NO3) and a benzene ring. It is a lipophilic, volatile, pungent, hydrophobic, colorless, and odorless white crystalline powder [5]. CAP increases lipid peroxidation which decreases the amount of adipose tissue, and serum triglycerides. CAP has also an effect to inhibit the glycogen metabolism in skeletal muscles [6].

Leptin, which is known as an anorexigenic hormone, has an appetite suppression. It does this function by connecting its receptors in the hypothalamus. There is a relationship between leptin levels and some diseases such as cardiovascular diseases, chronic kidney disease, and DM [7,8]. It has been understood that it plays a role in the regulation of all system functions because leptin receptors are almost found in all organs [9]. A kind of leptin resistance can occur with leptin receptor downregulation, and leptin does not work properly in this condition [10,11].

Ghrelin is an orexigenic hormone that increases appetite so it has the opposite effect of leptin [12]. Ghrelin is found in many organs such as the stomach, liver, hypothalamus, pancreas, and kidney [13]. The ghrelin receptor is also expressed in the kidneys [14]. Ghrelin stimulates insulin secretion in the pancreas [15]. While there is insulin resistance in DM, ghrelin upgrades insulin signaling [16].

The purpose of this study is to investigate the effect of CAP on leptin and ghrelin by immunohistochemical method and on the histological structure by histochemical method in diabetic and non-diabetic rat kidneys.

Material and Methods

Ethical Statement

We received approval from Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK: 2019-057) for the applications performed on rats in the study.

Animals and Experimental Design

The study was started after the rats were adapted to the environment for one week. After this process, 30 female Wistar albino rats (three months aged, weighing 200-220 g) were separated into 5 groups: control, sham, diabetes, CAP, and D+CAP. The control group was left untreated, only a citrate buffer (0.1 M, pH 4.5) was injected via i.p. into the rats in the sham group, CAP was injected into the rats in the CAP and D+CAP groups and Streptozotocin (STZ) was injected to the rats in diabetes and D+CAP groups. Experimental applications were performed at Kafkas University Experimental Animal Application and Research Center. Rats were kept in standard cages in a room that had 22±2ºC room temperature, and 12 h of light and dark cycle. They were fed *ad libitum* with a standard rat diet and water except 8 h before STZ injection and taking blood samples. The duration of the study lasted for 14 days and the study started with STZ injection which was accepted the day 0. After 14 days, cervical dislocation was applied to the rats under ether anesthesia, and kidney tissues were taken.

Preparation of STZ and Induction of Diabetes

STZ (Sigma S0130-100 MG) was dissolved in a citrate buffer (0.1M at pH 4.5). Then it was administered intraperitoneally to each animal in diabetes and D+CAP groups as a single dose of 45 mg/kg [17]. Blood samples were drawn from all rats 72 h after STZ and citrate buffer administration. Before blood samples were taken, all subjects in the study were left fasting for 8 h. Rats with blood glucose levels of 200 mg/dL or higher in the test made with a glucometer (Accu-Chek-Go, Roche) were considered diabetic [18].

Preparation and Application of CAP

CAP (Sigma, St Louis, MO, USA) was dissolved in 10% ethanol, 1% Tween 20, and 80% distilled water and administered subcutaneously to each rat in the CAP and D+CAP groups as a dose of 1 mg/kg on the 3rd day and following every day until the end of the study.

Histological Procedure

The collected kidney samples were stored in 10% formalin solution. Then the tissues were prepared by going through routine histological procedures. Slides were sectioned from the kidney tissues by using a microtome (Leica-RM2125 RTS, Germany). Crossman's triple staining, Periodic Acid Schiff (PAS), and Hematoxylin & Eosin (H&E) staining were applied to the sections taken for histological examinations. A light microscope (Olympus Bx51, Japan) was used to evaluate the slides histologically.

Immunohistochemical Procedure

Avidin-Biotin-Peroxidase complex (ABC) technique [19] was applied to examine the immunolocalization of leptin and ghrelin antibodies in kidneys. The slides were deparaffinized with xylene, rehydrated with alcohol, and washed with phosphate-buffered saline (PBS), respectively. After those processes, the slides were incubated for 10 min in 3% H_2O_2 (prepared in 0.1 M PBS) to inhibit endogenous peroxidase activity. After washing with PBS (3x5 min), the slides were kept in the microwave at 800 watts for 10 min to reveal antigens. The slides were washed with PBS (3x5 min) again, and incubated for 10 min with UV block serum (10%, Ultra V Block, Fremont CA) to prevent non-specific binding. The kidney tissues on slides were incubated with anti-leptin primary antibody (Ob Antibody (A-20): sc-482; Santa Cruz) (1:400), and anti-ghrelin primary antibody (Phoenix-H-031-31) (1:400) at room temperature for one hour. After the slides were washed with PBS, a secondary antibody (Biotinylated Goat Anti-Rabbit [Lab. Vision, 510.991.2800]) was added to the sections and kept at room temperature for 30 min. The slides were washed with PBS (3x5 min), then slides were incubated with streptavidin-horseradish peroxidase (Ultravision Detection System Large Volume Anti-Polyvalent, HRP, Thermo-scientific, UK) at room temperature for 15 min. Diaminobenzidine-hydrogen peroxidase (DAB) [20] was used to apply chromogen to the slides after the slides were washed with PBS again (3x5 min). Hematoxylin was used as a counterstain. Covered slides with entallen were examined under a light microscope (Olympus Bx51, Japan). The immunoreactivity scores of leptin and ghrelin in proximal tubules were determined according to the reaction intensity (strong=3, mild=2, weak=1, negative=0). For this aim, 20 tubules of each subject in each group were randomly selected, and the same histologist scored a total of 120 tubules in each

group. In order to understand whether it was the specific immunoreactivity, a negative control was prepared by performing all steps without adding a primary antibody.

Statistical Analysis

In our study, the number of proximal tubules samples used for Leptin and Ghrelin immunoreactivity scoring was subjected to G*Power (Universität Düsseldorf, Düsseldorf, Germany) analysis, and was confirmed with 96% reliability. Immunoreactivity scoring data were analysed statistically by using IBM SPSS Statistics Version 22 (IBM Corp., Armonk, NY, USA). Normality test (Shapiro-Wilk) and variance homogeneity test (Levene) were performed. P<0.05 was found for both tests and for both leptin and ghrelin. Since parametric assumptions were not met, Kruskal Wallis analysis of variance was performed. Kruskal Wallis test found P<0.05, then a One-Way ANOWA (Post-hoc, Tamhane) test was performed to find out which group caused the difference.

Results

Histological Results

Histologically the kidney tissues were seen as normal in the control, sham, and CAP groups. In the diabetic rats, it was observed that the glomerulus of Malpighi bodies in the cortex were scattered, and there were narrowing in the Bowman's capsules and glomerular contraction. Different cell sizes, cytoplasm disorders, cell scattering, and cell shedding were locally observed in the tubular epithelial cells of the diabetes group. Lymphocyte infiltration was also found between some tubules *(Fig. 1)*. In the D+CAP group, the structure of Malpighi bodies was similar to the control group. However, vacuolization and different cell

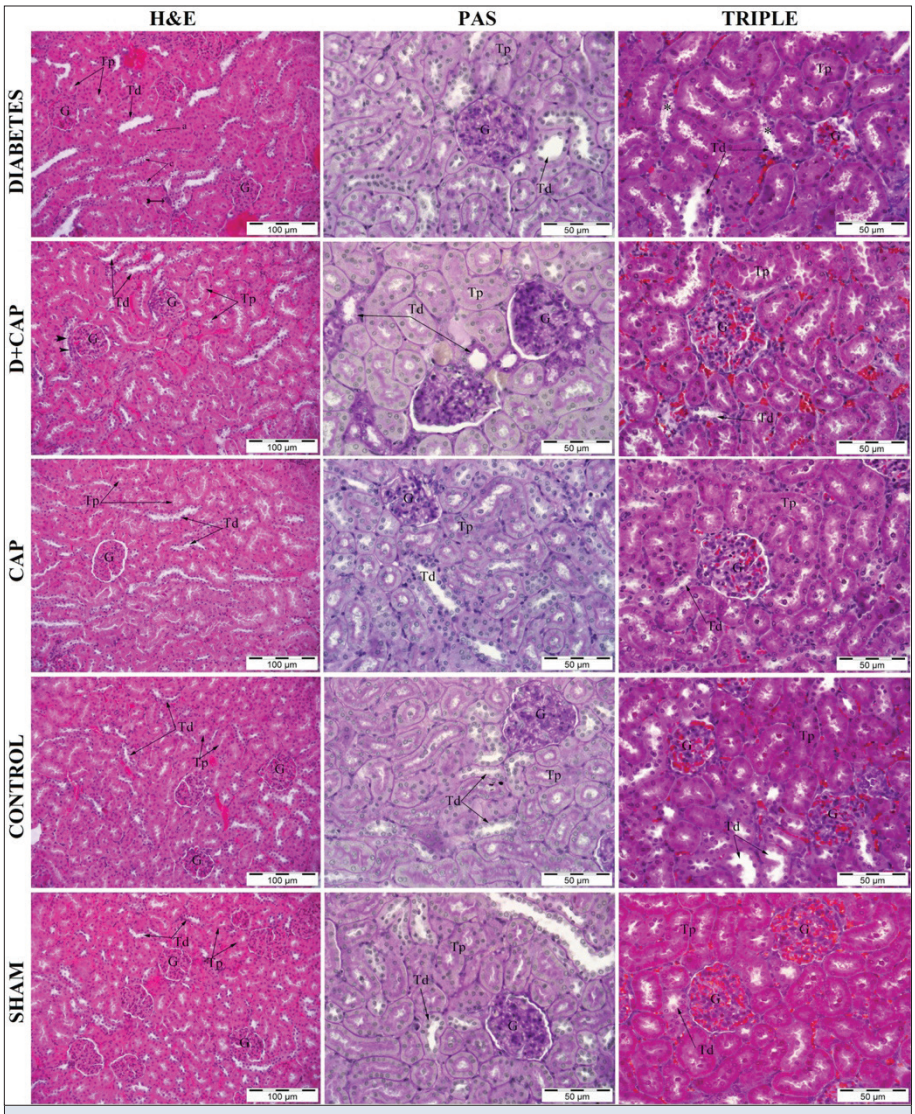

Fig 1. Histochemical staining micrographs of kidney tissues belonging to the groups. Cell irregularities *(star),* different cell size (c), cell scattering and shedding (a) in triple and H&E staining of diabetes group. Macula densa *(arrowhead),* Lympocyte cells *(tailed arrow),* Glomeruli (G), Distal tubules (Td), Proximal tubules (Tp). H&E, Scale bar: 100 µm; PAS, Scale bar: 50 µm; Triple, Scale bar: 50 µm

Table 1. *The Comparison of immunoreactivity for leptin and ghrelin in proximal tubules*

Groups	N	Number of Proximal Tubules	Immnoreactivity Score of Leptin (Mean±SD)	Immnoreactivity Score of Ghrelin (Mean±SD)
Control	6	120	1.85±0.6[a]	2.98±0.1[a]
Sham	6	120	1.87±0.6[a]	2.96±0.1[a]
CAP	6	120	1.02±0.5[b]	1.82±0.5[b]
Diabetes	6	120	2.96±0.1[c]	1.17±0.5[c]
D+CAP	6	120	1.09±0.6[b]	1.10±0.6[c]
P-value			0.001*	0.000*

[a,b,c] *Values within a row with different superscripts differ significantly at P<0.05;* ***SD:*** *Standard deviation; * Statistically significant*

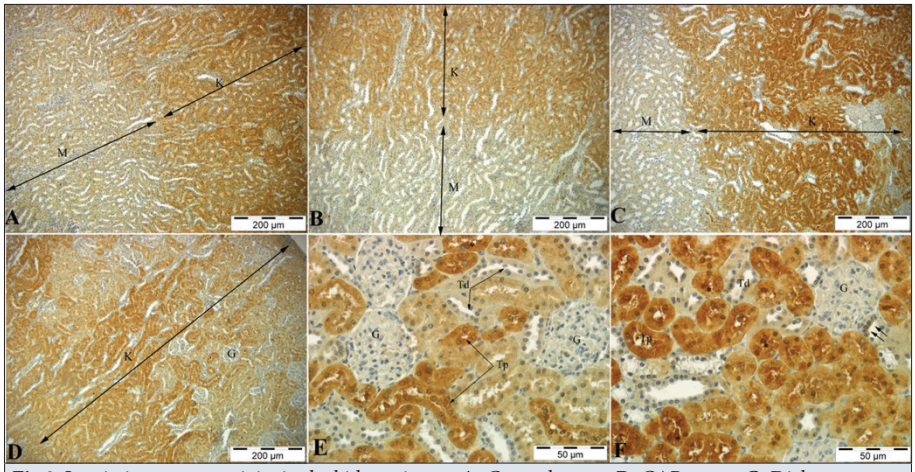

Fig 2. Leptin immunoreactivity in the kidney tissues. **A-** Control group, **B-** CAP group, **C-** Diabetes group, **D-** D+CAP group, **E-** Control group, **F-** Diabetes group. General localization of leptin immunoreactivity in groups (A, B, C, D). Glomeruli (G), Distal tubules (Td), Proximal tubules (Tp), Macula densa *(double arrows),* Nuclear Leptin immunoreactivity *(star)*

sizes were observed in the epithelial cells of the tubules in the D+CAP group *(Fig. 1)*.

In the PAS staining, we determined PAS+ areas in the basal membrane of tubules, brush border of the proximal tubules, and cytoplasm of the distal tubules in all groups. PAS positivity of glomeruli and cytoplasm of the distal tubule cells was more distinct in the D+CAP group than that of the other groups. It was also found that PAS positivity was higher in the D+CAP group and the lower in the diabetes group compared to others *(Fig. 1)*.

Immunohistochemical Results

In general, we observed that leptin immunoreactivity in the kidney was stronger in the cortex than in the medulla *(Fig. 2-A,B,C,D)*. It was seen as weaker in the distal tubule and macula densa compared to the proximal tubule. However, it was not seen in Malpighi bodies in the cortex *(Fig. 2-E,F)*. We determined leptin immunoreactivity both in cytoplasm and in nuclei but leptin showed only cytoplasmic immunoreactivity in some cells. Leptin immunoreactivity was not detected in the wall of the blood vessels.

Leptin immunoreactivity was the strongest in the diabetes group, while it exhibited the weakest immunoreactivity in the CAP and D+CAP groups, and mild immunoreactivity in the control and sham groups *(Table 1)*. In diabetes and D+CAP groups, leptin immunoreactivity was stronger in the inner cortex and weaker in the outer cortex than that of other groups in which leptin immunoreactivity had a homogeneous distribution in the cortex *(Fig. 2)*.

We observed that ghrelin immunoreactivity was widely distributed in the kidney tissue, and it was weaker in the medulla compared to the cortex. The intensity of ghrelin immunoreactivity was seen especially in the part of the cortex close to the organ capsule *(Fig. 3-A)*. Bowman capsule cells and some cells in the glomeruli had weak ghrelin immunoreactivity. Distal tubule epithelial cells had weaker immunoreactivity than that of the proximal tubule. It was noted that the ghrelin immunoreactivity in the proximal tubule epithelial cells was located and concentrated in their basal part *(Fig. 3-B,C)*. The ghrelin immunoreactivity was usually observed in cytoplasm, but in some cells, it was seen in both cytoplasm and nuclei. In addition, ghrelin immunoreactivity was determined to be widespread in the medulla, but weaker than in the cortex *(Fig. 3-D,E,F)*.

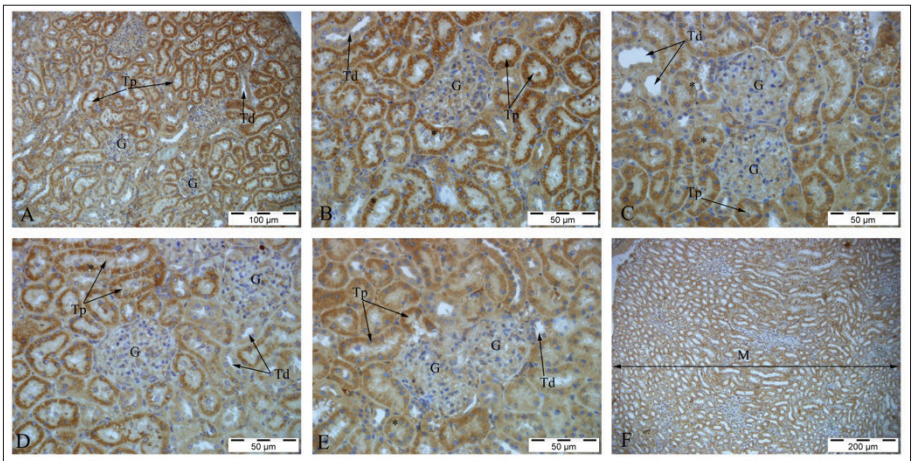

Fig 3. Ghrelin immunoreactivity in the kidney tissues. **A**- Control group, **B**- Control group, **C**- Diabetes group, **D**- CAP group, **E**- D+CAP group, **F**- Sham group. Medulla (M), Glomeruli (G), Distal tubules (Td), Proximal tubules (Tp), Nuclear ghrelin immunoreactivity *(star)*

It was found that control and sham groups had the strongest, CAP group had moderate, diabetes and D+CAP groups had the weakest ghrelin immunoreactivity when groups compared with each other (P<0.05) *(Table 1)*.

Discussion

DM is a chronic and metabolic disease characterized by the inability to produce or use insulin, and it is one of the major global health problems [21]. In our study, we aimed to examine the effects of CAP application to diabetic rats on both leptin and ghrelin immunoreactivity in the kidney, and also its effects on the kidney histological structure.

In previous studies, researchers said that STZ elevated the blood glucose level in rats at a dose of 45 mg/kg and caused DM [22,23]. STZ caused high blood glucose levels and DM by injuring the pancreas β cells [24]. In our study, STZ increased the blood-glucose level in rats at a dose of 45 mg/kg as reported in the previous studies.

Tubular atrophy, thickening of the tubular basement membrane, and interstitial inflammation may be some of the histological changes in the diabetic kidney [25]. One of these changes is the result of hyperglycaemic conditions of DM because glucose binds to the amino groups of proteins in the early phase of diabetes during hyperglycemia [26]. Under diabetic conditions, all cells of the kidney may be affected, including endothelial cells, tubule interstitial cells, podocytes, and mesangial cells. On the other hand, damage or dysfunction in any cell type can spread to all kidney cells and ultimately affect kidney function [27]. In a study, the researchers declared that the PAS stain was strong in the mesangial matrix of glomeruli in diabetic animals [28]. We observed glomerular thickening, irregularity in tubular epithelial cells, an increase in some epithelial cell size, and a narrowing as a result of thickening of the basal membranes of the tubules in the tubulointerstitial areas in diabetic kidney tissue. We also determined that the PAS reaction in the mesangial matrix of glomeruli and in the brush border of tubules was strong in the D+CAP group and the kidney histological structure of this group was similar to the control group.

Leptin and ghrelin are the main hormones by working together in opposite directions and regulating the appetite and hunger sensations [29,30]. While leptin is considered an endogenous anorexigenic hormone, ghrelin is considered the most potent endogenous orexigenic peptide, and both of them play important roles in glucose homeostasis [30,31].

A team of researchers reported that leptin immunoreactivity was observed in the distal and proximal tubules, and its immunoreactivity was found at the same location in all groups but weaker immunoreactivity in diabetic kidneys [32]. We determined intense leptin-immunoreactivity in the cortex compared to the medulla and in the proximal tubules compared to the distal tubules and macula densa. In our study, there was not any leptin immunoreactivity in the glomeruli, Bowman capsule cells, and vascular endothelial cells. In addition, unlike the previous study mentioned above, we determined more intense leptin immunoreactivity in the diabetes group than that of the others. We think that CAP decreases leptin expression when diabetes increases leptin expression because of our finding of the weakest leptin immunoreactivity in the CAP and D+CAP groups and highest leptin immunoreactivity in the diabetes group.

In a study, it was stated that ghrelin immunoreactivity was found clearly in the macula densa, which is a part of the distal tubules, and also found in the ascending loops, but not in the proximal tubules, descending loops, collecting ducts, glomeruli and interstitial cells [33]. In another study related to diabetic nephropathy, the researchers declared that ghrelin immunoreactivity was weak in the collecting

ducts, mild in the distal tubules, and negative in the glomeruli and proximal tubules. In addition, they noted that ghrelin immunoreactivity in the diabetes group was found stronger than that of the control group [34]. Unlike in the previous studies, we found ghrelin immunoreactivity in the proximal tubules and glomeruli, and even it was strong in the proximal tubules. We also determined that ghrelin immunoreactivity was located in the basal part of the proximal tubule cells. Because of this result, we consider that proximal tubules can be mistaken for distal tubules in terms of ghrelin immunoreactivity localization. In addition, we found that ghrelin immunoreactivity was weaker in the medulla compared to the cortex, and Bowman's capsule cells and some cells in the glomeruli had weak ghrelin immunoreactivity. We determined strong ghrelin immunoreactivity in the control and sham groups, mild immunoreactivity in the CAP group, and weak immunoreactivity in the diabetes and D+CAP groups. We consider that CAP has a suppression effect on ghrelin expression but diabetes has more suppression effect on its expression.

In conclusion, diabetes has bidirectional effects on appetite because it both increases leptin and decreases ghrelin. In our study, the leptin immunoreactivity increased in the diabetes group compared to the control and sham groups, while the ghrelin immunoreactivity decreased in the diabetes group compared to the control, sham, and CAP groups. We considered that the application of CAP can prevent loss of appetite by reducing the leptin expression during diabetes, and CAP cannot protect ghrelin from the adverse effects of diabetes.

Declarations

Availability of Data and Materials: The findings of the current study are available from the corresponding author (SA Bingöl) upon reasonable request.

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

Author Contributions: S.A.B, T.D, S.K.T, and S.A designed and performed the study; S.I performed immunohistochemical stain and scored immunoreactivity degree; D.G.E performed histochemical stains; S.A.B, T.D and S.I wrote the results; S.A.B. reviewed the literature and wrote the article.

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Research Article

Seroprevalence and Assessment of Risk Factors Associated to *Borrelia burgdorferi* Infection in Egyptian Horses

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Abstract

Borrelia burgdorferi sensu lato can infect horses. However, the extent to which Egyptian horses get infected and seroconvert owing to this pathogen is unclear. Thus, this study aimed to determine the seroprevalence of *B. burgdorferi* and estimate its associated risk factors. A total of 385 serum samples collected from asymptomatic horses reared in three governorates in Egypt were screened using a commercial IDEXX SNAP 4Dx Plus kit. The results revealed that 58 (15.1%) out of 385 horses had antibodies against *B. burgdorferi* with Giza governorate showing the highest prevalence rate (30/130; 23.1%). According to univariate logistic regression analysis, locality, age, breed of examined horses and application of hygienic measures in house were significantly associated with *B. burgdorferi* infection. Furthermore, the multivariate logistic regression study revealed that thoroughbred horses older than four years old and raised in Giza governorate under unsanitary conditions, were more likely to be infected with *B. burgdorferi* than others. The serological evidence of *B. burgdorferi* in Egyptian horses and assessment of the risk factors associated with infection, necessitate wide range screening of this disease in other areas as well for efficient diagnosis and control.

Keywords: *Borrelia burgdorferi*; SNAP test; Risk factors; Horses; Egypt

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Introduction

Horse Lyme borreliosis (LB) was first reported in Wisconsin state at USA, an area where *Borrelia burgdorferi* is widespread [1]. *B. burgdorferi* is spread by the biting of hard ticks i.e. *Ixodes* spp., whereas mammals and birds act as reservoirs [2,3].

There is no typical symptom of Lyme disease among horses [4], but some may show meningitis, encephalitis [5], radiculoneuritis [6], arthritis, lameness [7], and panuveitis [1]. Human infection is usually associated with erythematous rash, neurological abnormalities, and arthritis, but some patients do not show any symptoms, as with horses [8].

The LB in horse is difficult to diagnose since specific antibodies against the *B. burgdorferi* sensu lato complex are not necessarily associated with clinical signs and symptoms [9]. Depending on the distribution of *Ixodes*

spp. ticks infected with *Borrelia burgdorferi*, equine LB seroprevalence varies across geographic regions. However, it is not clear whether high antibody levels correlate with clinical signs in infected hosts, or whether clinical signs are unrelated to antibody levels [10-12].

A number of previous studies on *B. burgdorferi* seroprevalence found 47.8% seropositive horses in Slovakia [13], 29.0% in Denmark [14], 25.6% in Poland [15], 6.8% in Sweden [16], 16.1% in Germany [17], 6.3% in Turkey [18], 34% in Mexico [19], 33% in France [20], 58.7% in Minnesota [12], 63% in Wisconsin [21], 60% in New Jersey [22] and 60-90% in Austria [23]. The available information concerning the seroprevalence of *B. burgdorferi* s.l. in horses in Egypt is rare.

Although both direct and indirect pathogen detection methods are available, direct detection of *Borrelia* spp. by culture is challenging and costly [24]. Although polymerase chain reaction (PCR) can detect *Borrelia* specific DNA during most stages of the disease, various in-house PCR tests have not been validated under standardized study conditions as methods for detecting *Borrelia* spp. from various sample materials. As a result, the use of PCR for detecting *Borrelia* spp. in routine diagnostics remains restricted [25,26]. Serological approaches for identifying specific antibodies against members of the *B. burgdorferi* complex remain a sensitive, low-cost, and quick laboratory diagnostic tool in both human and veterinary medicine [24].

Therefore, the purpose of this study was to determine the seroprevalence of *B. burgdorferi* s.l. among horses residing in three governorates in Egypt.

Material and Methods

Ethical Approval

The Benha University ethics committee for animal studies authorized all operations, including the collection and processing of blood samples. The animals' owners gave their informed consent for the collection of samples. The Benha ethics committee ensured that all operations followed all applicable laws and rules. The ARRIVE criteria were followed throughout the study process.

Study Area

The research was conducted in three Egyptian governorates: Giza, Kafr ElSheikh and Qalyubia which are located at 29.9870°N 31.2118°E, 31°06′42″N 30°56′45″E and 30.41°N 31.21°E, respectively *(Fig. 1)*.

Egypt has mostly hot and dry climate characteristics (Köppen climate classification BWh). Giza has a desert-like climate, which is characterized by warm summers with temperature range between 25-45°C and moderate winters with low rainfall (100 mm). Kafr ElSheikh and Qalyubia governorates situated at the Nile Delta, have hot desert climates (Köppen: BWh), like most of Egypt. In the Nile Delta, temperatures are relatively moderate, but they rarely reach 35°C during the summer. The delta region receives about 100-200 millimeters of rainfall on average per year, most of which falls in the winter. A variety of habitats, including lakes, marshes, grass pastures, and agricultural areas, may be found in the study area. These habitats together support many biological species and the proliferation of ticks.

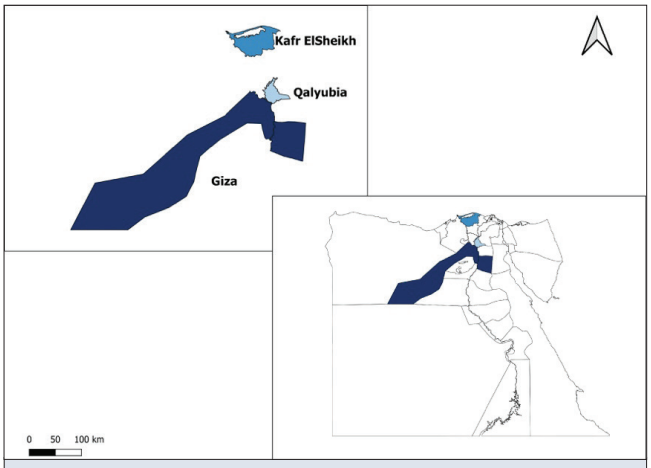

Fig 1. MAP showed the studied governorates (MAP generated by QGIS software)

Sample Size and Sampling

A cross-sectional study was carried out from January to December 2022. A sample size of 385 was calculated based on an estimated 50% prevalence. The samples were collected from asymptomatic horses distributed in three governorates (130 from Giza, 140 from Kafr El Sheikh and 115 from Qalyubia) under the study. These horses were raised by individual farmers and did not suffer from any disease or clinical signs.

Blood sample (10 mL) was collected from jugular vein of each horse, transferred immediately to Veterinary Diagnostic Laboratory, Benha university and centrifugated at 1500xg for 10 min to separate the sera. The sera samples were kept at -20°C till serological analysis.

Questionnaire

The examined horses included those kept for drafting and others being raised for breeding. These animals living in indoor house and some of them have outdoor yard. Each horse owner was asked to fill out a questionnaire that assessed each horse's farm management and demographics. Demographics included: sex (male or female), age (<2, 2-4 and >4 years) and breed (Arabian, Thoroughbred and mixed). Management related questions included: if owners apply regular cleaning of stable every week and application of acaricides (every month) or not.

Serological Analysis

The IDEXX SNAP 4Dx Plus (IDEXX Laboratories) was used in accordance with the manufacturer's instructions to test sera for the existence of antibodies against *B. burgdorferi*.

Three drops of serum and four drops of conjugate are added to the sample tube, the tube is inverted four times and then the entire contents of the tube are poured into the "sample well" of the SNAP device. Results are read 8 minutes after the test is activated. According to the published data by Chandrashekar, Daniluk [27], this test was validated for detection of antibodies to *B. burgdorferi* by 100% sensitivity and 95% specificity.

In addition, all examined samples with IDEXX SNAP 4Dx Plus were confirmed by by Viramed Biotech AG - Borrelia B31 IgG ViraStripe (Vuramed Biotech AG, Planegg, Germany).

Data Analysis

The IBM SPSS Statistics program (SPSS Inc., Chicago, USA, version 24) was used to conduct the statistical analysis. The variations in seroprevalences of variable groups were analyzed using the chi-square test. P-values under 0.05 were regarded as significant. The relationship between *B. burgdorferi* infection and the relevant risk variables was evaluated using univariate analysis.

All variables (locality, age, breed and hygienic measures) with *P* values less than 0.25 in the univariate analysis were subjected to multivariable logistic regression analysis [28-31].

To determine the strength of the correlation between the presence of *B. burgdorferi* and other factors, odds ratios (ORs) and 95% confidence intervals (CIs) were computed. The model's fit was assessed using the Hosmer and Lemeshow goodness-of-fit test.

Results

Using ELISA, 58 (15.1%) of 385 horse blood samples tested positive for *B. burgdorferi* with IDEXX SNAP 4Dx Plus and Borrelia B31 IgG ViraStripe®. The seroprevalence *B. burgdorferi* was varied significantly between different localities, Giza had the greatest prevalence (23.1%), followed by Kafr ElSheikh (12.1%) and Qalyubia (9.6%) *(Table 1)*.

There was no significant difference between sex and *B. burgdorferi* seroprevalence and females showed higher seroprevalence (16.7%) than males. In addition, the univariate analysis showed age, breed and hygienic measures had significant effect (P<0.05) on seroprevalence of *B. burgdorferi* in horses. Horses older than four years were more likely to be positive (24.5%) than horses of a middle age or younger than two years *(Table 1)*.

Regarding to breed, the seroprevalence of *B. burgdorferi* in thoroughbred horses (25.7%) was higher in comparison with Arabian horses (6.9%) especially in the absence of hygienic measures (17.5%) *(Table 1)*.

A multivariate logistic regression model was performed on the factors with a *P* value less than 0.25 in univariate analysis. The study found that horses raised in Giza, had

Table 1. *Seroprevalence of B. burgdorferi in horses in relation with different variables*

Variable		No of Examined Horses	Distribution (%)	No of Positive	No of Negative	% of Positive	95% CI	Statistic
Locality	Giza	130	33.8	30	100	23.1	16.67-31.03	χ2=10.175 df=2 P=0.006*
	Kafr ElSheikh	140	36.4	17	123	12.1	7.72-18.58	
	Qalyubia	115	29.9	11	104	9.6	5.43-16.32	
Sex	Male	115	29.9	13	102	11.3	6.72-18.38	χ2=1.812 df=1 P=0.178
	Female	270	70.1	45	225	16.7	12.7-21.58	
Age	<2 years	99	25.7	10	76	10.1	5.58-17.6	χ2=6.374 df=2 P=0.041*
	2-4 years	192	49.9	25	167	13.0	8.98-18.51	
	>4 years	94	24.4	23	84	24.5	16.9-34.05	
Breed	Arabian	145	37.6	10	135	6.9	3.79-12.23	χ2=16.893 df=2 P<0.0001*
	Thoroughbred	105	27.3	27	78	25.7	18.31-34.82	
	Mixed	135	35.1	21	114	15.6	10.41-22.62	
Hygienic measures	poor	286	74.3	50	236	17.5	13.52-22.3	χ2=3.910 df=1 P=0.048*
	good	99	25.7	8	91	8.1	4.15-15.14	
Total		385		58	327	15.1	11.83-18.98	

** The results with P value less than 0.05 considered significant*

Table 2. *Multivariate logistic regression analysis for the factors associated with B. burgdorferi in horses*

Variables		B	SE	OR	95% CI for OR		P Value
					Lower	Upper	
Locality	Giza	1.086	0.399	2.96	1.36	6.47	0.006
	Kafr ElSheikh	0.224	0.425	1.25	0.54	2.88	0.039
Age	2-4 years	0.359	0.412	1.43	0.64	3.21	0.038
	>4 years	1.230	0.436	3.42	1.46	8.03	0.005
Breed	Thoroughbred	1.647	0.415	5.19	2.30	11.72	0.000
	Mixed	0.992	0.420	2.70	1.18	6.14	0.018
Hygienic measures	Poor	0.744	0.427	2.10	0.91	4.86	0.081

B: Logistic regression coefficient, SE: Standard error, OR: Odds ratio, CI: Confidence interval

a three-fold greater likelihood (OR=2.96, 95% CI: 1.36-6.47) of being infected with *B. burgdorferi* than horses in other areas *(Table 2)*. Moreover, Thoroughbreds were more likely to have *B. burgdorferi* infection (OR=5.19, 95% CI: 2.30-11.72), especially older examined horses more than four years (OR=3.42, 95% CI: 1.46-8.03), *Table 2*. Furthermore, absence of hygienic measures (OR=2.10, 95% CI: 0.91-4.86) was identified as a risk factor for *B. burgdorferi* infection in horses *(Table 2)*.

Discussion

Horses are frequently infested by ticks and might be directly infected by tick-borne diseases [32-35]. Lyme borreliosis is one of the most important tick-borne diseases in horses and is caused by *B. burgdorferi*. The serological detection of *B. burgdorferi* has been reported in dogs in Egypt but there is no available data about the disease in Egyptian horses.

Thus, the present study investigated the presence of antibodies against *B. burgdorferi* and the associated risk factor for infection.

The overall seroprevalence of antibodies against *B. burgdorferi* was 15.1% using IDEXX SNAP 4Dx Plus and the presence of IgG-specific antibodies against *B. burgdorferi* were confirmed by *Borrelia* B31 IgG ViraStripe.

The reported prevalence of the study lies in similar range (15-16%) reported by Tsachev et al.[36] among horses in Northern Bulgarian using method of rapid ELISA test (SNAP 4Dx Plus Test, IDEXX Laboratories Inc., USA) and in German horse [17] and in Northern Algeria using ELISA [37]. The seroprevalence rate in this study was higher than those reported in Sweden 6.8% [16] and Turkey 6% [18]. In addition, seroprevalence found in this study is lower than those reported in Mexico 34% [19], France 33% [20], Minnesota 58.7% [12], Wisconsin 63% [21], New Jersey 60% [22] and Austria 60-90% [23].

Various factors influencing seroprevalence between different countries that may explain the discrepancies in the values reported, include the number of surveyed horses, hygienic measures, environmental conditions, and the frequency of exposure to ticks [14,33,38].

In addition, the seroprevalence of *B. burgdorferi* was highest in Giza in comparison with other studied governorates which might be attributed to warmer weather of Giza which is significant for tick ecology [39-45].

There is no significant variation in seroprevalence between both sexes with a higher trend in females. This is consistent with previous findings which have been found by Lee et al.[46] and Laamari et al.[37].

The seroprevalence of *B. burgdorferi* increased significantly with age in horses, which concurs with a study conducted by Hansen et al.[14]. However, other studies found no significant difference between age groups and a higher presence of antibodies in young animals [37,46]. It may be attributed to horses being exposed to ticks frequently with age, increasing their chances of contracting Lyme disease [3,10,13,20].

Several studies have indicated that the breed is a significant risk factor for *B. burgdorferi* infection, and in the current study, the thoroughbred horses were found more likely to contract the infection than other breeds, consistent with previous results of Lee et al.[46]. However, Laamari et al.[37] found no significant correlation between breed and seroprevalence of *B. burgdorferi* and Arabian horses were found more frequently infected than others [41,47].

There is no clear explanation for the higher *B. burgdorferi* seroprevalence found in our study among thoroughbred horses compared to other breeds, but it might be attributed to a difference in management. Compared to Arabian breed horses that are kept in stables and are more protected from ticks than thoroughbreds, which are housed outdoors [48,49].

Additionally, the horses were not generally well cared

or did not kept in clean houses, which made them more vulnerable to ticks and *B. burgdorferi* infection, as previously reported by Neely et al.[50].

Limitation of the present study was sample selection bias and bias in timing of the sample.

To the best of our knowledge, this study is the first to reveal the countrywide seroprevalence of *B. burgdorferi* in Egyptian horses. This study concludes that *B. burgdorferi* exists in Egyptian horses and that the frequency is highest in the Giza governorate. Furthermore, the multivariate logistic regression analysis revealed that the locality, sex, breed and hygienic measures were identified as risk factors for *B. burgdorferi* infection in horses.

Declarations

Availability of Data and Materials:

The data used in this article will be provided by the corresponding author (MM and AS) upon request.

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Research Article

Bone Marrow Mesenchymal Stem Cells Facilitate Alveolar Bone Remodeling in Periodontitis Rats During Orthodontic Tooth Movement Through STAT3/β-Catenin

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Abstract

We aimed to explore the performance of bone marrow mesenchymal stem cells (BMSCs) in modulating signal transducer and activator of transcription 3 (STAT3)/β-catenin to facilitate alveolar bone remodeling in periodontitis rats in the process of orthodontic tooth movement. Flow cytometry was employed to identify BMSCs isolated, and then their osteogenic capacity was examined by alizarin red staining assay. BMSCs group (n=10), periodontitis + orthodontic tooth movement (PO+OTM) group (n=10), BMSCs+Static group (n=10) and negative control (NC) group (n=10) were set up for random allocation of 40 rats. The PO+OTM group had significantly decreased bone mineral density (BMD), trabecular number (Tb.N), bone volume/total volume (BV/TV), trabecular thickness (Tb.Th), phosphorylated STAT3 (p-STAT3) ratio in alveolar bone tissues, but increased receptor activator of nuclear factor-κB ligand protein expression, trabecular space (Tb.Sp), cement-enamel junction to alveolar bone crest, and osteoclast count in comparison to those of the NC group ($P<0.05$). In contrast with the PO+OTM group, the BMSCs group had significantly increased BMD, Tb.N, BV/TV, Tb.Th, p-STAT3/STAT3 ratio, and protein expressions of β-catenin and osteoprotegerin, but decreased Tb.Sp ($P<0.05$). For periodontitis rats, BMSCs can promote osteogenic differentiation to facilitate alveolar bone remodeling in the process of orthodontic tooth movement.

Keywords: Alveolar bone loss, Bone marrow, Mesenchymal stem cells, Periodontitis

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Introduction

As a most frequent-occurring oral disease in clinical practice, periodontitis exhibits a prevalence rate of about 11% according to global data [1]. Periodontitis leads to injuries of periodontal tissues, usually accompanied by pathological tooth migration, which injures occlusion to speed up periodontal deterioration plus the loss of alveolar bone. Finally, secondary malocclusion is developed, severely affecting the oral health and facial aesthetics of people, which often requires orthodontic treatment [2,3]. Orthodontic treatment can reduce the mobility degree of teeth with periodontal disease, during which, however, there is a risk of alveolar bone loss. Hence, discovering methods able to effectively avoid the loss of alveolar bone in the period of orthodontic therapy for periodontitis is a hot research topic. Belonging to mesenchymal cells extracted from bone marrow, bone marrow mesenchymal stem cells (BMSCs) are potentially capable of not only differentiating into osteoblasts but also promoting bone tissue generation [4,5]. BMSCs are characterized by rich sources, convenient acquisition, multiple differentiation abilities, and anti-inflammatory and immunomodulatory properties, so they have been widely applied in clinical practice. BMSCs are of great significance for the repair of bone defects [6]. Signal transducer and activator of transcription 3 (STAT3) is a vital player in bone metabolism. After knockout of STAT3 in osteoblasts, the bone morphology of mice becomes obviously abnormal, and STAT3 can also mediate the osteogenic effect of osteoblasts on mechanical stress loading [7]. It is therefore speculated that STAT3 may be involved in regulating orthodontic tooth movement and bone remodeling. For periodontitis patients, β-catenin, a core regulatory factor

in the downstream of the typical Wnt pathway, has been proven to have a high expression in gingival tissues and a relation to periodontal destruction [8]. However, whether the STAT3/β-catenin pathway in periodontitis rats can act as a mechanism promoting alveolar bone remodeling throughout orthodontic tooth movement has not been reported yet.

In this study, therefore, whether BMSCs can promote alveolar bone remodeling in the course of orthodontic tooth movement by modulating the STAT3/β-catenin pathway in periodontitis rats was explored.

Material and Methods

Ethical Approval

This study has been approved by the ethics committee of Tai'an 88 Hospital on November 15th, 2022 (approval No. 2022-273).

Laboratory Animals

Liaoning Changsheng Biotechnology Co., Ltd. provided 40 SPF-grade male Sprague-Dawley (SD) rats [license No.: SCXK (Liaoning) 2020-0001], with an age of 6-8 weeks old plus a weight of 220-240 g. Besides, 31-week-old female SD mice weighing 18-20 g were bought. The rats and mice were kept in a unified animal house under 45-55% indoor relative humidity and 22-26°C room temperature, with a 12 h/12 h light/dark cycle, which were permitted to eat food and drink water freely during 1 week of adaptive feeding.

Reagents and Apparatus

Static, a STAT3 small molecule inhibitor, was bought from Selleck (USA). Wuhan Boster Biological Technology Co., Ltd. (China) supplied the antibodies against receptor activator of nuclear factor-κB ligand (RANKL), STAT3, phosphorylated STAT3 (p-STAT3), osteoprotegerin (OPG), β-catenin, and alkaline phosphatase (ALP), and rabbit anti-rat cluster of differentiation 29 (CD29), CD34, CD44 and CD45 antibodies were provided by Shanghai Qiming Biotechnology Co., Ltd. (China). A kit for bicinchoninic acid (BCA) was offered by Beijing Solarbio Science & Technology Co., Ltd. (China). The instruments used in this study included a dynamometer (Hangzhou Aosu Dental, China), a desktop micro-computed tomography (CT) scanner (Bruker, Siemens, Germany), and an optical microscope (Guangzhou Micro Domain Optical Instrument Co. Ltd., China).

Isolation and Identification of BMSCs

Bilateral femurs of the 3 1-week-old mice were taken out, from which the metaphyseal red bone marrow was aspirated, followed by culture in IMEM (containing 20 μg/mL heparin). Next, bone marrow cells were resuspended and subjected to 10 min of centrifugation at 1500 rpm. Afterwards, following the absorption and discarding of the supernatant, Dulbecco's modified Eagle medium was applied to dilute bone marrow cells to 10 mL, which were sieved using a 90-mesh filter screen. Thereafter, bone marrow cells were cultured in a 37°C incubator containing 5% CO_2 for 24 h. With the 80-90% confluence achieved, trypsin (0.25%) was added for cell digestion, and then the cells were passaged (1:2), followed by observation of morphology of the third- to fifth-generation cells under the microscope. The third-generation BMSCs were harvested for 5 min of 1000 rpm centrifugation. Next, monoclonal antibodies against CD29, CD90, CD45 and CD34 were added to each tube in turn and incubated in a dark ice box for 45 min. After that, 1% BSA-containing PBS was used for cell resuspending, and CD45, CD29, CD34, and CD90 as the cell surface markers were detected by a flow cytometer.

Alizarin Red Staining

BMSCs passaged to the third generation were harvested, inoculated in each well of a 6-well plate after adjusting the density to 2×10^5/cells and subjected to 24 h of incubation. Next, osteogenesis induction medium (10 mm/L β-glycerophosphate, 10 mL of fetal bovine serum, 50 μm/L antithrombotic, 89 mL of Dulbecco's modified Eagle medium/F12, 1 mL of double antibody, and 100 nm/L dexamethasone) was utilized to substitute the conventional culture medium, followed by 21 d of continuous culture, during which the medium was replaced once every 3 d. Afterwards, the original culture medium was discarded, and then paraformaldehyde solution (4%) was added for 30-min fixation of washed cells. Thereafter, alizarin red staining was performed on the cells for 3 min, followed by cell washing. Finally, cells were observed under the microscope for their osteogenic capacity.

Grouping and Modeling

A random number table was employed to set up groups for totally 40 rats, namely model group (n=30) plus negative control (NC) group (n=10). Then models of periodontitis were established using all rats from model group according to the following steps. Firstly, 10% chloral hydrate was injected intraperitoneally into rats for anesthesia. Then the first molar on the left maxilla of rats was peeled off with a probe, a 0.2 mm deep retention groove was ground in the gingival sulcus near the mesial tooth neck of the molar with a rapid hand grinding machine, and the first molar was ligated with a 0.2 mm ligature. Next, rats were fed with high sugar water (100 g/L) and softened feed for 1 month. Red and swollen gums, bleeding on probing and periodontal pocket formation suggested successful modeling of periodontitis [9].

All the 30 rats were successfully prepared into models

of periodontitis, and the model of orthodontic tooth movement was prepared. In brief, after anesthetizing rats, a retention groove with a depth of 0.2 mm was ground on the distal surfaces of the necks of the two maxillary central incisors. Then a tension spring was utilized, with one end connected to the molar of rats and the other end connected to the incisors of rats. The force of tension spring was adjusted to 50 g by the dynamometer, and the force applying device was removed after force application for 21 d. Next, the first molar and two incisors of rats were ligated with ligature wires to keep the first molar at the position after movement. One day before the removal of the force applying device, the 30 rats were then evenly and randomly divided into periodontitis + orthodontic tooth movement (PO+OTM) group, BMSCs group and BMSCs+Static group. No treatment was conducted on rats in NC group.

Drug Intervention

One day before the removal of the force applying device, 15 μL of BMSC suspension with a cell density of 1×10^7 cells/mL was injected into the distal local gingival tissues and buccal and palatine mucosae of the left maxillary first molar in BMSCs group. For BMSCs+Static group, based on the treatment in BMSCs group, 10 μL of Static solution (prepared as follows: Dimethyl sulfoxide was added to dissolve the STAT3 small molecule inhibitor Static, whose final concentration of 50 μmol/L was reached through normal saline dilution) was injected into the buccal and palatine mucosae of the maxillary first molar once every two days for 14 consecutive days. In NC group and PO+OTM group, an equal volume of normal saline was injected at the same site. Following 14 d of drug intervention, all rats were anesthetized by the intraperitoneal injection of 3% pentobarbital sodium and sacrificed through cervical dislocation. Then alveolar bone tissues of the left maxillary first molar were separated, some of which were quickly frozen for 30 min by liquid nitrogen for -80°C preservation using a refrigerator. Finally, 4% paraformaldehyde solution was utilized for fixation of the remaining tissues, which were decalcified for 30 d.

Micro-CT

The maxillary alveolar bone tissues were collected, followed by removal of skin and muscle tissues. Next, the alveolar bone was scanned with the micro-CT scanner under the following conditions: tube voltage: 70 kV, current: 353 μA, and scanning thickness: 8 μm, followed by three-dimensional image reconstruction. Afterwards, the degrees of alveolar bone loss and tissue damage were evaluated. The three-dimensional images were used to measure the distance from cement-enamel junction to alveolar bone crest (CEJ-ABC), bone mineral density (BMD) of rats was calculated for all groups, and trabecular thickness (Tb.Th), trabecular space (Tb.Sp), bone volume/total volume (BV/TV), and trabecular number (Tb.N) were measured.

Hematoxylin-Eosin (HE) Staining

Decalcified alveolar bone tissues were collected for paraffin embedding and tissue section (thickness: 5 μm) preparation using a slicer. Then tissue sections were deparaffinized in xylene, dehydrated with ethanol gradient, and treated with HE staining. Afterwards, they underwent dehydration together with transparentization, followed by observation under the microscope. The degree of bone defect repair was scored according to the Lane-Sandhu histological scoring criteria [10], and a higher score indicated a higher repair degree.

Tartrate-Resistant acid Phosphatase (TRAP) Staining

After deparaffinization plus rehydration, the tissue sections were put in glycollate solution and incubated in the dark at 37°C for 1 h. Thereafter, tissue sections were stained in accordance with the instructions of TRAP/ALP staining kit. Afterwards, 5 sections were randomly selected, and 5 clear positive visual fields were selected at the root of each section to count the number of osteoclasts. The cytoplasm of positive osteoclasts was pink to carmine.

Measurement of Protein Expressions of STAT3, p-STAT3, β-catenin, RANKL and OPG in Alveolar Bone Tissues by Western Blotting

The alveolar bone tissues were taken, and the BCA assay was carried out to determine the concentration of total protein therein. The protein specimens with a final concentration of 2 μg/mL were boiled in hot water for 10 min, which were subsequently preserved by the -20°C refrigerator. Next, protein samples (50 μg) were collected, mixed with loading buffer, and denatured in boiling water. Thereafter, SDS-PAGE separation and PVDF membrane transfer were adopted for the protein specimens. Afterwards, 5% skim milk was supplemented to seal the membrane for 1 h, followed by membrane washing with Tris-buffered saline solution with Tween (1 mL/L). Afterwards, primary antibodies were added for overnight incubation (4°C) of the membrane, and then cleaning agent was used for rinsing. Thereafter, secondary antibodies (1:5000) were employed for room-temperature incubation of the membrane for 2 h. Then electrochemiluminescence liquid was added for development and exposure. Finally, Image Lab software was adopted to analyze the relative expression of each protein, with GAPDH as the internal reference.

Statistical Analysis

Experimental data were subjected to statistical analysis

through GraphPad Prism 8.0 software. Mean ± standard deviation (X±s) was used as the expressing format of all measurement data with normal distribution. The measurement data underwent statistical comparison among groups by one-way ANOVA and between groups through the LSD-t test. The difference of statistical significance was denoted with P<0.05.

Results

Microscopically, BMSCs passed to the third generation were mostly spindle-shaped and arranged radially *(Fig. 1-A)*. The results of flow cytometer exhibited a positive expression rate of 98.3%, 99.1%, 2.3%, and 3.6% for CD29, CD90, CD45, and CD34 antibodies, respectively, which are in line with the surface markers of mesenchymal stem cells, implying that the isolated cells were BMSCs *(Fig. 1-B-E)*.

According to alizarin red staining assay, BMSCs had more mineralized nodules and a significantly increased alizarin red stained area than Control cells (P<0.05), indicating that BMSCs possess osteogenic capacity *(Fig. 2)*.

Compared with those in NC group, Tb.Th, BMD, Tb.N, and BV/TV significantly declined while CEJ-ABC plus Tb.Sp significantly rose in PO+OTM group (P<0.05). In contrast with PO+OTM group, BMSCs group had significantly elevated BMD, Tb.Th, Tb.N, and BV/TV but significantly dropped CEJ-ABC and Tb.Sp (P<0.05). BMSCs+Static group presented significantly lower BMD, Tb.N, BV/TV, and Tb.Th, as well as significantly higher Tb.Sp and CEJ-ABC than BMSCs group (P<0.05) *(Fig. 3)*.

In NC group displayed neatly arranged periodontal membrane fibers together with smooth alveolar bone and root surfaces and no distinct osteoclasts. In PO+OTM group, periodontal membrane fibers were disorganized, and the alveolar bone was observed with scattered bone absorption lacunae on the pressure surface in addition to active osteoclasts on the noncontinuous surface, with a marked elevation in the number of osteoclasts compared with NC group (P<0.05). As for BMSCs group, periodontal ligament fibers exhibited gradually regularized arrangement, and by contrast to PO+OTM group, it had a significantly smaller number of osteoclasts (P<0.05). According to *Fig. 4*, BMSCs+Static group displayed similar changes to PO+OTM group, with obviously disordered or even broken periodontal ligament fibers and an obvious increase in the number of osteoclasts in comparison to BMSCs group (P<0.05).

PO+OTM group had lower p-STAT3/STAT3 ratio and protein expressions of β-catenin and OPG and a higher protein expression of RANKL in alveolar bone tissues

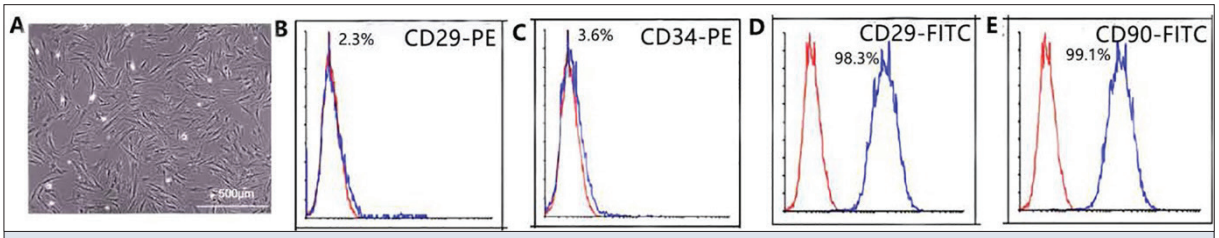

Fig 1. Morphology and identification of BMSCs. **A:** BMSCs typically spindle-shaped and arranged radially, **B-E:** Identification of cell surface markers by the flow cytometer

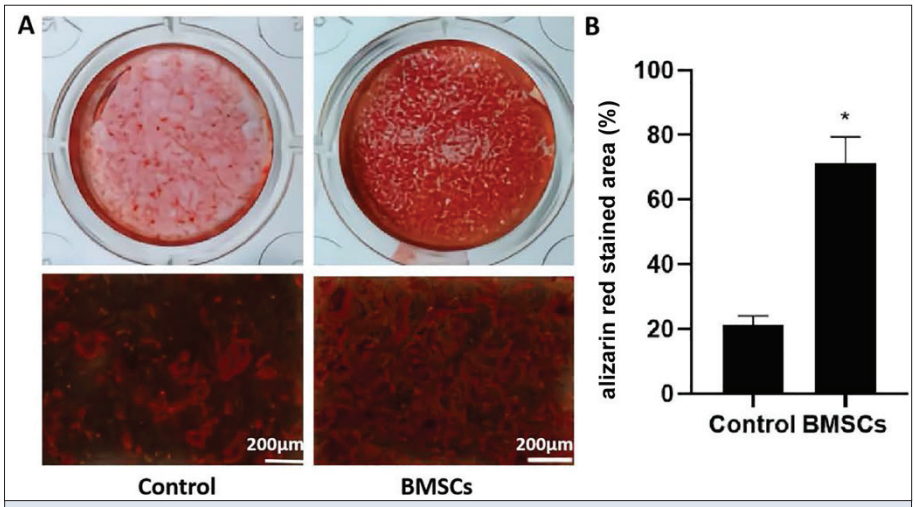

Fig 2. Identification of osteogenic capacity of BMSCs. **A:** Alizarin red staining assay, **B:** Comparison of alizarin red stained area. *P<0.05 *vs.* NC Group

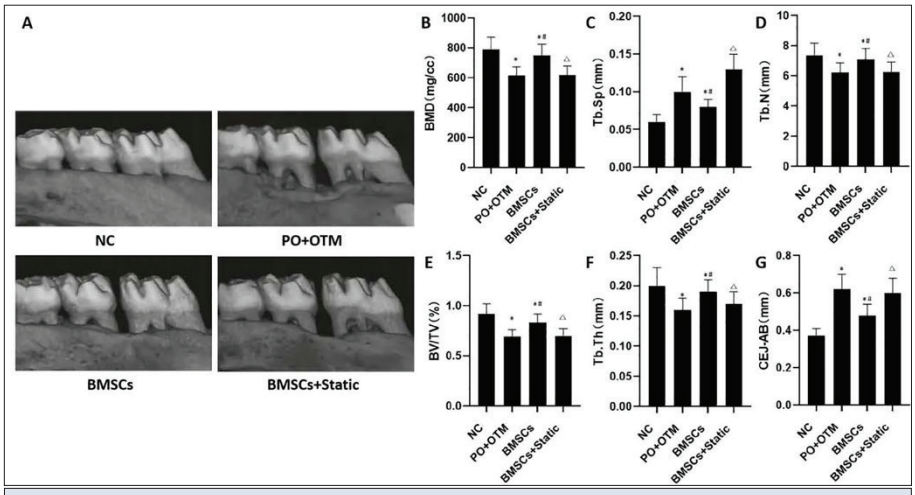

Fig 3. Alveolar bone remodeling of rats. **A:** f Alveolar bone under micro-CT scan, **B-G:** Comparisons of Tb.Th, BMD, BV/TV, Tb.Sp, Tb.N, and CEJ-ABC in alveolar bone. *P<0.05 *vs.* NC Group, #P<0.05 *vs.* PO+OTM Group, #P<0.05 *vs.* BMSCs Group

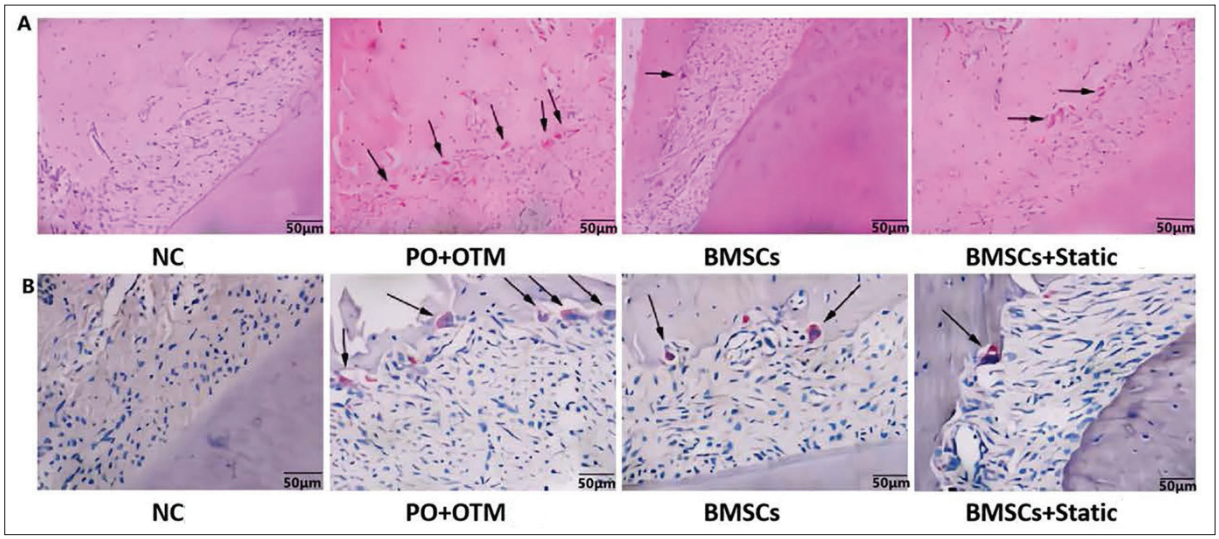

Fig 4. HE + TRAP staining assays on alveolar bone tissues. **A:** HE staining assay on alveolar bone tissues (×200), **B:** TRAP staining assay on alveolar bone tissues (×200). The black arrow represents osteoclasts

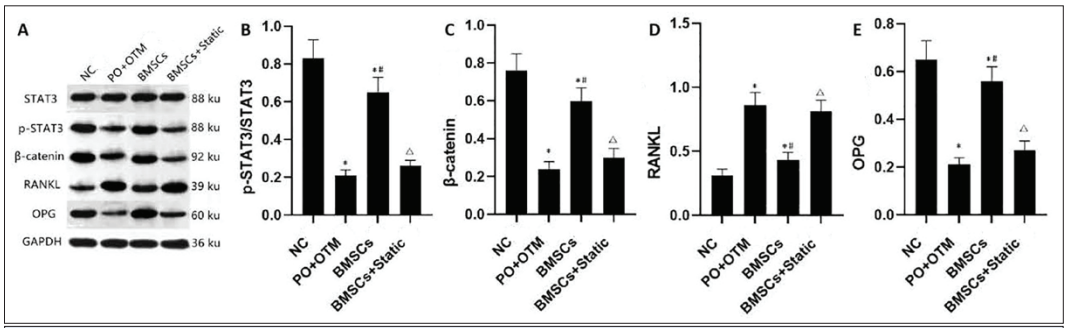

Fig 5. Expressions of STAT3, p-STAT3, β-catenin, RANKL and OPG proteins in alveolar bone tissues of rats. **A:** Protein bands of STAT3, p-STAT3, β-catenin, RANKL and OPG, **B:** Comparison of p-STAT3/STAT3 ratio in alveolar bone tissues, **C:** Comparison of protein expression of β-catenin from alveolar bone tissues, **D:** Comparison of expression of RANKL protein from alveolar bone tissues, **E:** Comparison of expression of OPG protein from alveolar bone tissues. *P<0.05 *vs.* NC Group, #P<0.05 *vs.* PO+OTM Group, △P<0.05 *vs.* BMSCs Group

than NC group (P<0.05). Compared to PO+OTM group, BMSCs group displayed elevated p-STAT3/STAT3 ratio and protein expressions of β-catenin and OPG and a decreased protein expression of RANKL in alveolar bone tissues (P<0.05). BMSCs+Static group possessed significantly lower p-STAT3/STAT3 ratio and protein expressions of β-catenin and OPG in alveolar bone tissues besides significantly higher protein expression of RANKL than BMSCs group (P<0.05) *(Fig. 5)*.

Discussion

As a chronic inflammation of periodontal tissues, periodontitis refers to gingivitis-induced inflammation spreading to the periodontal membrane, alveolar bone, cementum and so on, which can result in tooth loss in severe cases [11]. Orthodontic treatment can align dentition while bringing periodontitis under control, enabling the recovery of adjacent relationship and chewing function of teeth to normal levels [12]. However, alveolar bone loss or resorption occurs due to periodontitis-induced destruction of periodontal tissue, weakening tooth support and reducing the stability of tooth movement, which leads to the instability of teeth during orthodontic treatment to easily result in bad movement such as inclination and rotation.

Stem cell transplantation has become a new strategy for the treatment of bone defects due to the rapid development of tissue engineering [13,14]. BMSCs are considered ideal bone repair cells by tissue engineering due to such characteristics as easy isolation, convenient acquisition, abundant sources, stable biocompatibility, and osteogenic differentiation potential. BMSCs have become a hot spot in the repair of bone defects in tissue engineering in recent years since they can differentiate into many types of cells and have the potential of multi-directional differentiation. BMSCs are capable of differentiating into osteoblasts by proper induction [15], and can exert potential curative effects in the treatment of bone defects and soft tissue injuries by releasing various osteogenically active factors. Sun et al.[16] proved that BMSCs were beneficial for alveolar bone defects in rats in terms of regeneration and repair. Likewise, we herein found that BMSCs in periodontitis rats promoted alveolar bone remodeling throughout tooth movement in orthodontic treatment.

The regeneration and repair of alveolar bone defects are realized through a series of repeated tissue resorption and formation processes, during which osteoclasts are responsible for absorbing bone tissue, whereas osteoblasts are responsible for forming new bone tissue. The two kinds of cells are in a dynamic equilibrium state due to the mutual suppression between them [17]. Osteoclasts are a kind of highly differentiated cells, which are derived from hematopoietic stem cells and can be fused by peripheral blood mononuclear cells and osteoclast precursor cells. However, there are too many osteoclasts in the alveolar bone due to the inflammation caused by alveolar bone defects, and their function is abnormal, jointly giving rise to excessive bone resorption and thus weakening osteogenic function. As a result, the normal osteogenesis-osteoclast balance is destroyed, and thus the repair process of alveolar bone defects is hindered [18]. In this study, the micro-CT results shows that BMSCs significantly elevated BMD, Tb.Th, Tb.N, and BV/TV but significantly dropped CEJ-ABC and Tb.Sp. Moreover, BMSCs promoted OPG to express by impeding the production of RANKL, thus decreasing the osteoclast count in alveolar bone tissues while facilitating alveolar bone remodeling.

The Wnt/β-catenin pathway is able to mediate bone formation, i.e. bone formation can be triggered by activating the Wnt/β-catenin pathway, while it is hindered by repressing this pathway. The Wnt/β-catenin pathway can modulate osteogenesis plus osteoclast differentiation by keeping OPG/RANKL balance [19]. Lan et al.[20] reported that activating the Wnt/β-catenin pathway facilitated periodontal ligament stem cells to differentiate into osteoblasts. Besides, STAT3 may influence the Wnt/β-catenin pathway to modulate bone formation [21]. In this research, STAT3 attenuated the Wnt/β-catenin pathway from activation, inhibition of osteoclasts and promotion of alveolar bone remodeling by BMSCs in PO+OTM rats.

In conclusion, for periodontitis rats, BMSCs can promote osteogenic differentiation to facilitate alveolar bone remodeling in the process of orthodontic tooth movement, of which the action mechanism may be related to activating the STAT3/β-catenin signal to suppress osteoclast differentiation.

Declarations

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

Ethical Approval: This study has been approved by the ethic committee of our hospital (approval No. 2022-273), and great efforts have been made to minimize the animals' suffering.

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Conflict of Interest: There is no conflict of interest.

Authors' Contributions: H. Wang and F. Gong designed the study and drafted the paper; Z. Wu, D. Lai, and K. Wu performed and analyzed the experiments; X. Tian designed the study and significantly revised the paper. All authors have approved the submission and publication of this paper.

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RESEARCH ARTICLE

The Effect of *Ferula communis* L. on Body-Relative Organ Weight, Serum and Tissue Oxidative Status, Biochemical and Pathological Changes in Rats Exposed to Continuous Light

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Abstract

The goal of this study was to investigate the effects of *Ferula communis* L. on growth performance, relative organ weights, tissue and serum oxidative status, and biochemical and histopathological changes in rats exposed to continuous light. Rats exposed to continuous light for 10 days were given *F. communis* orally. Daily body weight was recorded, and rats were euthanized by cervical dislocation. Blood was collected to determine relative organ weights, followed by immediate biochemical and histopathological analysis of the organs. Serum and tissue oxidative status were measured. Continuous light exposure in rats resulted in weight loss, decreased ovary, uterus, and kidney weights, diminished total antioxidant status (TAS) along with increased cortisol, total oxidant status, and oxidative stress index. However, *F. communis* treatment reduced serum cortisol concentration and alleviated oxidative stress by increasing TAS even under prolonged light exposure conditions. Neither continuous light exposure nor F. communis treatment caused significant changes in malondialdehyde and glutathione in organ tissues. Although continuous light caused an increase in the number of cystic follicles, *F. communis* treatment did not seem to have a positive effect on cystic follicle formation. In conclusion, continuous light exposure stressed the rats and increased cortisol, as well as stimulated oxidative stress and cystic follicle formation. *F. communis* treatment can help alleviate the harmful effects of constant light exposure.

Keywords: Body weight, Continuous light stress, Cortisol, Cystic follicle, *Ferula*, Ovary, Oxidative stress

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INTRODUCTION

Ferula includes more than 170 genera, with the majority of them being found in Central Asia, the Mediterranean, and North Africa. In order to ascertain their pharmacological characteristics, *Ferula* spp. have been employed in both previous and contemporary studies on humans and animals [1]. In Türkiye, the genus *Ferula* is known as "Çakşır" and is represented by 25 species. The root components are renowned as potent aphrodisiacs in eastern Türkiye [2].

In the genus *Ferula*, components such as coumarin (ferulenol, galbanic acid, and umbelliprenin), coumarin esters (ferulon A, B), sesquiterpenes (germakrans, himacalans, carotenes, humulans, guaians, daucane esters farnesiferol A and B) and sesquiterpene lactones, monoterpene (α-pinene, β-pinene), monoterpene coumarins, prenylated coumarins, sulfur-containing derivatives, phytoestrogens (ferutinin), flavonoids, carbohydrates (galactose, glucuronic acid, arabinose, rhamnose) are well defined. This genus is utilized in traditional medicine for skin infections, psychiatric conditions, high cholesterol, diabetes, arteriosclerosis, gastrointestinal problems, osteo-

porosis, rheumatism, human immunodeficiency virus, type A influenza, cancers (uterine cancer), muscle relaxants, headache, hypertension, toothaches, and vertigo because of its abundant chemical content [2-4].

Studies to identify the phytochemical characteristics of *Ferula* have increased over the last 20 years [3,5-8]. The antioxidant qualities of extracts derived from *Ferula* species have also been the subject of certain studies. *Ferula* extracts are usually thought to have considerable antioxidant activity, according to the research [4,7,9]. *F. communis* extracts have powerful antioxidant capabilities and exhibit no cell toxicity in both traditional *in vitro* assays and *in vitro* cellular models (HL-60). It also possesses intracellular and extracellular scavenging properties. The presence of considerable amounts of phenolic compounds and the interesting and powerful antioxidant capabilities of *F. communis* were also discovered [9].

One of the most significant environmental factors influencing animal behavior is light. The temporal variation of light alters numerous physiological processes in various animal species. The major circadian oscillator in animals is located in the hypothalamic suprachiasmatic nucleus (SCN), and the retinohypothalamic pathway directly links ambient light to SCN neuronal activity. The SCN controls circadian sleep patterns, body temperature, mobility, and nutrition, and these functions may be interfered with when the SCN is negatively impacted by light. Circadian rhythms are synchronized by the length of light and darkness during the day. Circadian rhythm patterns are greatly impacted by variations in the length of the light-dark period. In primates, transitory sleep patterns and body temperature are variably influenced by ambient light, and the SCN controls these circadian cycles [10,11].

Problems including jet lag disorder, delayed sleep phase disorder, and irregular sleep-wake rhythm are experienced as a result of variations in the 24-h circadian cycle's light/dark cycles or sleep-wake cycles [10,12]. These issues can be regarded as severe stressors because they alter the homeostasis of various physiological systems. Stress is brought on by the disruption of sleep and circadian rhythms. Since lighting conditions have a negative impact on sleep, they also have a big impact on stress [13,14]. Distressing lighting alters the physiological and behavioral responses of rats as well as increasing corticosterone levels [15]. Continuous light increases both stress and fear responses in chickens [16].

Animal behavior is orchestrated by a complex interplay of factors. In rodents such as rats, these factors include the estrous cycle in females, exposure to a novel environment, and light [17]. Continuous light exposure has been shown to impact the endocrine system in rats. Specifically, it can increase serum anti-mullerian hormone and potentially exert a stress effect on the endocrine system, as indicated by elevated corticosterone concentration [18]. Moreover, continuous light exposure can increase serum testosterone, luteinizing hormone to follicle-stimulating hormone (LH/FSH) ratio, and the number of cystic-like follicles [19]. Continuous light exposure also caused reproductive activity, endocrine system disorders, and metabolic problems (glucose intolerance) in rats. Similarly, ongoing exposure to light alters the gut flora and brings on reproductive problems like polycystic ovarian syndrome (PCOS) [18].

The objective of this study was to ascertain how *Ferula communis* L. affected the body weight and relative organ weights, tissue and serum oxidative status, and biochemical and pathological alterations in rats exposed to continuous light.

Material and Methods

Ethical Approval

The Kafkas University Local Ethics Committee for Animal Experiments (KAÜ-HADYEK/2021-153), Kars, Türkiye, gave its clearance before this study could be conducted. According to the *Laboratory Animal Care and Use Manual*, all procedures were carried out.

Animal

Thirty-two, 3-month-old female Wistar albino rats weighing 275-300 g were utilized. The groups were housed in standard conditions at 23±1°C and 50±5% humidity after a fifteen-day adaptation period. Commercial pellet feed (Bayramoğlu Yem®, Erzurum, Türkiye) and water were given *ad libitum* to the groups.

Vaginal Cytology

Vaginal cytology was performed on the rats to determine their estrous cycle stage prior to the initiation of the experiment. The study started in diestrus because the luteal phase lasts longer than the follicular phase. Swabs were collected for cytology using the vaginal lavage technique as described in the literature [20]. Swabs were examined under a light microscope (Olympus CX23® Binocular Microscope, Olympus, Japan) after being stained with Giemsa (Merck, Darmstadt, Germany). When a combination of a diminished large cell profile, an elevated neutrophil count, tiny and big nucleated epithelial cells, and a very low number of anucleated keratinized cells were observed in cytology, the time period was regarded as diestrus [20]. Rats in diestrus were used in the experiment.

Progesterone Analysis

Vaginal cytology was used to determine which rats were in diestrus, and blood was taken from the tail vein and centrifuged at 3000 rpm for 10 min to assess the serum

progesterone concentration. Using commercial kits, a Roche Cobas c501® autoanalyzer (Roche Diagnostics, Mannheim, Germany) was used to measure the serum progesterone concentration. Rats were included in the groups if their progesterone concentration was greater than 1 ng/mL.

Ferula communis L. Extraction and GCMS Analysis

The *Ferula communis* L. roots of a plant were gathered from Ağrı Province, located in the Eastern Anatolia Region of Türkiye, in 2021. The plant was verified by Kafkas University Department of Botanical Sciences in Kars, Türkiye. The root oil was extracted using hydro-distillation with a Clevenger-type instrument for 4 h, using 10 mL of n-Hexane (Merck, Darmstadt, Germany) as the collecting solvent. The oil was then dried with anhydrous sodium sulfate, filtered, and stored in sealed vials at 4°C, protected from light until it was analyzed.

Three samples of oil were obtained through hydro-distillation, and analysis was carried out with GCMS using a capillary column of HP-5MS at 70eV (J & W Scientific, Folsom). The instrument was set up using the procedure developed by Nguir et al.[21]. Sigma-Aldrich, Milan, Italy, provided the standards for the component analysis. Authentic standards provided by the supplier (Sigma-Aldrich, Milan, Italy) and computer matching with the WILEY275, NIST05, and ADAMS libraries, as well as comparisons of mass spectra and retention indices (RI) to those described in the literature [22,23], were used to identify the volatile components. The percentage compositions of the oil components were calculated using electronic integration with flame ionization detection (FID, 280°C) by dividing the area of each component by the sum of the areas of all components separated under these conditions. The percentage values for volatile components were calculated using the average of three oil sample injections. The active compounds of *Ferula communis* L. roots are given in *Table 1*.

Experimental Design

The groups were formed to be balanced in terms of body weight. Pellet feeds were left in the cage to avoid casting shadows.

The experiment consisted of four groups of rats. The first group (G1) served as the control group and was not given any treatment. They were kept in 12/12 light/dark conditions for ten days. The second group (G2) was also kept in 12/12 light/dark conditions for ten days but was given *Ferula communis* L. orally by gavage at a daily dose of 150 mg/kg. The third group (G3) was kept in 12/12 light/light conditions for ten days with no treatment. The fourth group (G4) was also kept in 12/12 light/light conditions for ten days but was given *Ferula communis* L. orally by gavage at a daily dose of 150 mg/kg. Each group consisted of eight rats.

Body Weight

Rats were weighed daily for ten days.

Euthanasia

After intramuscular injections of xylazine HCl (15 mg/kg, Rompun®, Bayer, Germany) and ketamine HCl (75 mg/kg, Ketasol®, Richter Pharma AG, Austria), rats were euthanized by cervical dislocation at the end of the study. After that, rapid blood collection and organ sampling were performed.

Blood Collection

A gel-vacuum tube (BD Vacutainer® SST II Advance, Becton, Dickinson and Company, UK) was used to collect blood through a cardiac puncture. The blood was centrifuged (NF 400R®, Nüve, Türkiye) at +4°C for 10 min at 3000 rpm. Following centrifugation, the sera were separated into individual microcentrifuge tubes and kept at -18°C until biochemical analyses were performed.

Table 1. *The chemical composition of the essential oil from Ferula communis L. roots.*

No	Compounds	% Composition	No	Compounds	% Composition
1	Dillapiole	7.9	11	(E)-nerolidol acetate	3.4
2	Guaiol	7.3	12	(Z)-lanceol	3.1
3	Spathulenol	6.8	13	β-Barbatene	6.1
4	Myristicin	6.0	14	Epizonarene	6.1
5	T-cadinol	5.9	15	α-Zingibirene	4.7
6	β-Gurjunene	3.4	16	β-Bisabolene	5.1
7	(E)-β-farnesene	4.7	17	γ-Cadinene	4.8
8	(E)-nerolidol	3.5	18	Trans- Calamenene	3.9
9	T-Muurolol	3.0	19	Elemicin	6.0
10	α -Cadinol	2.8	-	-	-

Organ Weights

The stomach, liver, kidneys, spleen, total intestine, uterus, and ovaries were dissected immediately after euthanasia. The thorax was opened, and the heart and lungs were taken out and weighed. The eyes were also taken out and weighed. The aforementioned organs were removed from their surrounding tissues, and they were then instantly weighed to three decimal places (dual organs weighed together). The organs were divided for biochemical and histological analysis just after weighting. Separated for biochemical analysis were the right ovary, kidney, and eye, while for histopathological evaluation were the left ovary, kidney, and eye.

Biochemical Analyses

A fully automated ADVIA Centaur® XP analyzer (Siemens Healthcare Diagnostics, Tokyo, Japan) and a compatible commercial kit were used to assess serum 25-OH-vitamin D (vit D) and cortisol concentrations using the chemiluminescent immunoassay method.

Serum glucose, calcium (Ca), magnesium (Mg), and phosphorus (P) were measured by the photometric method using a fully automatic Abbot® ARCHITECT analyzer (Abbott Diagnostics, Lake Forest, IL, USA) and commercial kits.

Total antioxidant status (TAS) and total oxidant status (TOS) in serum were measured using a commercial kit (Rel Assay Diagnostics®, Mega Tıp, Türkiye) using the colorimetric method.

The oxidative stress index (OSI) was initially defined as the ratio of TOS to TAS. The resultant unit of TAS, mmol Trolox equivalent/L, was changed to µmol Trolox equivalent/L in order to do the calculation, and OSI was then determined using the formula below [24]:

OSI (Arbitrary unit) = TOS (µmol H_2O_2 Eq/L)/TAS (µmol Trolox Eq/L) x 100

For malondialdehyde (MDA) and glutathione (GSH) analyses, organ samples from the heart, kidney, uterus, ovary, and eye were homogenized with phosphate buffer (pH 7.4), and their homogenates were separated by centrifugation at 3000 rpm for 10 min. Before performing MDA and GSH measurements, homogenates were placed into separate microcentrifuge tubes and kept at -18°C. GSH and MDA analyses were performed on tissue homogenates using the methods described by Beutler et al.[25] and Yoshoiko et al.[26], respectively.

Histopathology

The tissues were fixed for eight hours in 10% buffered formaldehyde. Tissues were embedded in paraffin after a typical histological follow-up. Hematoxylin & Eosin was used to stain the tissues after they had been cut into 4 µm pieces and deparaffinized in an oven for 40 min at 62°C. Lastly, histopathologic features were assessed by light microscopy (Olympus BX46®, Olympus Corporation, Japan).

The left ovary sections were examined for the presence of Graafian follicles [27,28], cystic follicles, and corpus luteum [18,28].

Statistical Analysis

Before commencing the study, the sample size was determined through a power analysis using G*Power version 3.1.9.7 (University of Düsseldorf, Germany). The calculation was based on a test power of 0.95, a significance level of 0.05, and an effect size (d) of 1.83, as reported in a study [29].

The Shapiro-Wilk test was used to evaluate the normal distribution of the data. A one-way analysis of variance (ANOVA) was performed to compare normally distributed data. Following the check for homogeneity of variances with Levene's test, the Tukey honestly significant difference (HSD) test was used with the post-hoc comparison. When the variables were not normally distributed, Kruskal-Wallis ranked one-way analysis of variance was used for multiple comparisons, and pairwise comparisons of parameters with significance were evaluated using the Mann-Whitney U test. A Bonferroni correction was applied to the P value in this test result (adjustment significant P value). The chi-square test or Fisher's exact test was used to compare the ratios of Graafian follicles, cystic follicles, and corpora lutea in the groups. Data are given as mean ± standard deviation (SD). GraphPad Prism® (Version 8.0, GraphPad Software Inc., San Diego, CA, USA) and SPSS® (Version 26.0, SPSS Inc./IBM Group, Chicago, IL, USA) programs were used for statistical analysis. A P value of <0.05 was considered significant in the multiple or pairwise comparisons.

Results

Body Weight Changes

In terms of body weight change over time, there was no statistically significant difference among the groups ($P>0.05$). The control group's body weight did not alter noticeably (G1). Rats lost weight when exposed to constant light (G3). Gaining body weight was positively impacted by *F. communis* (G2). Continuous light's detrimental impact on body weight was not identified in G4. *Fig. 1* displays the change in body weight in rats.

Organ Weights

The effects of continuous light exposure on the weight of the liver, spleen, stomach, whole intestine, heart, lungs,

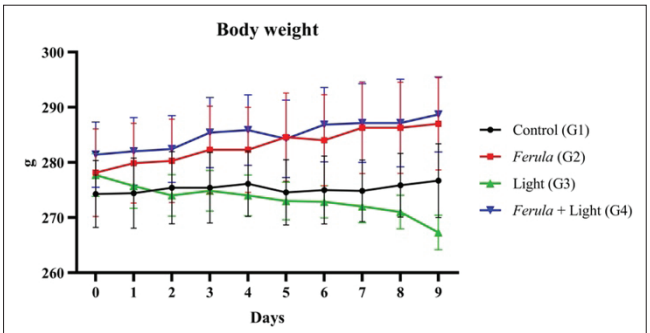

Fig 1. The differences in body weight among the groups. Across the groups, there was no statistically significant difference in body weight (P>0.05). Rats' body weight was affected negatively by continuous light but positively by *Ferula communis*

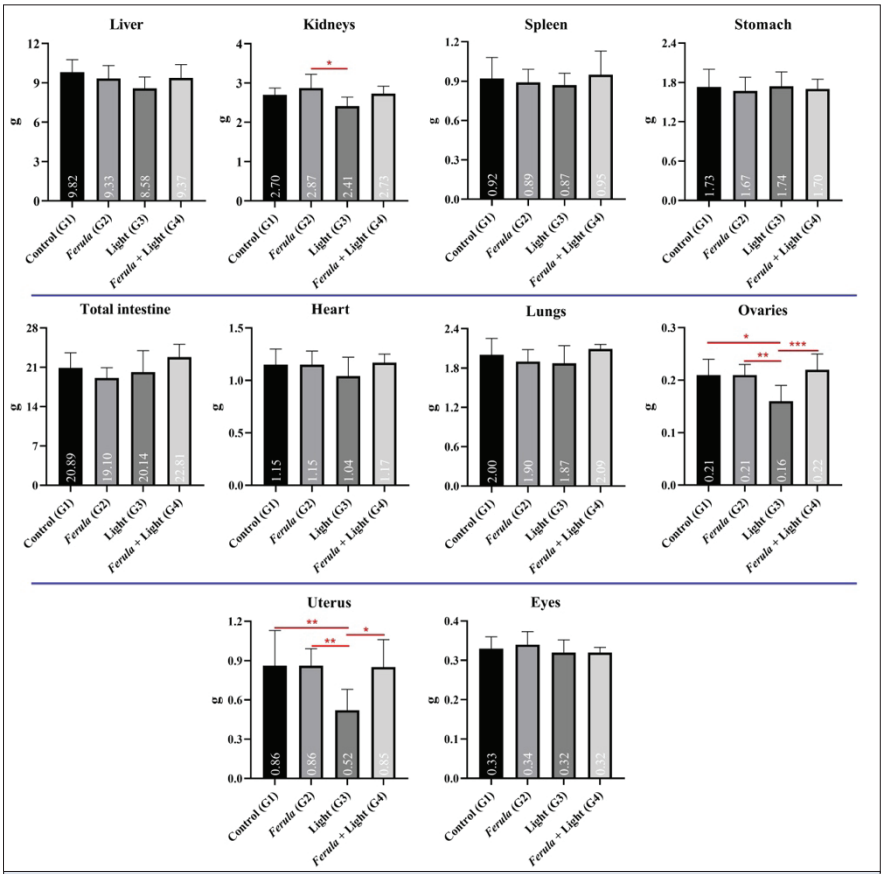

Fig 2. The changes in relative organ weights depending on the group. Continuous light exposure or *Ferula communis* treatment had no effect on the weight of the liver, spleen, stomach, total intestine, heart, lungs, or eyes (P>0.05). Kidney weight increased with *F. communis* treatment (G2) and decreased with continuous light exposure (G3). After exposure to light for an extended period of time, the ovary and uterus lost weight. Continuous light exposure had a deleterious impact on ovarian and uterine weight; however, this was mitigated by *F. communis* therapy. *: P<0.05, **: P<0.01, ***: P<0.001

and eyes were not statistically significant (P>0.05). The relative weights of the organs were unaffected by the *F. communis* treatment, either positively or negatively *(Fig. 2)*. The relative kidney weight was statistically different between the G2 and G3 groups, increasing in G2 but decreasing in G3 (P=0.017, *Fig. 2)*.

Regarding relative organ weight, prolonged exposure to light had the greatest impact on the uterus and ovaries *(Fig. 2)*. A statistical significance was found in the multiple comparisons of ovarian weight between groups (P=0.003). In G3 compared to G1, G2, and G4, ovarian weight was significantly decreased (P=0.023, P=0.01, and P=0.005, respectively). Relative uterine weight was changed by continuous light exposure, and multiple comparisons of groups were statistically significant (P=0.009). Uterine weight was lower in G3 than in G1 (P=0.02), G2 (P=0.02),

and G4 (P=0.025). Surprisingly, *F. communis* treatment (G4) seems to have removed the deleterious effects of continuous light exposure on both ovarian and uterine weight *(Fig. 2)*.

Biochemical Changes

The highest serum vit D concentration was measured in G3, there were no statistically significant differences among groups. (P>0.05, *Fig. 3)*.

Continuous light exposure in G3 generated a substantial increase in serum cortisol concentration compared to the other groups (G1, G2, and G4). Multiple comparisons for serum cortisol concentration were statistically significant (P=0.003). *F. communis* tends to lower serum cortisol concentrations even with prolonged exposure to light (G4). Surprisingly and remarkably, there was a statistical difference (P=0.04) in blood cortisol between G3 and

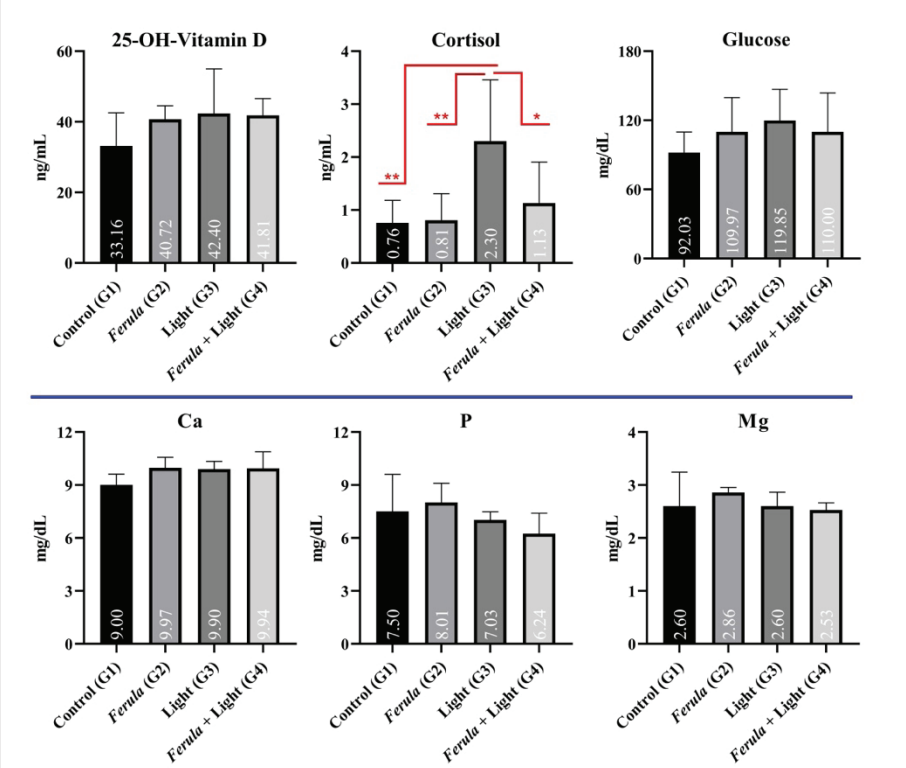

Fig 3. Group-based variations in several biochemical parameters. The difference in blood cortisol concentrations between G3 and G4 is crucial. Vitamin D, glucose, Ca, Mg, and P were unaffected by either continuous light exposure or *Ferula communis* treatment. *: P<0.05, **: P<0.01

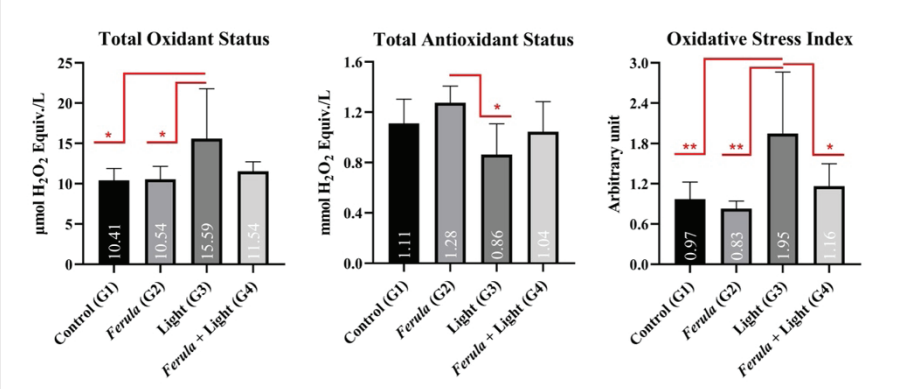

Fig 4. Change of oxidative status in groups. Continuous light exposure (G3) increased total oxidant status (TOS) and oxidative stress index (OSI) and decreased total antioxidant status (TAS). *Ferula communis* treatment, on the other hand, decreased TOS and increased TAS when exposed to continuous light, but these effects were limited. But when exposed to constant light, the *F. communis* treatment reduced OSI. OSI was statistically lower in G4 than in G3, suggesting that *F. communis* may be effective in reducing oxidative stress. *: P<0.05, **: P<0.0

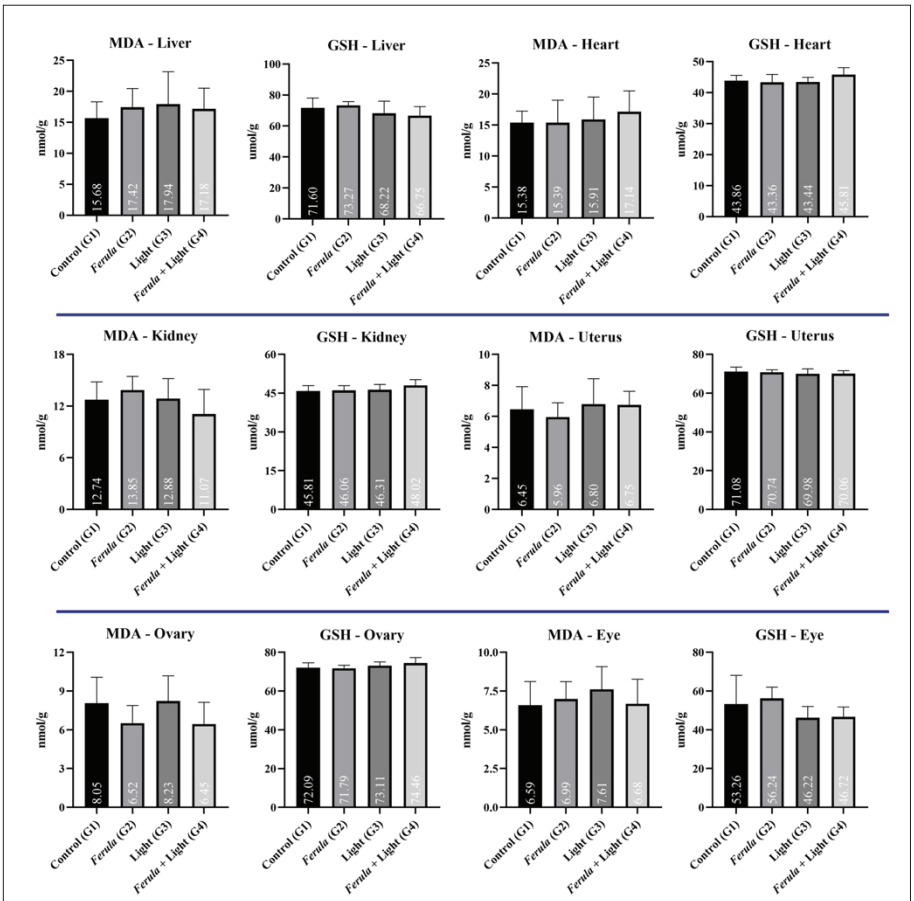

Fig 5. Change of tissue oxidative status in groups. Malondialdehyde (MDA) and glutathione (GSH) in the liver, heart, kidney, uterus, ovary, and eye were not significantly changed by continuous light exposure or treatment with *Ferula communis* (P>0.05)

G4 *(Fig. 3)*. Again, serum cortisol concentration was not altered in G4 exposed to continuous light. The absence of a significant difference between G1 and G4 shows that *F. communis* is able to avoid an increase in cortisol following continuous light exposure *(Fig. 3)*.

Serum glucose concentrations were not statistically significant among groups (P>0.05). The variation in serum glucose concentration according to groups is shown in *Fig. 3*. Continuous light exposure or *F. communis* treatment did not have a statistically significant effect on serum Ca, P, and Mg concentrations (P>0.05, *Fig. 3)*.

Serum Oxidative Status

There was a statistically significant difference in TOS between the groups (P=0.025). Constant light exposure enhanced TOS in rats. The increase in TOS in G3 was significantly different compared to G1 (P=0.037) and G2 (P=0.043) *(Fig. 4)*. The *F. communis* treatment (G4) reduced continuous light stress but was not statistically significant compared to G3 (P>0.05).

Although the *F. communis* therapy (G2) increased TAS compared to G1 and G4, it was not statistically significant. TAS decreased as a result of continuous light exposure (G3). Therefore, there was a statistically significant difference between G2 and G3 (P=0.005). Although there was no statistically significant difference, TAS appears to be higher in G4 than in G3 *(Fig. 4)*.

Continuous light exposure (G3) increased OSI in rats. OSI in G3 was statistically higher than in G1 (P=0.007), G2 (P=0.002) and G4 (P=0.04) *(Fig. 4)*. The *F. communis* treatment had a positive effect on the reduction of OSI. OSI was lower than G3 in G4 and statistically similar to G1 and G3 *(Fig. 4)*.

Tissue Oxidative Status

Rat liver, heart, kidney, uterus, ovary, and eye MDA and GSH levels were not significantly affected by continuous light exposure or *F. communis* treatment (P>0.05, *Fig. 5)*. Thus, continuous light stress or treatment with *F. communis* had no effect on the tissue's oxidative status *(Fig. 5)*.

Histopathology

The livers of all rats did not exhibit any signs of necrosis, apoptosis, or fibrosis. There was only mild

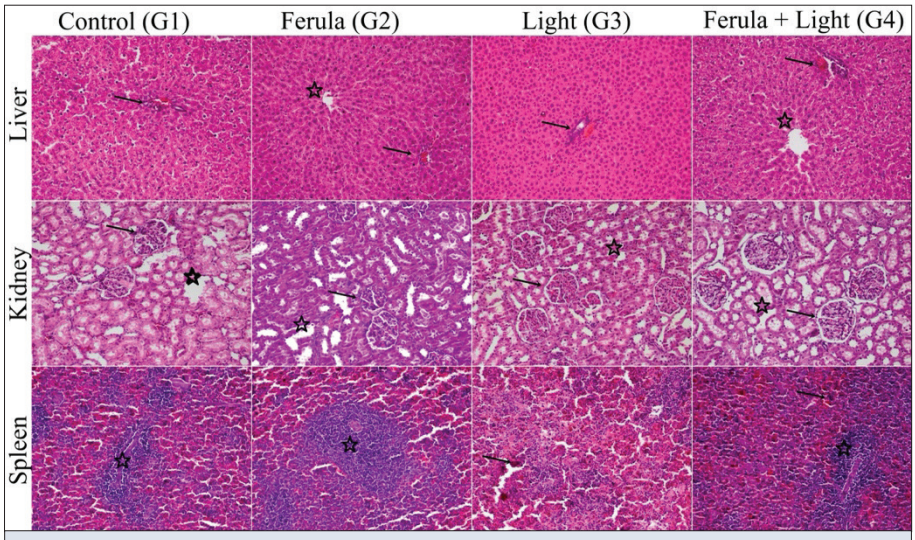

Fig 6. Histopathological changes in various organs (H&E x200). Liver - G1: Liver tissue of the control group (H&E, x200) in the usual histomorphology with the portal area indicated by the arrow. G2: Portal area shown with an arrow, central vein with an asterisk. Liver tissue of the *Ferula communis* group in usual histomorphology (H&E, x200). G3: The liver tissue of the light group with mild portal inflammation, the portal area is indicated by the arrow (H&E, x200). G4: Mild portal inflammation with portal area with arrow and central vein with asterisk, liver tissue of the Light + *F. communis* group (H&E, x200). Kidney - G1, G2, G3, and G4: Arrows represent glomeruli, stars represent tubules. Renal tissue samples in usual histomorphology (H&E, x200). Spleen - G1, G2: Samples of spleen tissue in usual histomorphology. Asterisks indicates white pulp (H&E, x200). G3: The arrow indicates one of the hemosiderin-loaded macrophages in the red pulp. Pigmentation of the macrophage represents the sign of bleeding (H&E, x200). G4: A star indicates white pulp, and an arrow indicates one of the hemosiderin-loaded macrophages in the red pulp. Pigmentation of the macrophage represents the sign of bleeding (H&E, x200)

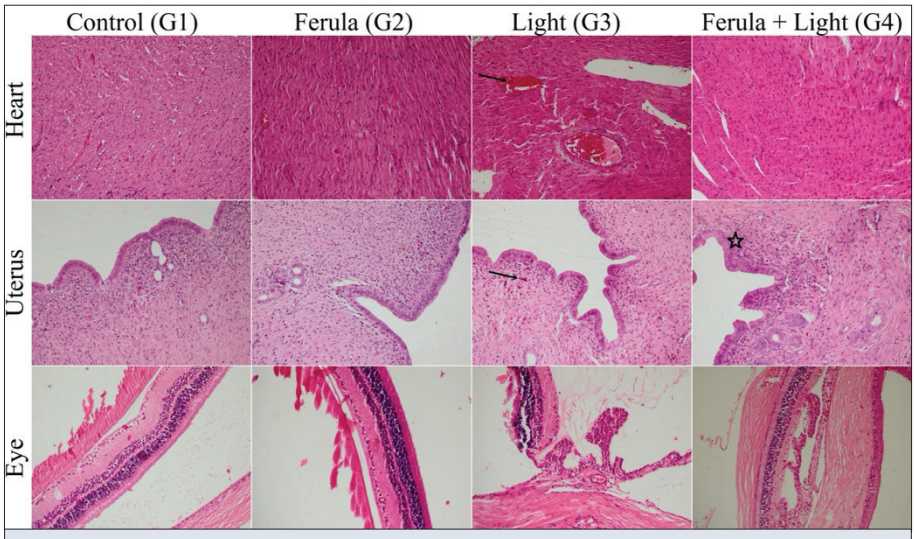

Fig 7. Histopathological changes in various organs (H&E x200). Heart - G1, G2, G3, and G4: Striated muscle cells in sections of the heart. G3: Slight congestion is observed in the vessels in the sections belonging to the light group with the arrow sign (H&E, x200). Uterus - G1, G2: Normal histomorphology shows endometrial glands, stroma, and myometrium in sections (H&E, x200). G3: One of the hemosiderin-loaded macrophages representing the bleeding sign in the light group is shown with an arrow (H&E, x200). G4: Signs of endometritis are observed in the focal area, with a few neutrophil infiltrations marked with an asterisk (H&E, x200). Eye - G1, G2, G3, G3: Retinal tissue samples in usual histomorphology (H&E, x200)

portal inflammation and congestion, and no observable difference in morphology between the groups. The kidneys had minimal congestion, hyaline cast deposition in some areas, and calcification in collecting ducts in a few tissues, but no evidence of necrosis, apoptosis, or inflammation. The spleen tissue of G1, G2, and G4 had areas of congestion and hemosiderin-loaded macrophages, but no other pathological findings. G3 showed more prominent bleeding and congestion, but not significantly different from the other groups *(Fig. 6)*. The heart tissues showed

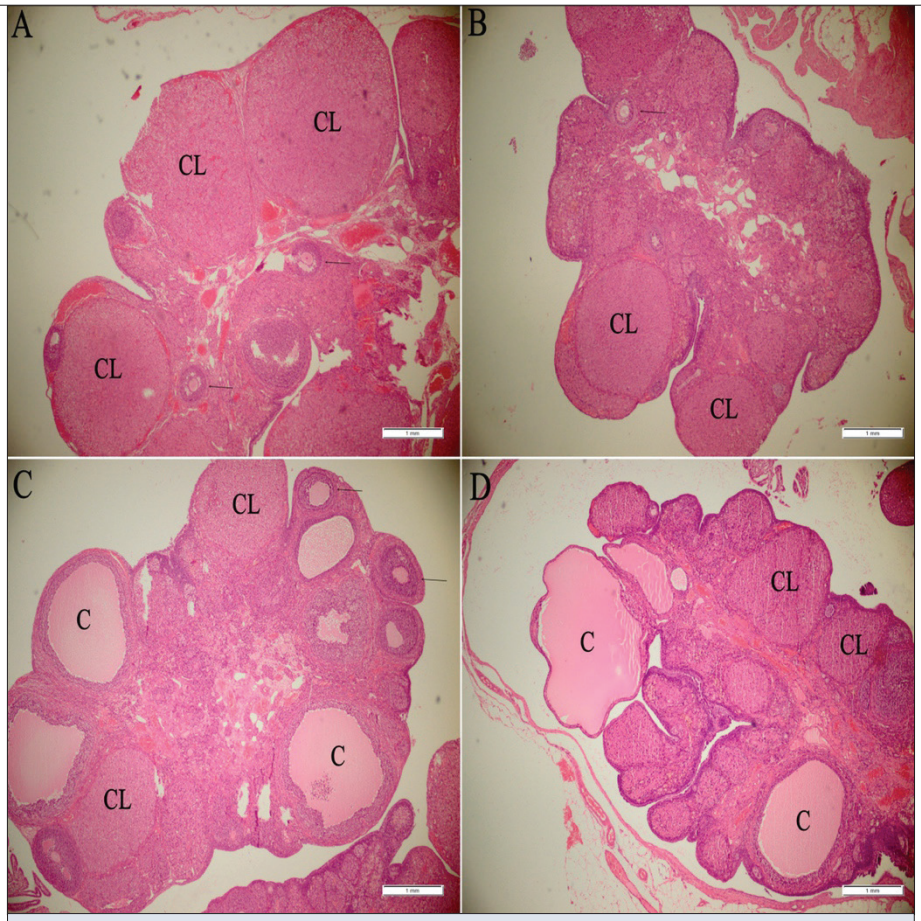

Fig 8. Ovarian histopathological alterations in the various groups. **A:** Numerous secondary follicles *(arrow)* and corpus luteum (CL) are seen in the G1 (H&E, x40), **B:** In G2, the corpus luteum (CL) and the primary follicle, which is marked by an arrow, are visible (H&E, x40), **C:** In G3, there are more secondary follicles *(arrow),* corpus luteum (CL), and follicular cysts (C) (H&E, x40), **D:** A considerably smaller corpus luteum (CL) and enlarged follicular cysts (C) in G4 (H&E, x40)

bleeding and congestion, but no other pathological finding. Uterine samples were examined for congestion, hemorrhage, endometritis, and hyperplasia, but none of the rats had hyperplasia. Some rats had focal endometritis and hemosiderin-loaded macrophages, indicating previous bleeding. Ocular tissue samples consistently showed the typical histomorphological appearance. The typical histomorphological appearance was consistently seen in the ocular tissue samples *(Fig. 7)*.

The ovary was examined in different groups of rats, and in G1, the structures looked normal, with a corpus luteum present and no cystic follicles seen in the ovarian sections *(Fig. 8-A)*. In G2, two rats lacked corpora lutea, while two others had cystic follicles *(Fig. 8-B)*. In G3, cystic follicles were found in almost all rats and were also present in the corpus luteum *(Fig. 8-C)*. Finally, in G4, all rats had both cystic follicles and the corpus luteum *(Fig. 8-D)*.

There were no significant differences between the groups regarding the presence of Graafian follicles or corpora lutea (P>0.05). However, while no cystic follicles were found in G1, the highest rate of cystic follicles was observed in G3 and G4 (P<0.05). Exposure to continuous light was found to increase the incidence of cystic follicles in rats. Interestingly, in G3 and G4, where the rate of cystic follicles was high, all rats still had corpus luteum. Although continuous light exposure increased the number of cystic follicles, treatment with *F. communis* did not have a positive effect on cystic follicle formation *(Table 2)*.

Table 2. *Ratio of Graafian follicle, cystic follicle and corpus luteum in groups*

Groups	Graafian Follicles % (+/-)	Cystic Follicles % (+/-)	Corpus Luteum % (+/-)
Control (G1)	100 (8/0)	0[a] (0/8)	100 (8/0)
Ferula (G2)	87.5 (7/1)	25[a] (2/6)	75 (6/2)
Light (G3)	100 (8/0)	87.5[b] (7/1)	100 (8/0)
Ferula + Light (G4)	100 (8/0)	100[b] (8/0)	100 (8/0)

[a-b] *Different letters in the same column are statistically significant (P<0.05)*

Discussion

Ferula communis exhibits a wide range of pharmacological effects both *in vitro* and *in vivo*, such as antidiabetic, antibacterial, antiproliferative, and cytotoxic actions. It comprises various bioactive compounds with diverse biological activities [4,21,30]. Likewise, several studies have shown that *Ferula communis* possesses potent antioxidant activity [9,31]. Therefore, our goals were to induce stress in rats by continuously exposing them to light and to evaluate the effects of Ferula communis treatment on body weight, relative organ weight, serum and tissue oxidative status, and biochemical and pathological changes. *F. hermonis* treatment had a positive effect on body weight gain in growing female and male rats [32]. Continuous light exposure has a limited effect on body weight [15,18,33]. Short-term continuous light exposure in our study slightly decreased body weight gain, but it was not statistically effective. However, if the light duration was extended, perhaps it would have an effect on body weight. The decrease in weight gain can be explained by the fact that continuous light exposure reduces feed consumption [34]. In the present study, rats exposed to continuous light exhibited a favorable response to *F. communis* treatment in terms of weight gain.

In our study, neither continuous light exposure nor *F. communis* treatment had a significant effect on most of the relative organ weights in rats, which was further supported by histopathological findings. Continuous light exposure did, however, adversely affect the relative weights of the ovaries and uterus in rats. The G3 group had significantly lower ovary and uterus weights compared to the other groups. Similar results were observed in a study where rats were exposed to continuous light for an extended period [35]. In this study, *F. communis* appeared to mitigate the negative effects of continuous light on ovary and uterus weight. It is possible that continuous light exposure disrupts the estrous cycle [18], leading to lower genital organ weights in G3. *F. communis* may have facilitated more regular estrous cycles through its estrogenic effects. A study supporting our findings reported that *Ferula* can be used as a therapy for female infertility [36]. In our study, kidney weight was not significantly affected by continuous light exposure but was lower in the *F. communis* group compared to the control group. Histopathologically, the kidney tissue was normal in all groups. This was an interesting finding. Perhaps continuous light exposure led to reduced water intake in rats, which in turn could have contributed to the decrease in kidney weight [34].

Long-term exposure to light can interfere with circadian rhythms, which has an impact on key physiological processes and organ systems [37]. Agonistic behaviors that disturb the in-group order are also brought on by constant light exposure in some animals [15]. Along with in-group restlessness, disrupted physiological processes and agonistic behaviors lead to stress, and the most significant consequence of this is an increase in corticosterone concentration [15,38]. As blood cortisol concentration was found to be statistically higher in G3 compared to the other groups, our study suggests that continuous light exposure (G3) induces severe stress. While the behavior of the groups was not directly observed, it is inferred that G3 experiences severe stress due to constant illumination. The results in G4 were also highly intriguing, as *F. communis* may have prevented the increase in serum cortisol concentrations following prolonged exposure to light. G4's serum cortisol concentrations was lower than that of G3 because it was comparable to that of G1 and G2. *F. communis* may include anticatabolic substances that function by inhibiting cortisol receptors, yet it is difficult to describe how *Ferula* carries out this activity [39]. Also, there is evidence to suggest that the ferulic acid found in *F. communis* lowers corticosterone concentrations [40].

F. communis possesses a large number of antioxidants and has been proven to be a potent radical scavenger in numerous tests [3,4,30]. The active component of *Ferula*, ferutinin, possesses antioxidant, antibacterial, anti-inflammatory, estrogenic, and anti-cancer properties [41]. *Ferula* contains flavonoids, which act as natural antioxidants and free radical scavengers [42]. Numerous studies have demonstrated that a wide range of diseases and inflammatory conditions trigger and exacerbate oxidative stress in the organism [43-45]. Stress was induced in this study by continuous light exposure, and an increase in serum oxidative stress parameters was also observed. Constant illumination increased TOS and OSI while decreasing TAS in G3. This was able to be changed by the *F. communis* treatment, which increased TAS in G4. Again, OSI decreased more in G4 than in G3. No appreciable variations in tissue oxidative status were discovered. In any event, prolonged exposure to light did not seriously damage the tissues. The ovary was found to be more sensitive to continuous light exposure than other organs in histopathological investigations. The results show that *F. communis* can be employed as a radical scavenger to defend against oxidative stress.

Continuous light exposure can form polycystic ovary-like structures in rats. Continuous light can prevent the formation of the luteinizing hormone peak in rodents, preventing ovulation from triggering. In earlier studies, this topic has been thoroughly examined [18,46,47]. In our study, continuous light induced cystic follicle formation. Cystic follicles were detected in both G3 and G4 groups. The presence of corpus luteum alongside these cystic follicles suggests that cyclic activity continued in the rats. While serum progesterone levels were not measured at

the study's conclusion, histopathological analysis revealed the clear presence of numerous corpus luteum. Despite the estrogenic properties of Ferula, it appears to have partially induced cystic follicle production. However, corpus luteum were not identified in two G2 rats. This may have been brought on by *Ferula*'s estrogenic effects. Moreover, the rate of cystic follicles was not sufficiently decreased with *F. communis* treatment. The percentage of cystic follicles in G4 was statistically similar to G3. The impact of *Ferula* on the cystic follicle may become clearer with continued use. It should be emphasized that cystic follicles are less frequent in G4 histopathologically. In patients with polycystic ovaries, *F. communis* treatment has already decreased the number of cystic follicles [48,49]. *F. communis* may have successfully treated it by suppressing the increased androgen concentration in the polycystic ovary with an estrogenic effect [48].

In conclusion, continuous light exposure can cause stress and increase cortisol concentration in rats. It can also lead to oxidative stress and cystic follicle formation. *Ferula* treatment can reverse the harmful effects of continuous light exposure with its antioxidant and estrogenic effects. There may still be some unknowns regarding *Ferula's* ability to eliminate cystic follicles. Therefore, detailed studies are needed to determine the effects of short-term or long-term use of *F. communis*.

Declarations

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

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Ethical Approval: Kafkas University Local Ethics Committee for Animal Experiments (KAÜ-HADYEK/2021-153), Kars, Türkiye, gave its clearance before this study could be conducted.

Conflict of Interests: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions: BBK, MM: Designing the study, MM, MK, FB, BBK: Implementation of the experimental protocol, data collection, BBK, TK: Data interpretation, data analysis and statistical analysis, MM, MK: Biochemical analysis, MÖ, TŞ: Extraction and GCMS analysis, GY, HB: Histopathology, BBK: Writing-Original drafting. All authors contributed to the article and gave final approval of the version to be submitted.

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Research Article

Probiotic Edible Film Added with Aqueous Clove Extract Milk Powder [1][2]

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Abstract

The integration of aqueous clove extract (ACE) and spray-dried probiotics into an edible film presents numerous health advantages, such as enhanced probiotic stability and antibacterial efficacy against harmful bacteria like *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. This study aimed to formulate a probiotic edible film with antibacterial properties by incorporating milk powder containing ACE and probiotic strains, namely *Streptococcus thermophilus* ATCC 19258, *Lactobacillus bulgaricus* ATCC 11842, and *Lactococcus lactis* MG1363. The milk powders, containing 7.5% (w/v) ACE with various probiotic strains, were combined with 2% (w/v) pectin biopolymer and 60% (w/w) glycerol plasticizer to produce the probiotic edible film with ACE. Antibacterial effects were investigated using disc diffusion, while probiotic film viability was assessed through the pour plate method. The study revealed higher viability in the probiotic strains *L. lactis* and *S. thermophilus* within the edible film, compared to *L. bulgaricus*. The specific strains exhibited increased antibacterial activity against *K. pneumoniae* and *P. aeruginosa*, underscoring the critical role of probiotic strain selection in determining functional properties. Notably, the edible film containing *L. lactis* and *S. thermophilus* demonstrated substantial antibacterial activity against both pathogens. However, further research is imperative to optimize industrial-scale production methods, assess efficacy in diverse food items, evaluate sensory properties, gauge barrier attributes, and investigate the impact of storage conditions on stability and performance.

Keywords: Probiotic edible film, S. thermophilus, L. bulgaricus, L. lactis, Viability, Antibacterial

Introduction

Pneumonia is an acute respiratory infection which affects the lungs, causing cough, nasal congestion, difficulty breathing, syanosis, hypoxia, fever and dizziness [1]. The World Health Organization states that in 2019, an estimate of 740 180 children under 5 years old lost their lives to pneumonia, which accounted for 14% of all deaths [2]. Pneumonia is typically caused by bacterial infection (90%), intoxication, and immuno-deficiency. Opportunistic pathogens *Puedomonas aeruginosa* and *Klebsiella pneumoniae* are known to cause major respiratory tract diseases, gastrointestinal infections, and bacteremia, and are particularly dangerous for those with AIDS, cancer, and severe burns. *K. pneumoniae* and *P. auroginosa* are two of the major pathogens causing pneumonia [3].

Incorporation of fermented products into one's daily diet, along with starter strains of *Lactobacillus bulgaricus* and *Streptococcus thermophils*, help to boost the immune system and can prevent respiratory infections. This is attributed to the immunoregulatory properties of these strains, which in turn strengthens a person's respiratory tract [4]. The study by Fauziah et al.[1] suggested that soyghurt with a concentration of 80% *L. bulgaricus* has the potential to exhibit bactericidal activity against *K. pneumoniae* strains, indicating its potential as a functional food product . Additionally, fermented milk is commonly used as a delivery system for various probiotics and bioactive compounds derived from medicinal herbs, as it can help to improve the viability and stability of probiotics and other bioactive compounds during storage and gastrointestinal transit. It can also provide a convenient and palatable means of delivering these beneficial compounds to consumers [5].

For centuries, clove *(Syzygium aromaticum* L.) has been a popular culinary spice and employed in traditional

medicine to treat different ailments. Clove ingredients have been found to possess antiviral, antibacterial and anti-inflammatory properties, particularly in relation to respiratory issues [6]. The study by Ogwaro et al.[7] reported that the use of clove extracts during fermentation and storage has the potential to improve the microbiological safety of fermented milk. The study found that the addition of clove extracts to fermented milk inhibited the growth of pathogenic bacteria such as *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhi*, which are known to cause foodborne illnesses. Clove extracts contain natural antimicrobial compounds such as eugenol, which have been shown to exhibit strong antimicrobial activity against various microorganisms. Therefore, the use of clove extracts in fermented milk could provide a natural and effective means of improving its microbiological safety and extending its shelf life.

The starter cultures *S. thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* are commonly used probiotics that have beneficial effects on gut health [8]. *Lactococcus* strains are also important probiotics that have been observed to produce bacteriocins, which are antimicrobial peptides that can inhibit the growth of pathogenic bacteria [9]. The use of edible films for delivering bioactive compounds and probiotics can help to protect these compounds from degradation during storage and transport, increasing their effectiveness and shelf life of probiotics. The incorporation of bioactive compounds and probiotics into edible films has the potential to transform food products into "functional" foods, which can provide additional health benefits beyond basic nutrition. This can add value to the food market and provide consumers with healthier options [10]. The European Food Safety Authority, and Food and Drug Administration have both allotted the group of lactic acid bacteria (LAB) and clove extract the category of "qualified presumption of safety" (QPS) and "generally recognized as safe" (GRAS) respectively, allowing for their use in the food industry [6,11].

Encapsulation is an advanced method for safeguard and increase the shelf life of probiotics, where the active component will be covered by a protective coating made of food-grade material during processing. Spray drying is one among that, spraying probiotic and protective material mixture into a heated chamber, the moisture evaporates and leave dry particles with probiotics [12]. To ensure the survival of probiotics, their insertion in edible films protects them from various physical and chemical hazards during processing and storage [13]. Because of the growing desire for minimally processed foods, edible film created from naturally derived biopolymers become highly popular; this can assist to extend probiotic shelf life and improve quality [14].

Functional probiotic microencapsulation with antibacterial potential is a promising alternative to antibiotics, as it allows the gradual release of functional compounds to prevent diseases [10]. Hence, the present work was aimed to produce fermented milk powder encapsulated in pectin biopolymer with ACE incorporated with *Streptococcus thermophilus* ATCC 19258, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 = JCM 1002, and *Lactococcus lactis* subsp. *tcremoris* MG1363, respectively, their viability in edible film and antibacterial effect against *K. pneumoniae* ATCC 700603 *and P. aeruginosa* ATCC 27853 were determined.

Material and Methods

Study Materials

The research utilized low-fat UHT milk sourced from Nestle products Sdn. Bhd. in Malaysia, *S. thermophilus* ATCC 19258, and *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 = JCM 1002, which were isolated from yoghurt by Malaysia Milk Sdn. Bhd. (MariGold, Malaysia). *L. lactis* subsp. *cremoris* MG1363 was obtained from the culture collection at the University of Putra Malaysia, Malaysia. All lactic acid bacteria were confirmed by 16S rRNA sequencing and the data was analysed by BLAST. Pathogenic strains *K. pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853 were obtained from the culture collection of UCSI University.

Preparation of ACE

Clove *(Syzygium aromaticum)* buds were procured from a local market in Cheras, Malaysia (Voucher no. KM 0042/22, Herbarium- Institute of Bioscience, University Putra Malaysia). These buds were oven-dried overnight at 60°C, and then powdered in a mixer and sieved. Subsequently, 10% (w/v) clove powder was added to sterile distilled water and kept at 50°C for 24 h. The resulting ACE was filtrated using a Whatman no. 1 filter paper, followed by a 0.22 µm syringe filter [15].

Preparation of Functional ACE Incorporated Fermented Milks

Fermented milks were prepared with slight modifications of method by Kanik et al.[16]. To produce functional fermented milk samples, 7.5 mL of ACE was added to 92.5 mL of low-fat UHT milk, which was homogenized for 5 min at room temperature. Then the mixture was inoculated with 1% fermented milk starter cultures containing *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, and *L. lactis*. The inoculated fermented milks were then incubated for 18 h at 42°C, 37°C, and 30°C, respectively. The fermented milks were then stored in the refrigerator at 4°C for 24 h before analysis.

Preparation of Functional ACE Incorporated Fermented Milk Powders

For functional fermented milk powder samples production, each fermented milks were spray dried at 4°C feed

temperature, 140°C inlet temperature and 30% pump speed with 100% aspiration (BÜCHI mini spray dryer, B-290, Switzerland) [17]. The fermented milk powder with *L. delbrueckii subsp. bulgaricus, S. thermophilus*, and *L. lactis*, respectively were then stored in the refrigerator (4°C) for 24 h prior to analysis.

Preparation of Functional Edible films with Fermented Milk Powder

Pectin edible film with specific fermented milk powder was prepared according to Ribeiro et al.[18] with slight modifications. The sterile casting solution was formulated using a combination of pectin biopolymer at a concentration of 2% (w/v) and glycerol plasticizer at a proportion of 60% (w/w). For functional edible film samples production, fermented milk powder (7.5%) with *L. delbrueckii subsp. bulgaricus, S. thermophilus*, and *L. lactis*, respectively were added. The inoculated edible films with each fermented milk powders were then dried in oven (Memmert, Germany) for 24 h at 30°C. Prepared edible films were then stored in the dedicator for further analysis.

Viability of Lactic Acid Bacteria

The viable counts of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, and *L. lactis* in samples were determined using the conventional pour plate count technique. *Streptococcus thermophilus* agar (HIMEDIA, India) [19] was used for the enumeration of *S. thermophilus* (37°C for 48 h), modified reinforced clostridial medium (mRCM) [20] for *L. bulgaricus* (37°C for 48 h), and GM17 agar [21] for *L. lactis* (30°C for 48 h). For each sample, 1 g was diluted with 9 mL of normal saline for serial dilution, and the plates were then incubated in an aerobic environment. The results were expressed as colony-forming units per millilitre (log CFU/mL).

Antibacterial Analysis

Mueller Hinton Agar (MHA) was placed on sterilised petri plates and allowed to completely solidify. A bacterial suspension of *K. pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853 (0.1 OD) was equally (100 µL per plate) dispersed onto the surface of the medium using a sterile coating rod [22]. To test the antibacterial activity of fermented milk supernatant, 10 g of fermented milks were centrifuged (MIKRO 220R, Germany) at 4.000 rpm (1431 x g) for 30 minutes at 4°C. The supernatant was then filtered using a 0.22 µm millipore membrane syringe filter before being used [23]. After 10 min in the treatment supernatant, each sterile disc was dried for one minute before being placed on the petri plates. The plates were incubated at 37°C and the diameter of the inhibition zone was measured using a Vernier caliper after 24 h using disc diffusion method [24]. Following that, a reconstituted fermented milk powder solution was made by dissolving 20% (w/v) of the powder in sterile distilled water. Sterile discs were then immersed in the solution for 10 min before being dried for one minute before being tested for antibacterial activity [25,26]. Then disc diffusion method was then used to detect the antibacterial activity of the edible film. The films were clipped into disk-shaped samples with a diameter of 6 mm for analysis [27]. Sterile blank disc and chloramphenicol (10%) were used as the negative and positive controls [28], respectively.

Statistical Analyses

Totally three trials were conducted and the data obtained were subjected to statistical analysis. To find significant differences between the samples ($P<0.05$), all data were subjected to one-way analysis of variance (ANOVA), followed by Tukey's post hoc test with SPSS statistics (IBM SPSS statistics 20 version).

Results

Antibacterial Effect of Aqueous Clove Extract (ACE)

Clove extract is an effective and natural alternative to synthetic preservatives, and is a valuable tool in the food industry for maintaining the safety and quality of food products. Clove has anti-inflammatory, antimicrobial, anti-thrombotic, antioxidant, antimutagenic, and anti-ulcerogenic attributes [29]. The effect of varying concentrations of aqueous clove extract on the antibacterial zone of inhibition (mm) against *K. pneumoniae* and *P. aeruginosa* is presented in *Table 1*.

Viability of Functional Fermented Milks, Fermented Milk Powders and Edible Films

Farmers incorporate different spices into milk either prior to or during fermentation, with the aim of enhancing the flavor and aroma of the fermented product [30]. Moreover, the inclusion of clove in fermented milk aids in its preservation and inhibits the proliferation of bacteria, which can cause food spoilage [7]. Researchers have found that the acceptable concentration of clove in yogurt,

Table 1. *The impact of varying ACE concentrations on the antibacterial zone of inhibition (mm) against K. pneumoniae and P. aeruginosa*

Concentration of ACE (%)	Zone of Inhibition (mm)	
	K. pneumoniae	*P. aeruginosa*
0% (blank)	6.00±0.00^{d}	6.00±0.00^{d}
2.5 %	7.67±0.58^{c}	7.33±0.58^{c}
5 %	9.33±0.58^{b}	9.00±0.00^{b}
7.5 %	11.00±0.00^{a}	10.33±0.58^{a}

Results were expressed as mean ± standard deviation, values are means of triplicates (n=3). ^{abcd} *means in the same columns followed by same uppercase letters are non-significant (P<0.05) via one way ANOVA with tukey test*

Table 2. *The viability and rate of survival of lactic acid bacteria in fermented milk with and without the presence of aqueous clove extract (ACE)*

Fermented Milks	*S. thermophilus*		*L. bulgaricus*		*L. lactis*	
	Viability (log CFU/mL)	Survivability (%)	Viability (log CFU/mL)	Survivability (%)	Viability (log CFU/mL)	Survivability (%)
Without ACE	13.83 ± 0.02^{Aa}	100.00	13.93 ± 0.03^{Aa}	100.00	13.22 ± 0.08^{Ab}	100.00
5% ACE	13.19 ± 0.017^{Ba}	95.35	13.21 ± 0.11^{Ba}	94.83	12.02 ± 0.07^{Bb}	90.92
7.5% ACE	12.21 ± 0.11^{Ca}	88.29	12.10 ± 0.16^{Ca}	86.86	10.78 ± 0.08^{Cb}	81.54

Results were expressed as mean ± standard deviation, values are means of triplicates (n=3). ^{ABC} means in the same column followed by same uppercase letters and ^{ab} means in the same rows followed by same lowercase letters are non-significant (P<0.05) via one way ANOVA with tukey test

Table 3. *Viability and reduction percentage of lactic acid bacteria in ACE-enriched fermented milk, spray dried powder, and edible film*

Sources	*S. thermophilus*		*L. bulgaricus*		*L. lactis*	
	Viability (log CFU/mL)	Reduction (%)	Viability (log CFU/mL)	Reduction (%)	Viability (log CFU/mL)	Reduction (%)
Fermented milk	12.21 ± 0.11^{Aa}	0.00	12.10 ± 0.16^{Aa}	0.00	10.78 ± 0.08^{Ab}	0.00
Spray dried powder	9.74 ± 0.10^{Ba}	20.23	9.39 ± 0.06^{Bb}	22.40	9.75 ± 0.05^{Ba}	9.55
Edible film	8.09 ± 0.06^{Cab}	33.74	7.95 ± 0.05^{Cb}	34.30	8.13 ± 0.08^{Ca}	24.58

Results were expressed as mean ± standard deviation, values are means of triplicates (n=3). ^{ABC} means in the same column followed by different uppercase letters and ^{ab} means in the same rows followed by different lowercase letters are significant (P<0.05) via one way ANOVA with tukey test separately

Table 4. *Antibacterial zone of inhibition (mm) of edible film, spray dried powder and fermented milk against P. aeruginosa and K. pneumoniae*

Source	Zone of Inhibition (mm)					
	S. thermophilus		*L. bulgaricus*		*L. lactis*	
	P. aeruginosa	*K. pneumoniae*	*P. aeruginosa*	*K. pneumoniae*	*P. aeruginosa*	*K. pneumoniae*
Fermented milk	8.67 ± 0.58^{Bb}	8.00 ± 0.00^{Ba}	9.33 ± 0.58^{Aab}	8.00 ± 0.00^{Aa}	10.00 ± 0.00^{Ba}	8.33 ± 0.58^{Ba}
Spray dried powder	9.67 ± 0.58^{ABb}	8.67 ± 0.58^{Ba}	9.33 ± 0.58^{Ab}	8.33 ± 0.58^{Aa}	11.67 ± 0.58^{ABa}	9.67 ± 0.58^{Ba}
Edible film	11.33 ± 1.15^{Aa}	11.00 ± 0.00^{Aa}	8.00 ± 0.00^{Bb}	8.67 ± 0.58^{Ab}	13.33 ± 1.15^{Aa}	11.67 ± 0.58^{Aa}

Results were expressed as mean ± standard deviation, values are means of triplicates (n=3). ^{AB} means in the same columns followed by different uppercase letters and ^{ab} means in the same rows followed by different lowercase letters are significant (P<0.05) via one way ANOVA with tukey test separately for P. aeruginosa and K. pneumoniae

as determined by its sensory attributes, was below the concentration required for microbial inhibition, resulting in a sublethal effect on both *Lactobacillus* sp. and the yogurt starter culture, without compromising the fermentation process. *Table 2* presents the viability and survivability of lactic acid bacteria in fermented milk without ACE and with varying percentages of ACE.

Numerous studies have demonstrated the benefits of microencapsulation in enhancing the survival of probiotics across various manufacturing techniques and unfavourable gastrointestinal conditions. Probiotic products can be sensitive to environmental factors such as temperature, moisture, oxygen, and light. Exposure to unfavorable conditions during processing, packaging, and storage can lead to a reduction in the viability of the probiotic microorganisms, which can, in turn, compromise the efficacy of the product. Edible films made from pectin can improve the quality of food products by acting as a barrier to moisture, oxygen, and other gases that can cause spoilage, resulting in an extended shelf life. Additionally, pectin-based films can provide a protective coating to encapsulate and preserve probiotics [31]. *Table 3* displays the viability of lactic acid bacteria in spray-dried fermented milk powder, and edible film.

Antibacterial Effect of Functional Fermented Milk, Fermented Milk Powders and Edible Films

K. pneumoniae and *P. aeruginosa* are capable of inhabiting the oral cavity, pharynx, and gastrointestinal tract of humans, and are highly virulent and antibiotic resistant. Currently, *K. pneumoniae* is the predominant cause of pneumonia acquired in hospital settings [32]. *Table 4* displays the impact of edible film, spray dried powder, and fermented milk on the antibacterial zone of inhibition (mm) against *P. aeruginosa* and *K. pneumoniae*. In this study, the antibacterial activity of edible films, spray dried powder, and fermented milk containing *S. thermophilus, L. bulgaricus*, and *L. lactis* against *P. aeruginosa* and *K. pneumoniae* was examined. The edible film with *S.*

thermophilus and *L. lactis* shows a significantly higher zone of inhibition against both *P. aeruginosa* and *K. pneumoniae* in the present study, measuring 11.33±1.15 mm and 13.33±1.15 mm, respectively, and 11.00±0.00 mm and 11.67±0.58 mm, respectively. The literature findings reported four categories of active compound inhibition in bacteria, with the diameter of the inhibition zone classified as weak (5 mm), moderate (5-10 mm), strong (10-20 mm), and very strong (20-30 mm) [28].

Discussion

A significant increase in the antibacterial zone of inhibition (mm) against *K. pneumoniae* and *P. aeruginosa*, with values of 11.00±0.00 and 10.33±0.58 respectively, was observed for 7.5% of ACE, in comparison to 5% and 2.5%. In vitro testing was conducted to assess the antibacterial effects of clove ethanolic extract on *K. pneumoniae* and *P. aeruginosa*, resulting in maximum zones of inhibition of 16mm and 14mm, respectively [33]. These results were consistent with previous research by which showed that clove water extract inhibited the growth of *P. aeruginosa*. Furthermore, antibacterial properties were observed in clove extracts prepared from both aqueous and ethanol-based solutions against both *K. pneumoniae* and *P. aeruginosa*, with the most significant activity being observed against *K. pneumoniae* [34].

The viability of lactic acid bacteria in fermented milk was significantly reduced with the addition of ACE, as indicated by the results. The fermented milk without ACE had the highest viability of lactic acid bacteria. These findings suggest that the addition of ACE negatively affected the viability of lactic acid bacteria in fermented milk. The viability of *S. thermophilus*, *L. bulgaricus*, and *L. lactis* fermented milk with 7.5% ACE was observed to be 12.21±0.11, 12.10±0.16, and 10.78±0.08 (log CFU/mL), respectively. Although there is no agreement among the global scientific community regarding the ideal dosage of probiotics needed to obtain health benefits, various studies have suggested that minimum doses ranging from 10^6 to 10^9 CFU/day may be necessary to produce therapeutic effects [11]. For probiotic foods to provide health benefits, they must contain a minimum of 10^7 cfu/g of probiotic bacteria. According to the findings gathered thus far, it can be inferred that fermented milk containing 7.5% ACE integration has demonstrated adequate viability, making it a viable candidate for both spray drying and edible film production, thereby enhancing the longevity of its viability according to Pourjafar et al.[35].

In the present study, it was observed that the viability of *S. thermophilus* and *L. lactis* in fermented milk powder and their respective edible film was significantly higher than that of *L. bulgaricus*. The viable count of edible films prepared with *S. thermophilus*, *L. lactis*, and *L. bulgaricus* in pectin biopolymer were 8.09±0.06 log CFU/mL, 8.13±0.08 log CFU/mL, and 7.95±0.05 log CFU/mL, respectively. Similarly, for spray-dried powder, the viable count was 9.74±0.10 log CFU/mL, 9.75±0.05 log CFU/mL, and 9.39±0.06 log CFU/mL, respectively. These results were consistent with previous reports that *S. thermophilus* was less susceptible to spray-drying than *L. delbrueckii* subsp. *bulgaricus*. In addition, probiotic yogurt powder containing *Lactobacillus paracasei* and *S. thermophilus* had viable counts exceeding 10^8 CFU/g, while the less heat-tolerant *L. delbrueckii* subsp. *bulgaricus* survived only at 10^5 CFU/g of powder. Also the coatings made with *S. thermophilus*, *L. bulgaricus*, and *L. acidophilus* exhibited the best protective properties against microbial spoilage, higher viability (10^8 CFU/g), and increased shelf life [36].

According to Gul & Atalar [37] the viability of *Lactobacillus* sp. reduced by 0.43-1.62 log on spray drying, where *L. paracasei* NFBC 338 and *L. rhamnosus* GG survived spray-drying of reconstituted skim milk at approximately 80% and 60%, respectively. Similarly, the viability of *Lactobacillus* strains after spray-drying process shows 1.69-1.99 log-reduction [38]. Correspondingly, fresh yoghurt *Lactobacillus* viability also dropped from 7.42-6.15 log CFU/g on spray drying, indicating a 17.1% loss in viability [39]. In comparison to different film-forming solutions, edible films containing *Lactobacillus* strains showed a decrease in viability ranging from 0.4 to 2 log CFU/g [40].

Furthermore, Ma et al.[41] reported that the *Lactococcus* strain showed the best viability and antimicrobial activity when added to edible film. A survival rate of 21.6% *for Lactococcus lactis* ssp. *cremoris* was observed to be higher when spray drying was carried out at 130°C, utilizing lactose and sodium caseinate as the drying agents. Various inlet temperatures were used, resulting in a loss of viability ranging from 0.60 to 1.22 log cycles for *Lactococcus* [42]. The study suggested that the reason for the high retention of *Lactococcus* survivability 93.14±2.74%, in edible film, could be linked to the utilization of a lower drying temperature of 30°C and an extended adaptation time of one day [43]. The results were consistent with the present study.

As a result of atypical encapsulation procedure, it is possible to reduce the viability of the *Lactobacillus* and *Lactococcus* strains, which could have consequences for their prospective application of probiotics. The ability of lactic acid bacteria to survive in edible film can be influenced by the base material of the film, nutritional content, initial viability of bacteria, pH, and osmotic pressure. Further research is required to enhance the encapsulation procedure to reduce the loss of viability while maximizing the potential advantages.

Lactococcus has been found to contain antibacterial compounds that inhibit the growth of *Pseudomonas,* making it a potential preventative and treatment option for *Pseudomonas* infections [44]. To ensure the safety of food, researchers have investigated the antimicrobial properties of *Lactococcus* strains found in spontaneously fermented camel milk and the elimination mechanism of *K. pneumoniae* in pasteurized camel milk. All milk samples that were inoculated with a *L. lactis* strain were devoid of *K. pneumoniae* after 75 h of testing [45]. Hence, the antimicrobial activity observed in *L. lactis* cannot be ascribed to the organic acids or hydrogen peroxide generated by the culture, as the inhibitory effects remained unchanged even after neutralization or treatment with catalase in the cell-free supernatant preparations.

P. aeruginosa is an opportunistic pathogen that can cause food spoilage through its lipolytic and proteolytic activities, although this can be prevented by the hydrogen peroxide produced by *Lactobacillus* species. Furthermore, according to Jamalifar et al.[46], *Lactobacillus acidophilus* strain exhibited strong (90 % inhibitory activity) anti-pseudomonal activity against multi-drug resistant clinical isolates. The current findings are in agreement with those of Pulusani et al.[47], *L. bulgaricus* was found to produce a robust zone of inhibition against the growth of *P. aeruginosa*, measuring over 25 mm, while *L. acidophilus* and *S. thermophilus* exhibited zones of inhibition ranging from 21-25 mm. Yerlikaya et al.[48] also reported the highest antimicrobial effect of *L. bulgaricus* (17 ± 1.7mm) than *S. thermophilus* against *P. aeruginosa*.

The current findings align with the outcomes presented by Fauziah et al.[1], signifies that *L. bulgaricus* found in soyghurt can impede the attachment of *K. pneumoniae* to HEp2 cell lines. Following a period of pre-infection, co-infection, and post-infection with *L. bulgaricus* at a concentration of 10^8 CFU/mL for 5 hours, the adhesion of *K. pneumoniae* ATCC 700603 decreased by 6.42%, 19.505%, and 35.405%, respectively. Similarly, the adhesion of *K. pneumoniae* S941 decreased by 10.11%, 37.845%, and 43.74%, while *K. pneumoniae* CT1538 showed a decline of 30%, 31.055%, and 55.875%. In addition, it has been demonstrated that strains of *L. bulgaricus* and *S. thermophilus* isolated from homemade yogurts exhibit antimicrobial activity against *K. Pneumoniae*. Furthermore, the impact of *L. bulgaricus* has been uncovered by researchers who observed that antimicrobial effect was attributed to the unique characteristics of *L. bulgaricus*, which resulted in the production of more advanced lactic acid [49]. Previous studies have demonstrated that lactic acid possesses significant antibacterial and antifungal properties [50].

Pseudomonas aeruginosa and *Klebsiella pneumoniae* are of multidrug-resistant gram-negative bacteria that are frequently associated with bacterial pneumonia. Clove has traditionally been used as a natural cure for a variety of diseases, including respiratory problems, headaches, and sore throats. Current study revealed that combining ACE with specific strains of *S. thermophilus*, *L. bulgaricus*, and *L. lactis* in fermented milk, spray dried powder, and edible films can potentially have an antibacterial impact against *P. aeruginosa* and *K. pneumoniae*. The recommended lactic acid bacteria viability for the final edible film made with these strains was 10^7 CFU/mL. *L. lactis* and *S. thermophilus* ACE integrated edible film and spray dried powder were found to have much higher viability and antibacterial action than *L. bulgaricus*. Lactic acid bacteria (LAB) are known to produce organic acids that reduce pH and enhance the formation of hydrogen peroxide and bacteriocins, both of which have antibacterial effects against a variety of pathogenic pathogens, including Gram-positive and Gram-negative bacteria. However, further research is needed, to fully understand the combined effects of multiple natural antimicrobial agents and lactic acid bacteria, its mechanisum of action in an actual food system to establish its potential value in preventing foodborne diseases.

Declarations

Availability of Data and Materials: The datasets used and/or analysed during the current study are available from the corresponding author (L. P. Pui) on reasonable request.

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Competing Interest: The authors declare no conflict of interest

Ethical Statement: Ethics committee approval is not required for this study.

Authors Contribution: Study design: LAKS and LPP; Experiments: LAKS; Data analysis: LAKS, LPP and KLN; Writing - Original draft preparation: LAKS; Writing - Review & Editing: all authors; All authors read and approved the final manuscript.

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Research Article

Response Surface Methodology Mediated Optimization of Medium Components for Growth Density and Rate of *Mycoplasma gallisepticum*

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Abstract

This study aimed to optimize the composition of a liquid medium for *Mycoplasma gallisepticum* growth rate and density through statistical approach. The growth concentration of *M. gallisepticum* was determined by plotting a standard curve using Real-Time Quantitative PCR. The one-factor-at-a-time method and the Plakett-Burman design were initially applied to identify the factors that influenced the biomass of *M. gallisepticum*. The steepest ascent experiment and response surface methodology (RSM) with Box-Behnken design was employed to simultaneously assess the effects of multiple factors. Finally, optimization of the initial pH and validation of the model were done. The optimum concentrations of the critical components were obtained as follows: 203.96 mL/L of horse serum, 9.64 g/L of glucose, and 9.49 g/L of PPLO broth. The nucleic acid copy number of *M. gallisepticum* reached $10^{10.5147}$ copies/mL and the viable cell count reached a maximum of $10^{9.8451}$ CCU/mL. This medium reduced the incubation time by approximately 6 h, and *M. gallisepticum* nucleic acid concentration and viable cell count were higher than those in the modified Frey medium (9.99 and 7 times, respectively). The new liquid medium is likely to improve productivity and reduce the production costs for vaccine-manufacturing companies in the future by reducing incubation times and increasing the growth rate and concentration of *M. gallisepticum*.

Keywords: Body weight, Continuous light stress, Cortisol, Cystic follicle, *Ferula*, Ovary, Oxidative stress

Introduction

Mycoplasma gallisepticum is a significant pathogen in poultry and wild birds [1]. *M. gallisepticum* can cause Chronic Respiratory Disease (CRD) in chickens [2], which results in high economic losses because of reduced feed conversion efficiency, slow growth, and decreased hatchability [3]. Both vertical and horizontal transmission of the pathogen is possible, and *M. gallisepticum* can also cause coinfections with other respiratory pathogens, which increases the mortality rate [4-6]. When chickens are infected with *M. gallisepticum*, antibiotics are commonly used to control the infection. However, completely clearing *M. gallisepticum* from infected chickens is impossible, and the concerns of increased drug resistance and antibiotic residues cannot be ignored [7,8].

Inoculating chickens with the *M. gallisepticum* vaccine is regarded as an acceptable method for providing protection [9]. Presently, these vaccines are primarily produced via microbial fermentation, the medium composition and the culture concentration of high-density fermentation culture are the critical factors that impact the cost of the vaccine [10]. It is uneasy to culture *M. gallisepticum* as it has limited synthesis capacity; therefore, it requires large amounts of nutrients from the external environment [11,12]. The most commonly used culture component in the *Mycoplasma* medium is based on pleuropneumonia-like organisms (PPLO) and is widely utilized [13]. The composition of the medium (carbon and nitrogen sources) is a crucial element influencing biological growth as fermentation conditions.

Hence, optimizing the medium and the fermentation conditions can aid in increasing biomass and reducing costs [14,15]. Response surface methodology (RSM) is an empirical statistical technique that uses mathematical modeling to determine the mutual effects of various process parameters on the response variable. Quantitative data generated from the design of experiments and the analysis of regression models and operational conditions can result in high-end performance [16]. Several statistically-based experimental designs have been used to optimize the fermentation medium, and these include full factorial design, partial factorial design, Plackett-Burman design (PBD), Box-Behnken design (BBD), and central composite design (CCD) [17]. Currently, PBD and RSM are successfully used to optimize the variables in the culture medium. These methods are used to enhance the production of industrially important metabolites by various microorganisms such as fungi, bacteria, and actinomycetes [18-20]. The concentration of viable bacterial cells of *Lactobacillus plantarum* Y44 cultured at 37°C for 16 h in the optimized medium was 3.363×10^{10} CFU/mL. This amount was 6.11 times higher than that in the MRS medium [21]. The yield of valine was 457.23±9.52 mg/L after optimizing the fermentation medium of *Streptomyces* sp. Zjut-iF-354 using RSM, which is the highest yield ever reported [22].

In this study, the *M. gallisepticum* R strain, which was preserved via lyophilization in our laboratory, was used for fermentation. Carbon and nitrogen sources that can increase the concentration of *M. gallisepticum* were screened using the one-factor-at-a-time method. Subsequently, the primary factors that affect the concentration of *M. gallisepticum* were optimized via RSM. Furthermore, a fermentation medium suitable for industrial production was screened and verified experimentally, which laid the foundation for further industrial application.

Material and Methods

Mycoplasma gallisepticum R Strain and Culture Medium

Mycoplasma gallisepticum R strain, which was freeze-dried and preserved by Shandong Lvdu Biotechnology Co., LTD. (Shandong, China), was used as the research object and was passaged at least three times before use. NaCl, KCl, $MgSO_4$·7H2O, Na_2HPO_4·12H_2O, KH_2PO_4, glucose, L-cysteine solution, arginine solution, penicillin, phenol red, and trehalose were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), yeast extract and yeast peptone was bought from Angel Yeast Co., Ltd. (Hubei, China), lactalbumin hydrolysate, peptone, heart infusion broth, beef extract, brain heart infusion, trypticase soy broth, and PPLO broth was bought from ThermoFisher Scientific (Waltham, MA, USA), NADH I was bought from Beijing Ruitaibio Co., Ltd. (Beijing, China), horse serum was bought from Tianjin Kangyuan Biotechnology Co., Ltd. (Tianjin, China).

Modified Frey medium: 5.0 g of NaCl, 0.4 g of KCl, 0.2 g of $MgSO_4$·7H2O, 1.6 g of Na_2HPO_4·12H_2O, 0.2 g of KH_2PO_4, 10.0 g of glucose, 5.0 g of lactalbumin hydrolysate, 5.0 g of yeast extract, 10 mL of 1% NADH I, 10 mL of 1% L-cysteine solution, 20 mL of 2% arginine solution, 10 mL of penicillin 80.000 U/mL, 1 mL of 1% phenol red, 100 mL of horse serum were added into 1000 mL sterile water. These ingredients were mixed and dissolved, the initial pH was adjusted to 7.6-7.8, and bacteria were removed via filtration through a 0.22-μm syringe filter (Guangzhou Jet Biofiltration Co., Ltd., China).

Fermentation Conditions for the *Mycoplasma gallisepticum* R Strain

This culture was inoculated aseptically (10% v/v) into 20 mL medium contained in a 100-mL glass bottle, incubated at 37°C until an orange-yellow color was obtained.

Measurement of the Nucleic Acid Concentration of *Mycoplasma gallisepticum*

Real-time quantitative PCR was performed to determine the nucleic acid concentration of *M. gallisepticum*. The standard curve was established according to the different concentrations of nucleic acid standard of *M. gallisepticum* and Ct value.

Sample Preparation

The fermentation medium cultured was heated at 95°C for 10-15 min and diluted 10 times for Real-Time Quantitative PCR detection.

Determination of Medium Composition Using the One-Factor-At-a-Time Method

Adequate nutrients are present in the culture medium of *M. gallisepticum*. Carbon and nitrogen sources and serum are essential for the growth of *M. gallisepticum*. To obtain a higher concentration of *M. gallisepticum*, eight basic media were configured, and the sources of carbon source (3 g/L of trehalose) and nitrogen sources (10 g/L of peptone, yeast peptone, heart infusion broth, beef extract, brain heart infusion, trypticase soy broth, PPLO broth) with different compositions were supplemented after the nitrogen source in the modified Frey medium was reduced to 50%.

Plackett-Burman Design (PBD)

The PBD was used to screen for and evaluate variables that exerted significant effects on the concentration of *M. gallisepticum*, but the results did not describe the interactions between these variables. According to the preliminary screening results, seven variables were selected, namely, brain heart infusion (X_1), yeast extract (X_2), lactalbumin hydrolysate (X_3), trypticase soy broth

Table 1. Plackett-Burman design factors and levels			
Factors	**Variable**	**Level**	
		Low Level (-1)	**High Level (1)**
X_1	Brain Heart Infusion	10	20
X_2	Yeast Extract	2.5	5
X_3	Lactalbumin Hydrolysate	2.5	5
X_4	Trypticase Soy Broth	10	20
X_5	Horse Serum	100	200
X_6	Glucose	10	20
X_7	PPLO Broth	10	20

(X_4), horse serum (X_5), glucose (X_6), and PPLO broth (X_7). The high and low levels of each factor were selected. The variables represented by high and low levels were coded as +1 and −1, respectively; the concentration of components in the initial medium was set to the low level (−1), and the high level was set to twice the low level. The factors and levels are listed in *Table 1*.

The Steepest Ascent Experiment

The steepest ascent experiment was designed based on the results of PBD. The appropriate direction and change steps were determined based on the influence values of each factor. According to the results of PBD, the direction and change steps of the steepest ascent experiment were estimated. The highest concentration of *M. gallisepticum* was rapidly approached based on the three main influencing factors, namely horse serum, glucose, and PPLO broth.

Box-Behenken Design (BBD)

BBD based on response surface analysis is a statistical-mathematical method that reflects the best matching conditions obtained when factors in a multifactorial system interact with each other to achieve the maximum response value. Based on the results of PBD, three factors (horse serum, glucose, and PPLO broth) at three levels (−1, 0, and 1) were selected for the next test, and Real-Time Quantitative PCR was used to determine the concentration of *M. gallisepticum* nucleic acid. The factors and levels in the experimental program are presented in *Table 2*.

Effect of Medium pH on Concentration

The effect of the initial pH of the culture medium on concentration was investigated to determine the optimal culture conditions for the *M. gallisepticum* R strain. The pH was adjusted using 1M NaOH (7.4, 7.6, 7.8, and 8.0) before filtration and incubated until an orange-yellow color was obtained. Subsequently, Real-Time Quantitative PCR was used to determine optimal fermentation conditions and provide process parameters for fermentation and cultivation in large-volume fermenters.

Table 2. Response surface experimental factors and levels				
Name	**Factors**	**Level**		
		-1	**0**	**1**
X_5	Horse Serum	200	205	210
X_6	Glucose	9	9.5	10
X_7	PPLO Broth	9	9.5	10

Model Validation

The optimized fermentation conditions were subjected to a model validation fermentation using response surface analysis. The concentration of *M. gallisepticum* was then determined using Real-Time Quantitative PCR and the color change unit (CCU) at the end of the fermentation, and the response values were compared with the predicted data.

Statistical Analysis

All experiments were repeated three times, and the data were presented as mean ±SD. The data were analyzed using one-way ANOVA and Duncan's post-hoc pairwise multiple range test to determine the magnitude of differences, and differences between sample mean values of $P<0.05$ were considered significant. The quality of fit for the polynomial model equations was quantified using the determination coefficient (R^2). The PBD and BBD data were analyzed using the Design Expert software (Version 10.0.3., USA), and GraphPad Prism 7.0 (GraphPad Software, USA) was used to perform all analyses.

Results

Rapid Detection Method for Nucleic Acid Concentration

Based on research previously conducted in our laboratory, a standard curve was plotted by correlating Real-Time Quantitative PCR Ct values with the copy number of *M. gallisepticum* nucleic acid samples *(Fig. 1)*. The equation of the standard curve was y = −3.646x+44.208, with a correlation coefficient R^2 of 0.998. It was utilized to

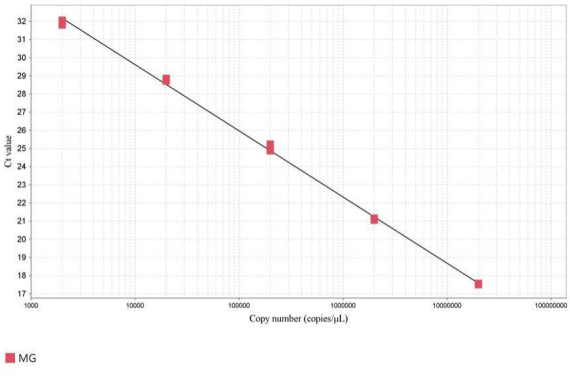

Fig 1. Standard curve of nucleic acid copy number of *M. gallisepticum* (MG: *Mycoplasma gallisepticum)*

rapidly identify the concentration of *M. gallisepticum* in the following fermentation process.

Effect of Different Nutrient Sources on the Concentration of *M. gallisepticum*

Nutrient requirements are crucial during the fermentation of *M. gallisepticum*. The findings demonstrated that different nutrients had a significant effect on the growth of *M. gallisepticum* (*Table 3*). The concentration of *M. gallisepticum* increased to $10^{10.1998}$ copies/mL when the PPLO broth was added; however, the addition of a carbon source (trehalose) did not have any effect (P>0.05) on the concentration of *M. gallisepticum*. Therefore, the three best nitrogen sources (brain heart infusion, trypticase soy broth, and PPLO broth) that increased the concentration of *M. gallisepticum* were selected for the subsequent experiments.

PBD to Screen the Main Effect Factors

To determine the factors with the highest influence on the concentration of *M. gallisepticum*, 12 different media were designed and formulated for testing using PBD. The observed responses are presented in *Table 4*, and the concentration of *M. gallisepticum* nucleic acid was found to vary from $10^{7.8143}$ copies/mL to $10^{10.2333}$ copies/mL (*Table 4*).

Regression analysis and ANOVA of the PBD were performed, as shown in *Table 4*. The regression was found to be significant (P-value 0.0459), and horse serum, glucose, and PPLO broth exhibited P-values of 0.0300, 0.0185, and 0.0359, respectively (*Table 5*). Prob>F values <0.05 denote that the model term holds significance. Thus, X_5, X_6, and X_7 were identified to be significant model terms and were deemed to be the variables with the highest effect on the nucleic acid concentration of *M. gallisepticum*. The

Table 3. *Results of the one-factor-a-time method*

Factors	Components	Copy Number (log copies/mL)
Unoptimized medium		9.3748
Nitrogen sources	Peptone	9.6181
	Yeast Peptone	9.8101
	Heart Infusion Broth	9.8126
	Beef Extract	9.8201
	Brain Heart Infusion	9.8439
	Trypticase Soy Broth	9.9114
	PPLO Broth	10.1998
Carbon sources	Trehalose	9.3217

Table 4. *Results of the Plackett-Burman design*

Run	Code Number							Copy Number (log copies/mL)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	
1	-1	-1	-1	-1	-1	-1	-1	10.1656
2	1	-1	1	1	-1	1	1	8.2194
3	1	1	-1	1	1	1	-1	8.7726
4	-1	1	1	1	-1	-1	-1	10.1209
5	1	-1	1	1	1	-1	-1	10.0577
6	1	-1	-1	-1	1	-1	1	9.7113
7	1	1	-1	-1	-1	1	-1	8.3793
8	1	1	1	-1	-1	-1	1	8.2723
9	-1	-1	1	-1	1	1	-1	10.2333
10	-1	-1	-1	1	-1	1	1	7.8143
11	-1	1	-1	1	1	-1	1	9.9415
12	-1	1	1	-1	1	1	1	9.1540

Table 5. *Analysis of the variance of PBD*

Source	Factors	Freedom	Coefficient Estimate	Sum of Squares	Mean Square	F-Value	P-Value
Model		7	9.24	8.24	1.18	6.41	0.0459*
X_1	Brain Heart Infusion	1	-0.33	1.34	1.34	7.32	0.0538
X_2	Yeast Extract	1	-0.13	0.20	0.20	1.10	0.3525
X_3	Lactalbumin Hydrolysate	1	0.11	0.14	0.14	0.73	0.4397
X_4	Trypticase Soy Broth	1	-0.082	0.082	0.082	0.44	0.5417
X_5	Horse Serum	1	0.41	2.00	2.00	10.88	0.0300*
X_6	Glucose	1	-0.47	2.70	2.70	14.71	0.0185*
X_7	PPLO Broth	1	-0.38	1.78	1.78	9.66	0.0359*

*R^2 = 91.81%; * Significant difference at P<0.05*

Table 6. *The design and results of the steepest ascent experiment*

Run	X_5 (mL/L)	X_6 (g/L)	X_7 (g/L)	Copy Number (log copies/mL)
1	200	10	10	10.4284
2	205	9.5	9.5	10.4973
3	210	9	9	10.4371
4	215	8.5	8.5	10.3987
5	220	8	8	10.3580

remaining variables exerted minimal and mathematically insignificant effects (P>0.05), as depicted in *Table 5*. Furthermore, the correlation coefficients for horse serum, glucose, and PPLO broth were 0.41, −0.47, and −0.38, respectively, which indicated that horse serum exhibited a positive impact and that glucose and PPLO broth exerted negative effects *(Table 5)*. Thus, X_5, X_6, and X_7 were selected for further optimization studies. In the next optimization step, the concentrations of horse serum, glucose, and PPLO broth in the fermentation liquid medium were increased.

The Steepest Ascent Experiment

To better approximate the domain of maximum response values for each major factor in the subsequent response surface analysis, a steepest ascent experimental design was utilized. The concentrations of horse serum, glucose, and PPLO broth were varied by 5 mL/L, 0.5 g/L, and 0.5 g/L, respectively, in the steepest ascent test, and the response values for the steepest ascent experimental design are presented in *Table 6*.

The nucleic acid concentration of *M. gallisepticum* exhibited a pattern of increase, which was followed by a decrease; hence, the design was reliable. During the second experiment, the concentration peaked at $10^{10.4973}$ copies/mL, which represented the maximum response value across all three factors. So, this point was selected to set the base concentration of BBD.

Optimization Using the BBD

Nutrient requirements are crucial during the fermentation of *M. gallisepticum*. Therefore, optimizing the nutrient composition of the medium is an effective way to increase its concentration. Response surface analysis is an effective approach to optimize fermentation parameters. This study was performed to assess the interaction of primary variables, such as X_5 (horse serum), X_6 (glucose), and X_7 (PPLO broth), using BBD to enhance the concentration of *M. gallisepticum*. The experimental design and obtained results are displayed in *Table 7*. The regression equation was obtained as follows: $Y = 10.49 - 0.1X_5 + 0.046X_6 - 0.025X_7 + 0.038X_5X_6 - 0.1X_5X_7 - (7.825E-003)X_6X_7 - 0.21X_5^2 - 0.071X_6^2 - 0.11X_7^2$

The statistical significance of the model equations was evaluated using the F-test for ANOVA, and the results are presented in *Table 7*. The ANOVA regression model showed a determination coefficient (R^2) of 0.9845, which indicated that the model accounted for 98.45% of the variance in the response *(Table 8)*. The adjusted coefficient of determination (R^2adj) was 0.9647 *(Table 8)*, and the high value implied the strong significance of the model. In this model, P<0.05, which signified that the regression was significant. Lack of fit (P>0.05) was not significant, which denoted that the model had a high level of confidence and could predict changes in the nucleic acid concentration of *M. gallisepticum (Table 8)*.

Table 7. *The results of the Box-Behenken design*

Run	Code Number			Copy Number (log copies/mL)
	X_5	X_6	X_7	
1	0	0	0	10.5092
2	-1	-1	0	10.3084
3	-1	0	-1	10.1945
4	1	0	-1	10.1833
5	0	0	0	10.4831
6	0	-1	-1	10.2473
7	0	0	0	10.5037
8	0	-1	1	10.2189
9	0	1	-1	10.4042
10	1	-1	0	10.0536
11	0	1	1	10.3445
12	-1	1	0	10.2769
13	0	0	0	10.4723
14	-1	0	1	10.3468
15	1	1	0	10.1732
16	1	0	1	9.9191
17	0	0	0	10.4688

Table 8. *The experimental results of the Box-Behenken design*

Source	Sum of Squares	Degree of Freedom	Mean Square	F-Value	P-Value
Model	0.44	9	0.049	49.52	< 0.0001*
X_5	0.079	1	0.079	80.21	< 0.0001*
X_6	0.017	1	0.017	17.33	0.0042*
X_7	5.000E-003	1	5.000E-003	5.05	0.0595
X_5X_6	5.708E-003	1	5.708E-003	5.76	0.0475*
X_5X_7	0.043	1	0.043	43.77	0.0003*
X_6X_7	2.449E-004	1	2.449E-004	0.25	0.6343
X_5^2	0.19	1	0.19	193.87	< 0.0001*
X_6^2	0.021	1	0.021	21.30	0.0024*
X_7^2	0.054	1	0.054	54.16	0.0002*
Residual	6.936E-003	7	9.909E-004		
Lack of Fit	5.603E-003	3	1.868E-003	5.60	0.0647
Pure Error	1.333E-003	4	3.333E-004		
Cor Total	0.45	16			

*R^2 = 0.9845; R^2 adj = 0.9647; * Significant difference at P<0.05*

The optimal level of interaction between any two variables was visualized using the 3D response surface. The 3D response surface plots illustrated the correlation among horse serum, glucose, and PPLO broth *(Fig. 2-A,B,C)*. According to the response surface analysis plots, the term X_5X_6 and X_5X_7 exerted a significant effect (P<0.05) on *M. gallisepticum* nucleic acid concentration *(Fig. 2-D,E)*. However, X_6X_7 did not have significant effects (P>0.05) on nucleic acid concentration of *M. gallisepticum* *(Fig. 2-F)*. At horse serum concentrations of 200-203.96 mL/L, the nucleic acid concentration of *M. gallisepticum* increased, but it decreased at concentrations 203.96-210 mL/L *(Fig.*

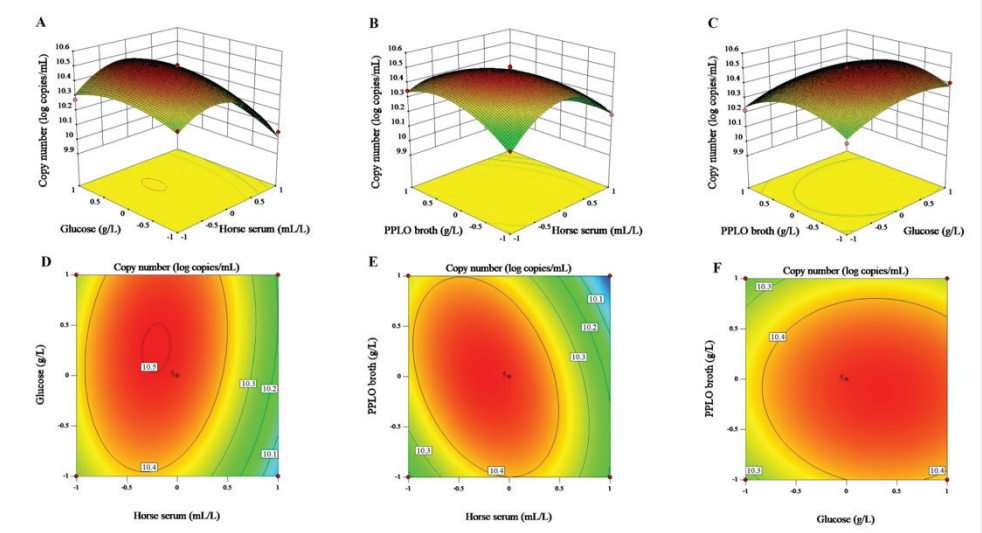

Fig 2. Response surface plots of horse serum (mL/L), glucose (g/L), and PPLO broth (g/L) on *M. gallisepticum* nucleic acid concentration. **(A)** The effect of horse serum and glucose levels, **(B)** The effect of horse serum and PPLO broth levels, **(C)** The effect of glucose and PPLO broth levels, **(D)** Horse serum and glucose levels, **(E)** Horse serum and PPLO broth levels, **(F)** Glucose and PPLO broth levels

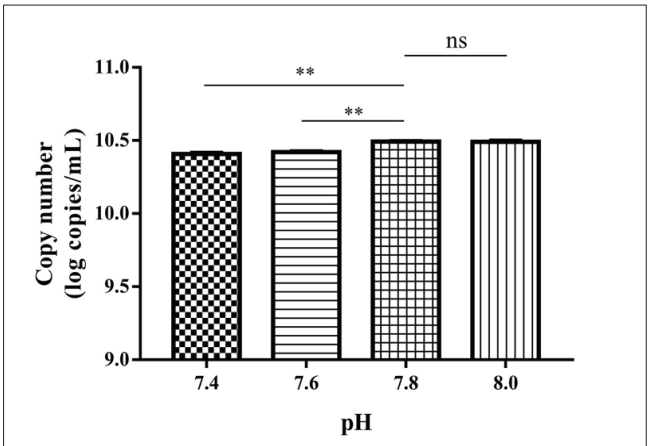

Fig 3. Nucleic acid copy number of *M. gallisepticum* under various pH values (*P<0.05, **P<0.01)

2-A,D). With an increase in the concentrations of glucose (9.00-9.64 g/L) and PPLO broth (9.00-9.49 g/L), the nucleic acid concentration increased and then dropped. When the concentrations of horse serum, glucose, and PPLO broth were 203.96 mL/L, 9.64 g/L, and 9.49 g/L, respectively, the regression model predicted a maximum value of $10^{10.504}$ copies/mL.

Effects of pH of the Medium on Nucleic Acid Concentration of *M. gallisepticum*

The nitrogen source and the pH value affected the growth of *M. gallisepticum* during fermentation in the medium. The growth of *M. gallisepticum* in the optimized medium was investigated to ascertain its response to the initial pH. As shown in *Fig. 3*, the results implied that the initial pH of 7.8 resulted in the maximum nucleic acid concentration of $10^{10.492}$ copies/mL, which was not significant at pH 8.0 (P>0.05). The maximum concentration of *M. gallisepticum* was attained when the initial pH was 7.8-8.0. Lower pH led to a decrease in the nucleic acid concentration of *M. gallisepticum*, which indicated a negative impact on its growth.

Validation of the Optimized Medium

Under the optimized conditions, the nucleic acid copy number of *M. gallisepticum* reached $10^{10.5147}$ copies/mL, which was close to the predicted value of RSM, thereby indicating that the experimental values agreed well with the predicted values *(Fig. 4-A)*. Compared with the unoptimized medium, the nucleic acid concentration of *M. gallisepticum* was increased by 9.99 times in the optimized medium *(Fig. 4-A)*. Furthermore, the optimized medium

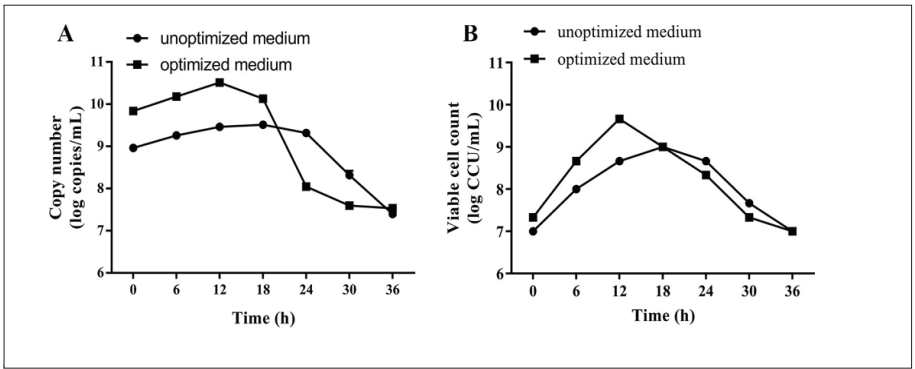

Fig 4. Growth curves of *M. gallisepticum.* **(A)** *M. gallisepticum* nucleic acid copy number growth curve, **(B)** *M. gallisepticum* CCU growth curve

exerted an effect on the viable cell count, which was up to $10^{9.8451}$ CCU/mL, signifying a 7-fold increase in the viable cell count compared with the unoptimized medium *(Fig. 4-B)*. The optimized medium enabled *M. gallisepticum* to reach the logarithmic growth stage earlier, which was approximately 6 h earlier compared with the unoptimized medium. The two growth curves demonstrated a rapid decline in nucleic acid concentration and viable cell count after *M. gallisepticum* reached maximum growth *(Fig. 4-A,B)*.

Discussion

Color change unit (CCU) is the conventional quantitative detection method for *M. gallisepticum* and this culturing step is time-consuming and difficult. In this context, Real-Time Quantitative PCR can be employed for the rapid determination of the concentration of *M. gallisepticum* [23]. This method serves as a time-efficient alternative to conventional quantitative assays, does not require further incubation, and accelerates the process of optimization. Therefore, this technique was used to rapidly determine the concentration of *M. gallisepticum*.

The addition of different nitrogen sources can increase the growth concentration of *M. gallisepticum*. Therefore, optimizing the nutrient composition of the medium is an effective way to increase the concentration of the bacterium. Response surface analysis is an effective approach to optimize fermentation parameters. PPLO broth, tryptic soy broth, and brain heart infusion media have been used for the cultivation of microorganisms, *and* PPLO broth improved both the quantity and quality of the harvested Apx toxins of *Actinobacillus pleuropneumoniae* [24]. Hwang [25] performed an experiment using CCD-optimized conditions and obtained 2.96 mg/L of *Mycoplasma* proteins, which was a three-fold increase over the unoptimized medium.

In the present study, the optimal carbon and nitrogen sources were determined using a unidirectional test, the main factors affecting *M. gallisepticum* growth were screened via PBD testing, and the fermentation parameters that resulted in the highest nucleic acid concentration were analyzed using a response surface analysis method. Under the optimized fermentation conditions, the nucleic acid copy number of *M. gallisepticum* reached $10^{10.5147}$ copies/mL, which was comparable to the predicted value. Therefore, the concentration of *M. gallisepticum* can be optimized using the RSM. The nitrogen source and the pH value affected the growth of *M. gallisepticum* during fermentation in the medium. The maximum concentration of *M. gallisepticum* was attained when the initial pH was 7.8-8.0. The growth curve of *M. gallisepticum* showed 9.99 times higher nucleic acid concentration and 7 times more viable cell count when compared with the unoptimized medium, which lowered the production cost. The logarithmic phase of *M. gallisepticum* was increased by approximately 6 h, which led to a 33.33% higher production efficiency. The new medium not only augmented the concentration but also shortened the fermentation time of *M. gallisepticum*. Nonetheless, attention must be paid to the harvesting time to avoid overculturing. Although the new medium increased the concentration of *M. gallisepticum*, serum costs remained high. Media containing 5% egg yolk, 10% horse serum, and 10% porcine serum all resulted in *M. gallisepticum* concentrations of up to 10^9 CCU/mL by the 3rd day, which could be used instead of serum. However, the number of viable organisms declined rapidly after the highest concentration was attained [26], which is in line with the results of the present study. Optimal lipid and albumin conditions established for *M. gallisepticum* were then used to propagate five different *Mycoplasma* spp. to growth levels that either equaled or surpassed those obtained with a medium containing 17% fetal bovine serum [27]. Lin [28] reported that the pH values of *M. gallisepticum* and *Mycoplasma synoviae* growth media were readjusted back to the original alkaline state when the pH reached 6.1 *(M. gallisepticum)* and 6.7 *(M. synoviae)*, and the medium was reincubated until the pH returned to 6.7-6.9. The *M. gallisepticum* and *M. synoviae* antigen yields were 43%

and 54% higher than those obtained at the usual harvest time. In the future, the concentration of *M. gallisepticum* could be increased and the cost of its medium could be reduced by adding supplements to replace the serum or by streaming NaOH solution. The cost of immunization for the farming industry could therefore be reduced.

In conclusion, this study optimized the fermentation medium for *M. gallisepticum* culture so that the nucleic acid copy number of *M. gallisepticum* reached $10^{10.5147}$ copies/mL. Compared with the unoptimized medium, the *M. gallisepticum* nucleic acid concentration in the optimized medium was increased by 9.99 times. Moreover, the viable cell count reached a maximum of $10^{9.8451}$ CCU/mL, which was a 7-fold increase compared with the unoptimized medium. The optimized medium may allow *M. gallisepticum* to reach the logarithmic growth phase earlier than the unoptimized medium, which could provide a basis for large-scale fermentation and cultivation for vaccine enterprises.

Declarations

Availability of Data and Materials: The datasets generated and/or analysed during the study are available from the corresponding authors and can be provided upon request.

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Authors' Contributions: YS performed the experiments, analysed the results, and drafted the manuscript. XZ and JZ assisted in the experimental design and summarized the experimental results. YY and XX put forward valuable suggestions for the revision and improvement of the paper. ZS, SL and LC conceived and designed the study, revised the manuscript and funded the study. All authors have read and agreed to the published version of the manuscript.

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Case Report

Persistent Left Cranial Vena Cava in a Cat

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Abstract

A 9-year-old-male British fold cat was presented for a general health check before anesthesia. Clinical and laboratory examinations were unremarkable. Echocardiography showed that the cardiac anatomy and function were normal, and the presence of a dilated coronary sinus (CS), highly suggestive of a persistent left cranial vena cava (PLCVC). Clinicians should keep in mind that PLCVC may be present when a dilated CS is noted by echocardiography, and it could be confirmed simultaneously by agitated-saline-study as a non-invasive and cost-effective diagnostic tool in practice. Further research is required to elucidate whether PLCVC is an accomplice or an innocent finding in cats.

Keywords: Cat, Persistent left cranial vena cava, Thoracic venous anomaly

Introduction

Dogs and cats normally have bilateral symmetrical cranial and caudal cardinal veins in fetal life. As the fetus develops, while the right cranial cardinal veins are directly fused and transformed into the right cranial vena cava, left cranial cardinal veins usually become atrophied, and left caudal cardinal veins develop into the coronary sinus (CS). When the left cranial cardinal veins are not atrophied and remain as a left common cardinal vein at the CS, it is described as a persistent left cranial vena cava (PLCVC) [1,2]. Four different types of PLCVC were reported and among them type 1 is the most frequently diagnosed [3,4].

The prevalence of incidentally detected asymptomatic PLCVC in dogs (2.6%) [5] is almost similar to that in humans (0.2-3%) [2], but not known yet in cats. Up-to-date, according to PubMed records, there are almost 1000 studies in humans, but nearly 20 cases in dogs, and only 5 cases in cats [1,6-9]. PLCVC cases are primarily asymptomatic and may be identified incidentally during transthoracic echocardiography and CT angiography [5]. However, some cases with PLCVC may be symptomatic when a dilated CS leads to left atrial (LA) compression and arrhythmias [2].

There are a limited number of studies showing a diagnostic algorithm for PLCVC in cats [1,9]. Thus, herein, we reported how the PLCVC was diagnosed and confirmed using transthoracic echocardiography and an agitated-saline study in an asymptomatic cat in a clinical setting and discussed whether PLCVC might be an innocent finding.

Case History

A 9-year-old male intact British Fold (4.6 kg) was presented with a clinical sign of halitosis due to dental tartar. Just after the owner approved the informed consent form, clinical and laboratory examinations were performed to decide whether the cat was suitable for anesthesia of a dental scaling procedure. There were no abnormalities of body temperature (38.3°C), heart (220 bpm) and respiratory rates (24 rpm), conjunctive mucous membranes, capillary refill time (2 sec), and lymph node palpation. Lung sounds were considered normal on auscultation, and there was no audible heart murmur. A six-lead electrocardiogram revealed a normal sinus rhythm with a normal QRS axis (CareWell 1101G, MVM Medikal, Istanbul, Türkiye). Thoracic radiography showed no thoracic abnormality. A complete blood count and serum biochemistry profile, including cardiac biomarkers, were unremarkable (data

Table 1. *Some echocardiographic measurements in a cat with persistent left cranial vena cava (PLCVC)*

Parameters		**Cat with PLCVC**	**Reference Ranges** [11]
Body weight (Kg)		4.6	4.5-5.0
Heart rate/beat per minute		220	<240
M-mode/RPSAx	RVDd (mm)	4.4	0.0-7.0
	IVSd (mm)	4.5	2.9-5.2
	LVIDd (mm)	15.1	12.7-20.3
	LVPWd (mm)	4.7	2.9-5.1
	IVSs (mm)	6.5	4.4-8.9
	LVIDs (mm)	8.1	5.7-13.4
	LVPWs (mm)	7.9	4.8-9.0
	FS % (Teich)	46.2	44.6-45.5
	EF % (Teich)	81.1	59.5-68.9
2-D/RPSAx - Aortic level	LA mm	9.7	8.2-14.9
	Ao mm	7.1	7.5-12.3
	LA/Ao	1.37	0.88-1.43
Doppler measurement/left apical 5ch	AV Vmax mm/s	82	80-150
	MV E/A	1.27	>1 - <2
	TV E/A	1.38	>1 - <2
	PV S/D ratio	0.99	0.9-1.2
	TDI MV annulus septal - E/E'	14.41	<15

RVDd: *Right ventricular diameter at diastole,* ***IVSd:*** *Interventricular septum at diastole,* ***LVIDd:*** *Left ventricular internal diameter at diastole,* ***LVPWd:*** *Left ventricular postwall diameter at diastole,* ***IVSs:*** *Interventricular septum at systole,* ***LVIDs:*** *Left ventricular internal diameter at systole,* ***LVPWs:*** *Left ventricular postwall diameter at systole,* ***LA:*** *Left atrium,* ***Ao:*** *Aorta,* ***LA/Ao:*** *Left atrium to Aorta ratio,* ***FS:*** *Fractional shortening,* ***EF:*** *Ejection fraction,* ***MPA Vmax:*** *Mean pulmonary artery maximal velocity,* ***AV Vmax:*** *Aortic valve maximal velocity,* ***MV E/A:*** *Mitral inflow E/A ratio,* ***TV E/A:*** *Tricuspid inflow E/A ratio,* ***PV S/D:*** *Pulmonary vein systole (S)/diastole (D) ratio,* ***TDI MV:*** *Tissue Doppler Imaging Mitral Valve,* ***RPSAx:*** *Right parasternal short axis*

not shown). Feline serum amyloid A, a non-specific inflammatory marker, was within the reference range (3.4 µg/mL, reference <5 µg/mL; Vcheck, Bionote, USA).

Cardiac anatomy and function were assessed by a standard echocardiographic protocol with a phased-array cardiac transducer (Vetus 7, Mindray, China) [10]. All measurements were found within the reference ranges *(Table 1)*. Through 2-D echocardiography, dilated CS was detected as a symmetric circular shape (diameter 0.97 cm and an area of 0.76 cm^2) just beneath the LA in the right parasternal long axis (RPLAX) view *(Fig. 1-A)*, and as an elliptic shape through LA free wall in the left apical 4-5CH view *(Fig. 1-B)*. PW Doppler showed the maximal velocity of the CS 0.49 milliseconds at systole and 0.41 milliseconds at diastole at the left apical 4CH view. After agitated-saline administration into the left brachial vein, there was immediate and sequential opacification of the dilated CS, RA, and right ventricle *(Fig. 1-C)*, confirming the presence

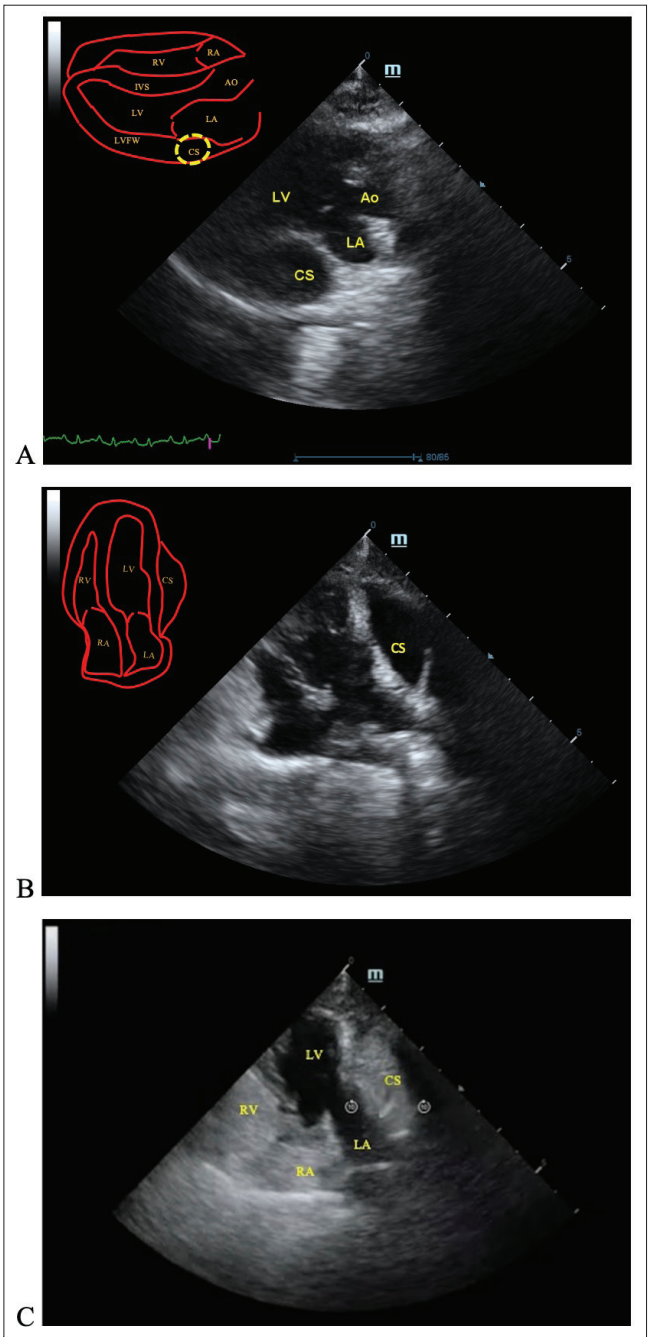

Fig 1. 2-D echocardiographic images showed a dilated coronary sinus (CS) just beneath of the left atrium (LA) at the right parasternal long axis view (A) and through the LA free wall at left apical 4-chamber views (B). CS was detected as a circular (A) or elliptic dark shape (B). 2-D echocardiographic images before (B) and just after agitated saline injection (C) at the left apical 4-chamber views. Microbubbles were seen firstly as hyperechoic content into the CS, and then right atrium (RA) and right ventricle (RV), confirming the presence of persistent left cranial vena cava (PLCVC). LV: left ventricle, Ao: aorta

of a PLSVC [1,9]. The same procedure was repeated from the right brachiocephalic vein, and microbubbles were seen firstly in the RA and not in CS, indicating the presence of normal venous return from the right superior vena cava into the RA. After the clinical evaluation, it was noted that the presence of PLCVC might not increase the risk of

anesthesia, and in case a central venous catheter would be placed, the right jugular vein should be preferred.

Discussion

The scarcity of research on this congenital anomaly in veterinary medicine, particularly in cats [1,6-9], may be attributed to the predominantly asymptomatic nature of PLCVC cases. According to PubMed records, the initial instances of PLCVC in cats were documented through necropsies, with Goodman reporting cases in 1950 [7] and Zeiner in 1957 [8]. After that, Heaney and Bulmer reported a case of PLCVC coexisted with cor triatriatum sinister in a kitten with a history of respiratory signs [6]. In recently published papers, PLCVC was detected with a polycystic renal disease in an older cat with anorexia [1] and a giant RA aneurysm in a young cat exhibited nasal stridor [9]. In the present study, the cat did not have any symptoms, except halitosis due to dental caries. Routine clinical and laboratory examinations did not show the presence of coexisting diseases.

In normal subjects, the CS is a small tubular structure just above the posterior left atrioventricular junction, and it is not remarkable during transthoracic echocardiography [2]. However, in the presented cat, CS seemed to be dilated just beneath the LA at RPLAX and through the LA free wall at the left apical-5ch view. There is no report indicating the diameter and Doppler flow characteristic of the CS in cats. Our measurement of CS (diameter = 0.97 cm) was comparable with that of a 24-year-old woman (1.5x1.6 cm) [12]. The range of potential diagnoses for dilated CS is extensive, primarily encompassing right-sided heart pathologies [2]. Specifically, in the context of CS, mild dilatation has been documented in patients with left ventricular systolic dysfunction, moderate dilatation in cases of PLCVC, and severe dilatation in PLCVC cases without a right superior vena cava (RSVC) [2,12].

The presented case had normal cardiac anatomy and functions despite a dilated CS, based on the echocardiographic evaluation. In the diagnostic workup, an agitated saline (bubble) transthoracic contrast echocardiography was performed to follow venous return from the main cranial veins to the RA in this cat [1,9]. When agitated-saline was injected into the left or right brachiocephalic vein, micro-bubbles appeared first in the CS or RA, respectively, confirming the presence of a PLCVC and normal RSVC, or double superior vena cava in this cat. PLCVC was classified as type 1 due to draining into the RA via the CS in our case; however, it drains into the RA through the CS and LA (unroofed CS) in type 2, directly into the LA in type 3, and connected to the left pulmonary vein in type 4 [2]. There is limited information on Doppler characteristics of CS in human and veterinary medicine. Spectral Doppler flow of CS in this cat was like those of humans and dogs and included two antegrade and a retrograde flow. Their maximal velocities were found lower than those of the previous study of a dog with PLCVC [2].

There are some limitations in this case. Firstly, in addition to agitated saline study, CT angiography could have been performed to demonstrate a connection between PLCVC and CS, as reported in a cat [1] and dogs [5]. However, the owner did not give permission for CT angiography because of anesthesia. Since there is a good correlation between both techniques diagnosis in practice. Secondly, left and right ventricular functions could have been evaluated by new echocardiographic methods, such as 2D speckle tracking.

In conclusion, accumulated evidence suggests that PLCVC may be more common in cats than previously reported in the literature. Therefore, clinicians should keep in mind that PLCVC may be present when a dilated CS is noted by echocardiography, and it could be confirmed by transthoracic echocardiography together with an agitated saline study as a non-invasive, safe, quick, and cost-effective diagnostic tool in a clinical setting. PLCVC was not associated with other clinical findings in the presented case. However, some cases with PLCVC may be symptomatic when a dilated CS leads to cardiopulmonary diseases. Future investigations should aim to elucidate whether PLCVC plays an active role or is merely an incidental discovery in feline cases.

Declarations

Availability of Data and Materials: The original images and video records obtained during transthoracic echocardiography in this case are available from the corresponding author (Z. Yilmaz) on request.

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

Author Contributions: Echocardiographic examination was performed by ZY, and the article was written by ZY, MK, and JK. All approved the final version of the paper.

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Case Report

Concurrent Occurrence of Aneurysmal Bone Cyst and Fibrous Dysplasia in the Mandibular Bone of a Neonatal Calf

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Abstract

This report documents the simultaneous occurrence of aneurysmal bone cysts and fibrous dysplasia in a neonatal calf's mandibular bone. The mandibular bones exhibited significant protrusion, cystic cavities, and thin woven bone spicules. Additionally, the bone trabeculae were underdeveloped with necrotic changes, and there was an absence of bone marrow and osteoblastic rimming in the woven bone. Aneurysmal bone cysts were characterized by blood-filled cystic spaces lacking epithelial or endothelial cells, separated by fibroblastic septa. In conclusion, a unique case of mandibular fibrous dysplasia displaying certain similarities to cherubism and ABC has been recorded in a calf.

Keywords: Aneurysmal bone cyst, Bovine, Congenital fibrous dysplasia, Mandibular bone

Introduction

Fibrous dysplasia (FD) of the bone is a congenital disorder characterized by replacing typical cortical and medullary bone with disorganized fibrous bone tissue in humans and animal species. This disorder disrupts the mechanisms of bone remodeling, leading to a developmental, non-neoplastic abnormality in bone growth [1]. The principal cause of FD in humans is associated with a somatic mutation in the GNAS gene (Guanine Nucleotide binding protein, Alpha Stimulating activity polypeptide) [2]. Significantly, FD is characterized by abnormal bone formations embedded within the fibrous tissues [1]. The incidence of FD in the animal population is reportedly rare, with documentation confined mainly to case reports including three canines [3-5], a single equine [6], multiple primate species [7,8], as well as a single instance in a kudu [8]. While it mainly impacts the craniofacial skeleton, there are recorded cases affecting the femur, tibia, and ribs in animals.

Aneurysmal bone cysts (ABCs) represent uncommon intraosseous lesions, predominantly impacting the axial and appendicular skeletons of both young animals and humans [9]. ABCs have been observed in a range of species, noted in canines [4], felines [10], equines [11], and bovines [12]. Morphologically, ABCs are distinguished by their expansive, osteolytic features, composed of intraosseous cavities of diverse sizes occupied by either blood or serosanguineous fluid [10].

The purpose of this report is to describe the morphological changes observed in a calf affected with both fibrous dysplasia and aneurysmal bone cyst of the mandibular bone in a calf.

Case History

Ethical Consideration

Information about necropsy procedures was provided to the animal owner before the necropsy, and a consent form was obtained from him (Protocol number: 5802).

Case Description

A neonatal male calf of mixed breed was presented to the Pathology Department, Veterinary Faculty of Fırat University for necropsy at two hours of birth. The calf experienced complications during birth, requiring the

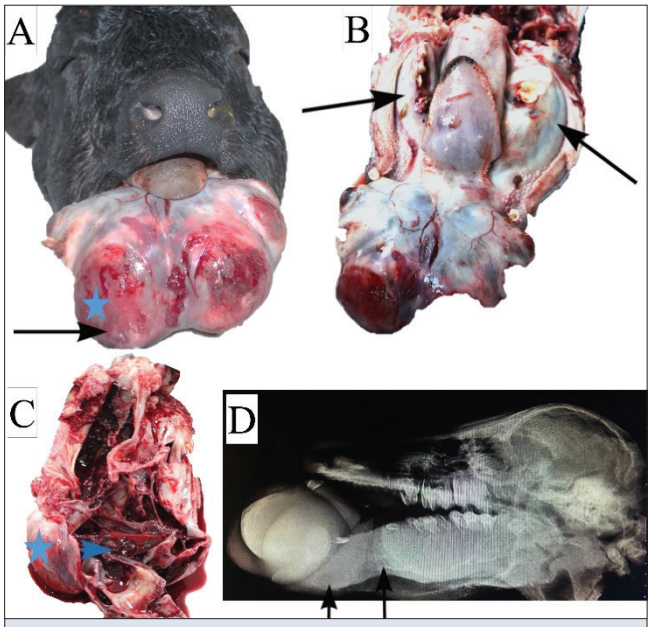

Fig 1. Gross and radiological images of the mandiblar changes. **A.** Anterior view of the head, highlighting bilateral expansion and bulging of the mandible *(arrow),* **B.** Anterior view of oral cavity, there was a noticeable enlargement and swelling *(arrows)* of the mandible, **C.** In the longitudinal section of the right hemimandible, multiple cystic spaces are evident *(arrowhead),* as well as stars marking their presence, **D.** The cystic spaces in the mandible, radiological examination, Lateral view

intervention of veterinary services. The dam had been purchased in her third trimester of gestation and did not exhibit any previous health problems. Prophylactic measures included vaccination against foot-and-mouth disease and administering antiparasitic agents roughly four months before parturition. The farm, maintaining a bovine population of 30 cattle, reported no prior occurrences of congenital anomalies in calves.

Gross Lesions

The entire mandible, extending bilaterally from the symphysis to the condyles, was swollen and filled with many cystic cavities *(Fig.1-A,B,C).* The swelling protruding outward from the mandibular symphysis measured 12x10x10 cm *(Fig. 1A).* The bone's thickness ranged from 2 to 3 mm, and the cyst-like spaces were filled with blood-tinged fluid *(Fig. 1-C).* The mandibular bone was so delicate that it could be cut with a scalpel. The affected areas exhibited a fragile and porous texture, attributable to the deterioration and enlargement. Consequently, this pathological process led to the extensive degradation of osseous tissue, forming numerous cystic cavities. Furthermore, this deterioration displaced the lower incisors and premolars from their respective alveolar lumens.

Radiological Findings

Radiographic examination revealed a mass encompassing the mandible, predominantly situated in the area typically reserved for the incisor teeth. Notably, the mandible exhibited cystic formations, particularly around the incisor zone *(Fig.1-D).* The lesion was multilocular, with well-defined boundaries consisting of multiple cysts or chambers.

Histopathologic Changes

Tissue samples from mandibula, kidney, liver, spleen, brain, lungs were fixed in a 10% formalin solution before being routinely processed and stained with hematoxylin and eosin (H&E) for light microscopic examination.

Microscopically, the most common lesion was numerous thin spicules of woven bone. They had a curved configuration, often forming circular patterns reminiscent of Chinese letters *(Fig. 2-A),* although occasionally they appeared linear. The woven bones were embedded in fibrous tissue containing fibroblastic cells. These trabeculae lack osteoblastic rimming. Bone trabeculae showed necrosis and remodeling and were surrounded by numerous multinucleated cells. *(Fig. 2-B)* Throughout the stroma, multinucleated giant cells were scattered throughout the stroma *(Fig. 2-C).* The foci of multifocal cystic degeneration were present focally *(Fig. 2-D).* There was no bone marrow nor rimming of osteoblasts present. Focal calcified foci were present. Lymphoid cells were infiltrated focally at the mass, and siderophages (iron-filled macrophages) were detected in the surrounding tissue. The subepithelial oral mucosa showed hemorrhage and hyalinization of the connective tissue.

Aneurysmal bone cysts were identified in a multifocal distribution in dysplastic bone tissue. These cysts are histologically characterized by the presence of blood-filled

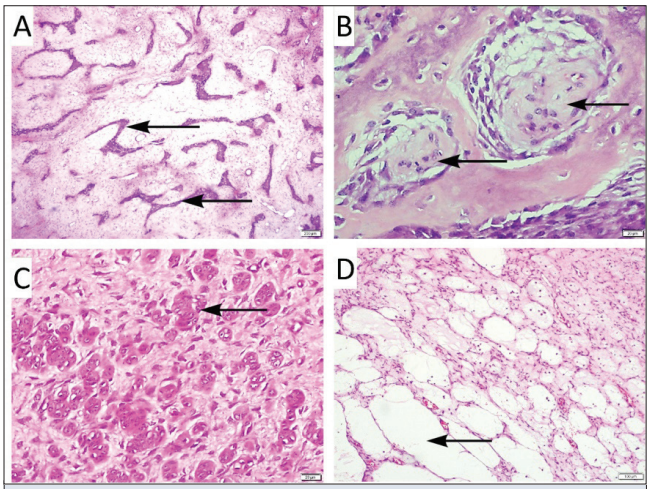

Fig 2. Histologic lesions of the fibrous dysplasia, x4, H&E, **A.** Branching and interconnecting immature bone spicules, both circular and irregular in shape (resembling Chinese characters) were embedded throughout the fibrous tissue, x40 H&E, **B.** Multinucleated giant cells *(arrows)* were situated in Howship's lacunae, x40 H&E, **C.** Diffusely distributed multinucleated cells *(arrow),* x20, H&E, **D.** Stromal cystic degeneration *(arrow),* x10, H&E

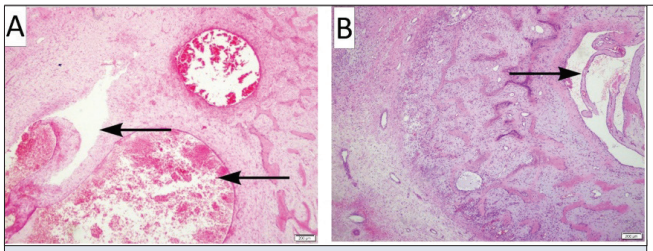

Fig 3. Histologic lesions of the bone, tooth, lungs, and eye, x4, H&E. **A.** Aneurysmal bone cysts *(arrows),* x4, H&E, **B.** Separation of tooth from alveolar bone, x4, H&E

cystic spaces devoid of epithelial or endothelial cells, which are separated by fibroblastic septa *(Fig. 3-A)*. The incisive teeth have become detached from the alveolar bone, with the periodontal ligament remaining in position *(Fig. 3-B)*. Some alveolar spaces showed deposition of meconium, hyperemia and interstitial edema. The optic nerve in the eyes showed mild papillary edema on the optic disc.

Examination of the lungs revealed marked emphysema in some lobes, atelectasis and interlobar edema.

Discussion

Fibrous Dysplasia and Aneurysmal Bone Cyst are two distinct pathological entities, each characterized by its unique pathogenetic mechanisms. A review of veterinary literature has shown no record of the simultaneous occurrence of these two conditions. Nevertheless, in humans, 36 reported cases of simultaneous fibrous dysplasia and aneurysmal bone cyst, which include four instances of mandibular involvement, as identified in literature [13] up to 2019. The simultaneous occurrence of these conditions is attributed to the hemodynamic disturbances arising from FD [14]. However, this concurrent presence is not specific to FD; ABCs may also occur secondarily within or adjacent to other tumours or malignant processes, like osteoblastoma. Therefore, any factor weakening the bone structure could potentially lead to the formation of ABCs [13]. An alternative explanation may be attributed to alterations in bone pressure or the redistribution of mechanical stress, stemming from structural changes within the bones. Contrary to what the name "ABC" might suggest, the term is somewhat misleading since the lesion does not present the typical features of an aneurysm or a cyst, particularly due to the lack of an endothelial lining [13]. At present, ABCs are recognized as benign osseous lesions that may undergo rapid enlargement and cause significant compromise to the integrity of the bones they occupy [13]. The differentials for ABC included hemangioma, hemangiosarcoma and osteosarcoma in this report. ABC is characterized by blood-filled spaces, delineated by connective tissue housing bone trabeculae or osteoid tissue. It also has irregular vascular channels devoid of endothelial lining. lacking malignant cells and endothelial cell. However, osteosarcoma contains malignant osteoid or bone forming tissue and hemangiosarcoma is characterized by malignant endothelial cells forming vascular structures

FD is characterized by the presence of underdeveloped bone segments, fibroblasts, hypertrophic osteoblasts, diverse trabecular bone patterns, multinucleated osteoclastic giant cells, and the absence of rimming osteoblasts in the surfaces of the trabeculae [6,7]. In differential diagnoses, the following lesions should be considered: non-ossifying fibroma, osteoma, adamantinoma, low-grade osteosarcoma, and Paget's disease [10]. The differentiation depended on the evaluation of clinical, radiological, and histopathological features in this report. In the clinical perspective, osteoma and ossifying fibroma present as palpable bony masses. Radiologically, fibrous dysplasia exhibits a distinctive ground-glass appearance. Conversely, ossifying fibroma displays a combination of radiolucent and radiopaque areas, often featuring a central calcified region. At the histopathological level, fibrous dysplasia manifests fibrous tissue with irregular trabeculae reminiscent of woven bone. In contrast, osteoma is characterized by the presence of lamellar bone [1,4]. Non-ossifying Fibroma has sheets of histiocytes, foam cells, and giant cells within a fibrous stroma. It does not show osteoblastic rimming, distinguishing it from fibrous dysplasia. Adamantinoma is characterized by epithelial-like nests and strands within fibrous stroma and is distinguished by the presence of epithelial components [10].

The current case, through its gross and histological findings, also exhibits characteristics similar to cherubism. This uncommon autoinflammatory bone disorder is characterized by the symmetrical expansion of fibro-osseous tissue in the mandible and/or maxilla, most commonly observed in children aged 2 to 5. Key features of the case -including bilateral mandibular involvement, bone tissue degradation, teeth dislodging from their alveoli, and the detection of osteoclast-like cells - indicate characteristics and are reportedly found in human cherubism [14]. Despite these similarities, the terminology "chimerism" remains unrecognized in veterinary literature. Nonetheless, a genetically developed animal model, specifically in mice, has been established [15].

In the present case, the diagnosis was based on the presence of woven bone spicules and the absence of osteoblastic rimming. The concurrent presence of fibrous dysplasia and cherubism presented here supports the theory that cherubism represents a hereditary form of fibrous dysplasia in humans [14]. The pulmonary lesions, including emphysema, atelectasis, and meconium aspiration, may have arisen due to the posterior displacement of the tongue caused by the effect of the mandibular mass. Similarly; separation of teeth from alveolar bone and edema of optic

nerve head were probably secondary lesions of fibrous dysplasia.

In conclusion, a unique case of mandibular fibrous dysplasia displaying certain similarities to cherubism and ABC has been recorded in a calf.

Declarations

Availability of Data and Materials: The data that support the findings of this case report are available from the corresponding author (Y. Eröksüz) upon reasonable request.

Conflict of Interest Statement: The author declares no conflict of interest

Author Contributions: Conceptualization, YE and CAI; Acquisition, analysis, and interpretation of data, YE and HE, CAI and MT; Writing - original draft preparation, Y.E.; Review and editing, H.E., CAI, MT. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part are appropriately investigated and resolved.

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Case Report

Retinoblastoma-Like Tumor with Brain Metastasis in a Border Collie

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Abstract

In this report, we described the clinical, ultrasound, contrast-enhanced T2-weighted brain magnetic resonance imaging (MRI), and histopathological findings of a retinoblastoma-like tumor with brain metastasis in a 3-year-old male Border Collie. Ophthalmoscopic and ultrasonographic examination revealed leukocoria associated with a solid mass of retinal origin in the left eye. Simultaneous contrast-enhanced T2-weighted brain MRI evaluation revealed solid masses at three different locations: the first one at the levels of the suprasellar cistern, third ventricle and chiasma opticum; the second one in the medulla oblongata adjacent to the caudal cranial fossa; and the third one in the left intraocular region. Histopathological examination of the extracted mass in the globe revealed a retinoblastoma-like tumor. The patient died before receiving radiotherapy treatment. In conclusion: this report highlights the importance of early diagnosis through ophthalmoscopic and ultrasonographic examinations. Emphasizing the brain as a potential secondary metastatic site, the report underscores the critical need to create a window for timely radiotherapy. Furthermore, the recommendation is made to evaluate dogs with leukocoria during ophthalmoscopic examination for both retinoblastoma and potential brain metastasis.

Keywords: Dog, Embryonal tumors with multilayered rosettes (ETMR), Ocular neoplasia, Primary neuroectodermal tumors (PNET), Retinoblastoma-like tumor

Introduction

The most common intraocular tumors in dogs are primary melanocytic and iridociliary neoplasms. The canine eye is usually the primary neoplastic focus [1,2]. However, it has also been reported that the anterior uvea of the eye is a secondary metastatic focus for tumors such as transmissible venereal tumor, lymphosarcoma, and hemangiosarcoma [1,3]. Primary neuroectodermal tumor (PNET) is a general term used to classify all embryonal neoplasms arising from the germinal neuroepithelium of the neural tube (neuroectoderm), and these tumors occur rarely in dogs [2]. PNETs have recently been terminologically designated as embryonal tumors with multilayered rosettes (ETMR) [4]. In humans, the majority of PNETs are observed in the pediatric population and recognized commonly as retinoblastoma arising from the primitive neuroepithelium of the retina and medulloepitheliomas arising from the primitive medullary epithelium of the ciliary body [5]. There are two types of retinoblastomas in humans; genetic and sporadic. Genetic retinoblastoma originates from retinal neurons in multiple locations in both eyes and is observed in infants under the age of 1 year [6].

In this report, ophthalmoscopic, ultrasonographic, contrast-enhanced brain MRI (CMRI), and histopathological findings of retinoblastoma-like tumor with brain metastasis in a dog were described. The importance of ophthalmoscopy and ultrasonography (USG) for early diagnosis of the patient and buying time to benefit from radiotherapy by considering the brain as a secondary metastatic site as well as the need to consider retinoblastoma in patients with leukocoria were emphasized.

Case History

For this case report, informed consent was obtained from the patient owner.

A 3-year-old male Border Collie was admitted to VetAmerikan Animal Hospital (İstanbul-Türkiye) with complaints of loss of appetite and vision. Hematologic

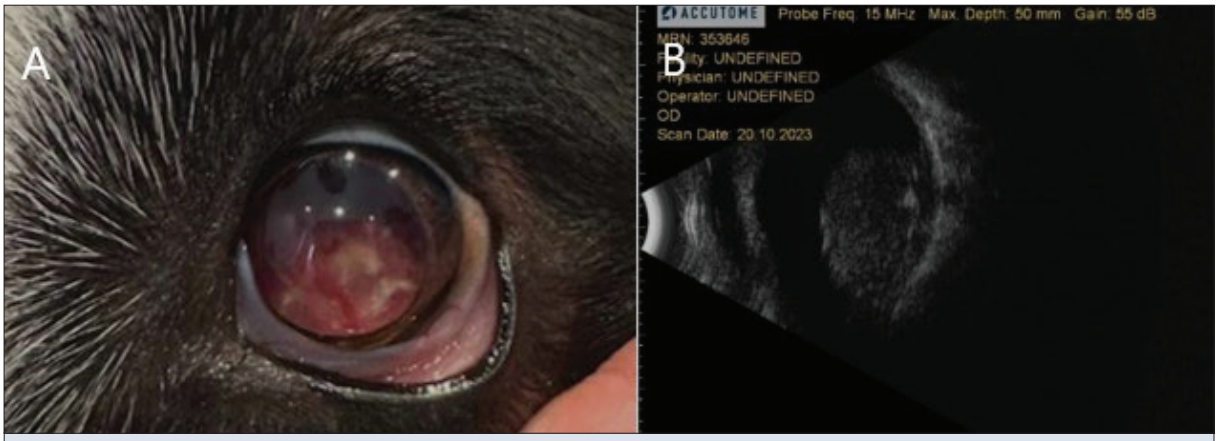

Fig 1. Ophthalmoscopic and ultrasonographic examination. **A.** Neoplastic mass enlarged into the posterior chamber and corpus vitreous, and leukocoria in the left eye internal angle, **B.** In the sonogram of the left eye, transversal sections showed an echogenic solid mass of retinal origin extending to the vitreous independent of papilla nervi opticus

and biochemical parameters were within normal limits. Ophthalmoscopic examination revealed that cotton ball test, menace response, and obstacle test were negative in both eyes. Direct and indirect pupillary light reflexes were positive in the right eye and negative and fixed mydriatic in the left eye, indicating N. oculomotorius damage. In the direct and indirect ophthalmoscopic examination, the fundus of the right eye was normal, and the leukocoria in the left eye was due to the presence of a solid intraocular mass, which was considered to be originating from the retina *(Fig. 1-A)*. Intraocular pressure (IOP) values were determined to be 21 mmHg in the right eye and 27 mmHg in the left eye. Background USG with a 15 MH probe in the left eye (Ophthalmic, Accutome, B-Scan Plus, Canada) suggested that the solitary mass of retinal origin was found to be independent of N. opticus *(Fig. 1-B)*.

Simultaneous T2 sagittal weighted evaluation of the patient with Contrast-enhanced brain MRI (1.5 Tesla, Siemens, Magnetom Sempra, Germany) revealed solid masses of 13.4 x 9.mm intraocular in the left bulbus oculi *(Fig. 2-A)*, 19.1 x 18.4 mm at the suprasellar cistern, third ventricle and chiasma opticum levels of the brain, and 4.0 x 6.7 mm adjacent to the caudal cranial fossa in the medulla oblongata *(Fig. 2-B)*. Contrast-enhanced computed tomography (CT) scan of the thorax and abdomen (Somatom go, Germany) showed no pathologic findings.

Enucleation of the left eye was performed with standard anesthesia and surgical protocol followed by histopathologic evaluation. The eyeball was cut sagittal. A partially solid mass of 13x9 mm in size, originating from the retina and expanding into the vitreous, with no connection to the lens, was found close to the nasal angle of the eye *(Fig. 3)*.

The sagittal sectioned eyeball was completely cassetted

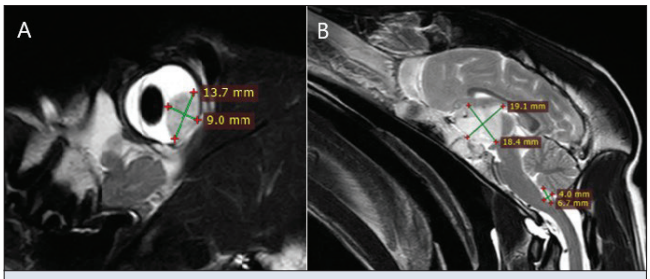

Fig 2. Contrast-enhanced brain MRI evaluation. **A.** Intraocular mass on T2-weighted sagittal plane in left bulbus oculi at the size of 13.7 x 9.0 mm, **B.** Solid masses on the T2-weighted sagittal plane at the suprasellar cistern, third ventricle and chiasma opticum levels of the brain (19.1 x 18.4 mm) and in the medulla oblongata adjacent to the caudal cranial fossa (4.0 x 6.7 mm)

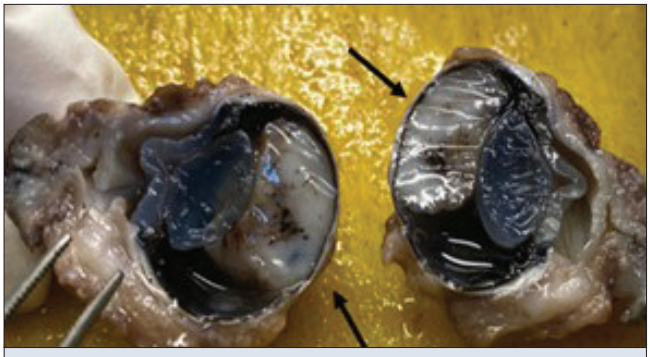

Fig 3. Nodular neoplastic tissue in the bulbus oculi (arrows)

and subjected to the usual routine procedures. The section surface and the tumoral mass were embedded in liquid paraffin, suitable for dissection. The prepared blocks were cut at a size of 4-5 micron thin with a rotary microtome and stained with hematoxylin and eosin (H&E) to be examined under a light microscope. The mass consisted of neoplastic cells originating from retina and expanding into the vitreous and attached to the retina at large areas *(Fig. 4-A)*. The mass had no connection with the lens and ciliar body *(Fig. 4-B,C)* in serial sections and there were

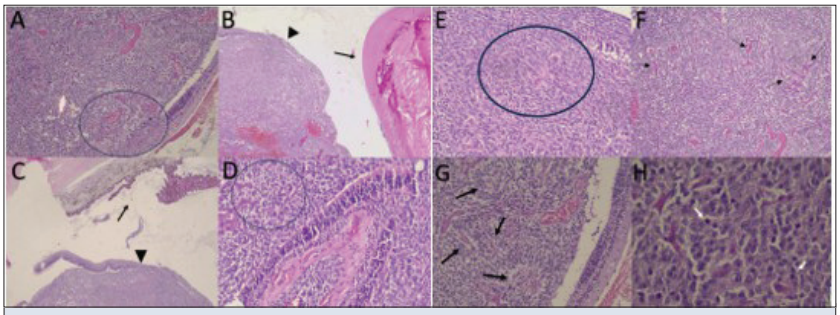

Fig 4. A. Neoplastic mass of retinal origin *(circled area)* HE x10, **B.** Lack of connection between neoplastic tissue *(arrowhead)* and the lens *(arrow)* HE x4, **C.** Lack of connection between neoplastic tissue *(arrowhead)* and the ciliary body *(arrow)* HE x 4, **D.** Atypical cells in neoplastic retinal tissue, some with vacuolated cytoplasm *(circled area)* HE x 20, **E.** Atypical neuroepithelial cells forming the neoplastic tissue *(circled area and its surroundings)* HE x 20, **F.** Atypical neuroepithelial cells forming neoplastic tissue with fibrovascular stroma *(arrows)* HE x 10, **G.** Imaginary rosette-like structures in neoplastic tissue *(arrows)* HE x20, **H.** Oval polygonal neuroepithelial cells forming the neoplastic tissue and mitotic structures *(arrows)* HE x40

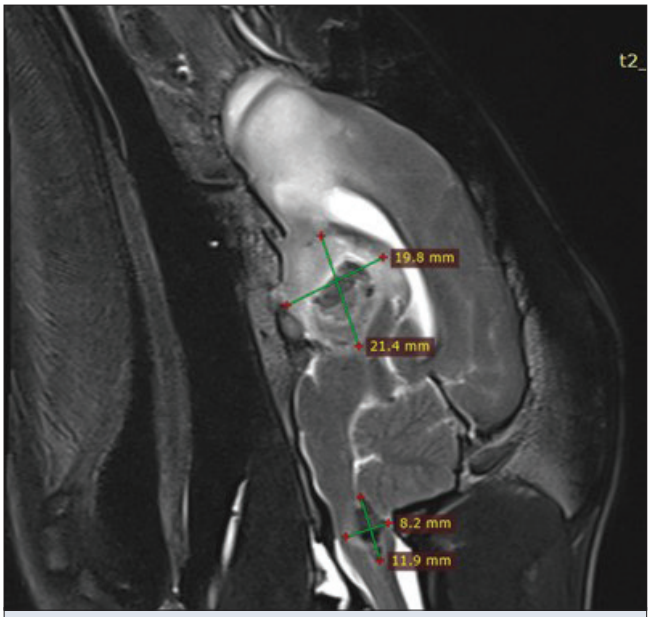

Fig 5. In the CMRI evaluation of the patient, on the T2-weighted sagittal plane, expansion of the mass in the suprasellar cistern, third ventricle and chiasma opticum levels of the brain to a size of 19.8 x 21.4 mm and the mass in the caudal cranial fossa adjacent to the medulla oblongata to a size of 8.2 x 11.9 mm

atypical cells in the mass, some with vacuolated cytoplasm *(Fig. 4-D)*. Atypical cells were oval, spindle or polygonal shaped cells of different sizes with hyperchromatic nuclei and little cytoplasm. These cells were similar to immature neuroepithelial cells of the retina *(Fig. 4-E)*. In different areas of the neoplastic tissue, the majority of atypical cells formed rosette like structures (Flexner-Wintersteiner) with based on imaginary appearance *(Fig. 4-F,G)* and 2-4 mitotic figure on average at x40 objective were observed in different areas of oval polygonal neuroepithelial cells forming the neoplastic tissue *(Fig. 4-G,H)*. Moreover, a focus of calcification within an area of atypical neoplastic cells was also detected. Histopathologic findings confirmed that the neoplastic tissue was a retinoblastoma-like tissue. Treatment was suggested as planning tomography followed by intensity-modulated external-beam radiation therapy (Trilogy /Varian Medical Systems, Palo Alto, CA) for both lesions in the brain. The patient died due to development of unconsciousness and pressure on the respiratory center because of rapid expansion of the tumor during intermittent follow-ups.

Contrast-enhanced brain MRI evaluation following death of the patient showed that the mass at the suprasellar cistern, third ventricle and chiasma opticum levels of the brain was enlarged to 19.5 x 21.4 mm, and the mass adjacent to the caudal cranial fossa of the medulla oblongata to 8.2 x 11.9 mm on the T2-weighted sagittal plane *(Fig. 5)*.

Discussion

Neuroblastoma, ependymoblastoma, retinoblastoma, and medulloepithelioma, which are classified in the PNET or ETMR classes, are observed in both humans and animal species, and they have been reported to occur in the peripheral and central nervous system or in the bulbus oculi [7-9]. The common feature of these tumors is that they have multilayered rosettes in histopathologic terms. Flexner-Wintersteiner rosettes are actual wheel-shaped rosettes in histopathologic evaluation and are known to be specific to retinoblastoma-like tumors and certain PNETs [10,11].

Syed NA found that the tumor contained areas of retinal photoreceptor and glial differentiation, unlike other tumors examined including Flexner-Wintersteiner rosettes, and the histopathological findings and differential staining characteristics of the retinal tumor were consistent with retinoblastoma, and they presented the first documented case of spontaneous retinoblastoma in an animal.

More recently, Regan et al.[9] diagnosed retinoblastoma-

like tumors in 4 out of 8 dogs with PNET and interestingly reported that these tumors were frequently observed in dogs ≤2 years of age, which was consistent with the clinical age presentation in humans, while the more common medulloepitheliomas occurred in dogs >7 years of age. They indicated that the characteristics of retinoblastoma include predominantly hyperchromatic small neuroepithelial cells, nuclei, scanty cytoplasm, and low mitotic index with smaller sized rosette formation, often characterized by a single row, as well as radially arranged neoplastic cells.

In the histopathological evaluation of our case, considering that imaginary rosette formation was observed, the tumors surrounded a central lumen containing small cytoplasmic extensions and were characteristic for retinoblastoma, and the fact that the tumor developed in a single eye and at a young age, the findings were remarkable, and the authors agreed on the diagnosis of spontaneous retinoblastoma-like tumor.

Retinoblastomas are malignant tumors arising from the primitive neuroepithelium of the retina while medulloepitheliomas arise from the primitive medullary epithelium of the ciliary body [5]. In our case, microscopic examination clearly showed that the neoplastic tissue originated from the retina, and the neoplasm was of retinal origin.

In a 10-year-old Golden Retriever, ETMR was considered in the histopathologic examination of a retrobulbar mass that pushed the globe to the dorsotemporal region and caused distortion. It has been reported that ETMRs are generally aggressive malignant tumors in both humans and animals, and no systemic metastasis of retrobulbar ETMRs has been reported [12].

In our case, MRI evaluation revealed solid mass metastatic areas of 19.1 x 18.4 mm in the suprasellar cistern, third ventricle and chiasma opticum levels of the brain and of 4.0 x 6.7 mm adjacent to the caudal cranial fossa in the medulla oblongata.

It has been emphasized that detailed ophthalmoscopic/background sonographic examination should be performed in the differential diagnosis of retinoblastoma; retinoblastoma should be differentiated from persistent hyperplastic primary vitreous, cataract, vitreous hemorrhage, retinal detachment, and meduloepithelioma, and that background USG is necessary to recognize the type of retinoblastoma tumor in the differential diagnosis. USG diagnosis of retinoblastoma is based on demonstrating calcifications within the lesion, which produce bright signals with high reflectivity and cause shadowing [13]. Similar reflections were observed on sonography performed with B-Scan mode and 15 MH probe, and a calcified focus was detected on histopathologic examination.

While most ophthalmic tumors are locally invasive and cause distortion of the globe, retinoblastoma has been rarely associated with pain and uveitis attack. In children with retinoblastoma, leukocoria (loss of normal fundus reflex) is related to the tumor filling the globe and is the most important clinical finding [14].

Unlike ciliary - uveal melanomas which may cause elevated intraocular pressure (IOP), uveitis, pain, and globe disorientation; in the present case, the neoplastic mass expanded into the vitreous inside the eye, and the animal did not develop any distress, pain, or uveitis associated with the globe position until it was visible from the outside. Leukocoria was determined as a typical finding in retinoblastoma.

Early diagnosis of ocular neoplasia is an important aspect of treatment success. It has been reported that retinoblastoma shows local invasion to choroid and sclera in humans and can metastasize hematogenously to the bone, liver, central nervous system, and other organs [14]. In the literature review, no case of brain metastasis associated with retinoblastoma was found in dogs, and our case was believed to be the only known case of brain metastasis associated with retinoblastoma. In addition, no malignant focus outside the brain was detected on CT evaluation. Hemangiosarcoma, mammary carcinoma and melanoma are known to be the most common metastatic intracranial neoplasms in dogs. The incidence rates of metastatic brain tumors and primary brain tumors in dogs are equal [15]. Early diagnosis of retinoblastoma increase survival in humans. Among the treatment options for retinoblastoma, the current approach is enucleation and external-beam therapy in humans [5,16]. The authors recommend examination of the brain with advanced imaging techniques in cases of suspected ocular neoplasia. When the MRI findings were evaluated in the patient that died after eye extirpation before the planned radiotherapy could be performed, it was observed that the first mass in the brain rapidly enlarged by approximately 1.2 mm^2, and the other mass by 21.84 mm^2 within 1.5 months. In the light of these findings, retinoblastoma should be considered especially in young dogs with unilateral leukocoria regardless of breed. Considering that the secondary metastatic site may be the brain and the primary and secondary foci grow very rapidly, it is believed that survival may improve with early-stage diagnosis and planned anticancer treatment.

Declarations

Availability of Data and Materials: The data used in this article will be provided by the corresponding author (E. Özgencil) upon request.

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

Author Contributions: Case examination and evaluation of clinical

findings were done by FEÖ, APG, OÜ and VÇ. FEÖ, ÖK, ND and VÇ performed the operation of the patient and provided postoperative care. EEÇ and EY interpreted MRI and CT images. FEÖ and APG contributed to the discussion. AG and DÖ performed the histopathological examination of the patient. FEÖ made the article writing and ND made the submission of the article. The authors have submitted the paper collectively.

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Case Report

Ethmoidal Meningoencephalocele in an Anatolian Shepherd Dog

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Abstract

Meningoencephalocele is an uncommon disorder in dogs, that has detrimental effects on both the duration and quality of life. Dogs afflicted by this condition may exhibit neurological deficits, epileptic seizures, and behavioral disorders. A 4-month-old female Anatolian Shepherd dog, weighing 13 kg presented for further investigation of a 3-week history of cluster, grand mal seizures. No abnormalities were evident on physical examination. A full neurological examination, including cranial nerve assessment was unremarkable, except a slight right proprioceptive deficit on the right front leg. MRI of the brain was performed. Protrusion of the right rostral rhinencephalon through the defect of the right cribriform plate and a right basal ethmoidal meningoencephalocele was detected. Due to the dog's age, aggressive temperament, and current clinical status, medical management was preferred, and the dog was managed on phenobarbital and levetiracetam. Seizures have remained well controlled 12 months post-diagnosis.

Keywords: Anatolian Shepherd Dog, Cluster seizure, Epilepsy, Meningoencephalocele, Magnetic resonance imaging, MRI

Introduction

In humans encephalocele refers to the abnormal protrusion of cranial contents beyond the normal confines of the skull. They may contain meninges (meningocele), brain matter and meninges (meningoencephalocele) or they may communicate with the ventricles (meningoencephalocystocele) [1]. A meningoencephalocele (MEC) is a protrusion of cerebral tissue and meninges through a cranial defect, whereas a meningocele (MC) is a herniation of the meninges only [2]. MECs protruding into the nasal cavity are named intranasal MECs [3].

This anomaly results from a focal failure of the neuroectoderm and surface ectoderm to separate during fetal development, occurring primarily during the 4th gestational week [4]. Genetic and environmental factors, including toxins, nutritional deficiencies, head traumas, and chronic intracranial hypertension, are identified as potential contributors to acquired cases [5-9]. Additionally, it has been reported that a meningoencephalocele formation occurred in a cat after the transfrontal craniotomy procedure. This situation indicates that this pathology can also develop iatrogenically [6]. In cases of congenital encephalocele, it has been observed that the meninges are often protruded [7].

Clinical presentations in both humans and dogs range from epileptic seizures to behavioral alterations. In the canine population, ethmoidal meningoencephaloceles are associated with diverse neurological signs, including aggressiveness, hyperactivity, and distinctive behaviors like stargazing or fly-catching [8]. Diagnosis necessitates a comprehensive approach involving clinical evaluation and advanced imaging techniques, with MRI being crucial for delineating intracranial compartments and herniated contents. Treatment options vary, with anti-epileptic drugs proving to be effective in some cases, while surgical intervention may be considered for drug-resistant cases or those presenting with cerebrospinal fluid rhinorrhea.

This report aims to comprehensively document and analyze a rare case of ethmoidal meningoencephalocele in a 4-month-old female Anatolian Shepherd dog presenting with cluster grand mal seizures. The investigation delves into the clinical presentation, diagnostic process utilizing MRI, and the subsequent management of the dog. The report emphasizes the challenges posed by the ethmoidal meningoencephalocele in a canine patient,

exploring the intricate relationship between anatomical anomalies, neurological manifestations, and treatment modalities. By providing detailed insights into this rare clinical scenario, the report contributes to the veterinary literature, fostering a deeper understanding of ethmoidal meningoencephaloceles in dogs and guiding clinicians in their diagnostic and therapeutic approaches to similar cases.

Case History

Informed consent was obtained from the animal owner to use the data obtained from the clinical examination.

Clinical Examination

A 4-month-old, intact female Anatolian Shepherd dog, weighing 13 kg, was admitted for evaluation of worsening cluster, grand mal seizures. The dog was adopted 3 months ago, medical history is unknown and was clinically healthy for the last 2 months. The first generalized seizure which lasted around 6 min was followed by 3 more seizures on the same day. No epileptic seizures occurred for the next 3 weeks, until the day before admission. The dog had 5 more grand mal seizures, each lasting around 4 min. During examination, the dog was bright, alert, and responsive. A full neurological examination, including cranial nerve assessment was unremarkable, except a slight right proprioceptive deficit on the right front leg. Complete blood count showed a mild increase in basophil (0.26 x 10^9/L; reference interval [RI] 0-0.1x10^9/L) and eosinophil (1.89 x 10^9/L; RI 0-1.6x10^9/L) counts. Serum biochemistry analysis also revealed a minor increase in ALP (139 U/L; RI 13.0-83.0 U/L) which is consistent with young age and cluster seizures. Serum TSH, total T4 and free T4 values were within the reference range.

Imaging, Diagnosis and Outcome

The dog was sedated with 10 µg/kg-IM medetomidine (Domitor®, Zoetis, Estonia) and 0.1 mg/kg-IM butorphanol (Butomidor®, RichterPharma, Austria) combination. Anesthesia was induced with 0.2 mg/kg-IV propofol (Propofol® 200 mg/20 mL, Abbott, Türkiye) and maintained by an inhalation anesthetic, isoflurane at a concentration of 1% (Forane®, liquid, Abbott, England) in 100% oxygen. MRI of the brain was performed using a 1.5 Tesla MRI system (GE Signa Excite 1.5T). T2-weighted fast recovery fast spin echo (frFSE), fluid-attenuation inversion recovery (FLAIR) in sagittal, dorsal, and transverse planes. T1-weighted fast spin echo (fSE) was performed with 4 mm slice thickness in transverse plane. Post-intravenous gadolinium contrast (0.1 mmol/kg - Gadovist®, Bayer, Türkiye) T1-weighted fat sat fast spin echo sequence (FS-T1-fSE) was obtained with 4 mm slice thickness, in transverse and dorsal planes.

MRI revealed protrusion of the right rostral rhinencephalon through the defect of the right cribriform plate, which was measured approximately 1 cm in diameter. Herniation of the neural parenchyma to the nasal cavity was measured at 22x19x12 mm. Bilateral deep ventricular white matter and the signal intensity of gray and white matter in bilateral cerebral hemispheres were evaluated as normal. The herniation of the neural parenchyma to the right rostral rhinencephalon was consistent with a right

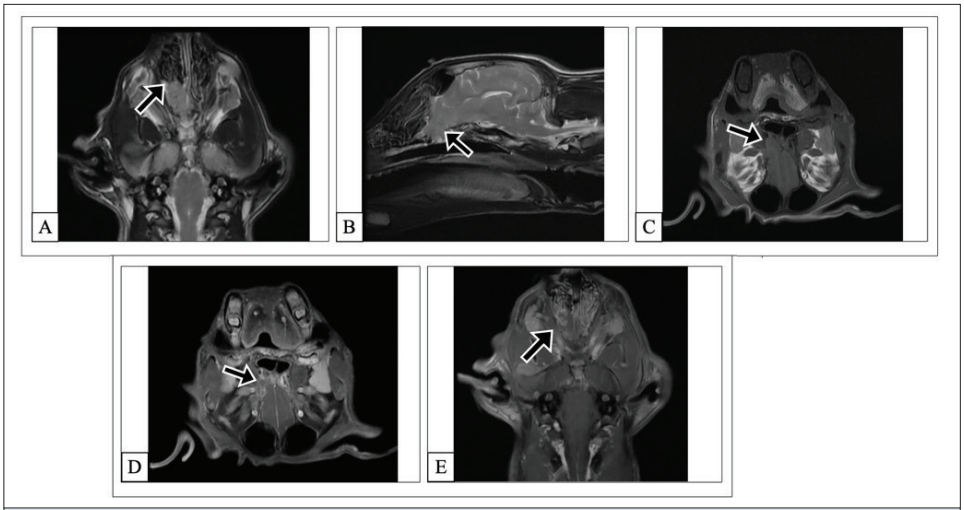

Fig 1. Magnetic resonance images of the dog's brain. **A**- Dorsal fast recovery Fast Spin Echo T2-weighted image. Brain tissue (rhinencephalon) and meninges *(black arrow)* and the absence of the normal structures in the caudal part of the right nose, **B**- Sagittal fast recovery Fast Spin Echo T2-weighted image through the skull. Rhinencephalon is seen in the right ethmoidal conchae *(black arrow)*, **C**- Transverse fast Spin Echo T-1 weighted image pre-contrast at the level of olfactory bulb and rhinencephalon *(black arrow)*, **D**- Post-contrast transverse fast Spin Echo T-1 weighted image with mild contrast enhancement of the meninges (black arrow), **E**- Post-contrast dorsal fast Spin Echo T-1 weighted image, mild contrast enhancement of the protruded meninges *(black arrow)*

ethmoidal MEC. In the post-contrast examination, mild meningeal contrast enhancement was observed around the protruding rhinencephalon tissue in both transverse and dorsal planes in T1- weighted sequences *(Fig. 1)*.

Cerebrospinal fluid analysis was performed after the magnetic resonance imaging process, which did not reveal any abnormalities. The dog recovered uneventfully from anesthesia. Conservative therapy with antiepileptic medication was preferred. Surgical correction of the meningoencephalocele and defect was not performed due to the dog's age, aggressive temperament, and current clinical status. The dog was discharged on the same day of diagnostic imaging.

Levetiracetam (Keppra, UCB Pharma, USA) was prescribed at 20 mg/kg PO q 8 h in combination with phenobarbital (Luminal, Bayer, Türkiye) 2 mg/kg PO q 12 h. 2 days after the patient was discharged, a single generalized epileptic seizure recurred, which was lasted less than 1 min. Phenobarbital and levetiracetam doses were adapted to the dog's changing weight each month and serum phenobarbital concentration was measured at 22.4 µg/mL in the fourth month and 19.7 µg/mL in the 9th month of therapy (range, 15-40 µg/mL). Seizures have been well controlled since last seizure and no seizure activity had been observed for 12 months post-diagnosis of the meningoencephalocele.

Discussion

A focal failure of the neuroectoderm and surface ectoderm to separate during fetal development results in congenital meningoencephalocele formation. In humans, it is considered a late neurulation defect taking place during the 4th gestational week may result in MECs [4]. In cases of congenital encephalocele, it has been observed that the meninges are often protruded [7].

The most common clinical signs were epileptic seizures, behavioral alterations including aggressiveness, hyperactivity, intermittent yelping, stargazing or fly-catching behavior in dogs [8]. In most cases, as outlined by Lazzerini et al.[11], seizures serve as the primary neurological manifestation in dogs with ethmoidal MEC, although it's important to note that seizure features don't consistently seem to align with the location of the defect. Hence, although the underlying causes of seizures in individuals with encephalocele are thought to stem from cortical traction or herniation, additional factors such as hemorrhage, white matter degeneration, or the presence of infectious or non-infectious inflammatory infiltrates may also contribute to abnormal neuronal excitability [12]. Additionally, cases of cerebrospinal fluid rhinorrhea have been documented in dogs with frontoethmoidal encephaloceles [11-13]. In Lazzerini et al.[11]'s research, when examining dogs with frontoethmoidal encephaloceles, neurological abnormalities such as circling, proprioceptive deficits, and reduced menace response were observed. Interestingly, this study revealed that 6 out of 17 dogs with frontoethmoidal encephaloceles displayed normal neurological examination findings.

The diagnosis of encephaloceles necessitates a dual approach, combining clinical assessment with advanced imaging methods. Following the clinical and neurological evaluation, an MRI becomes essential, while a CT scan can provide valuable insights into the craniofacial skeleton's visualization. Additionally, MRI serves the purpose of delineating the communication between intracranial compartments and the herniated contents [12].

Dogs affected by MECs may respond to medical treatment with anti-epileptic drugs [11]. However, the development of drug resistance can serve as an indication for considering surgical intervention [11-14]. In cases where surgery is not performed, the response to anti-epileptic drugs may vary. Notably, some dogs afflicted with frontoethmoidal encephaloceles may eventually be euthanized due to unmanageable seizures [11-12]. Within the Lazzerini et al.[11] study involving 11 dogs treated with anti-epileptic drugs, 2 out of the 11 dogs were seizure-free, while 3 out of 11 experienced a seizure reduction of at least ≥50%.

The only abnormal finding obtained during the neurological examination of the patient presenting with complaints of generalized epileptic seizures is a slight proprioceptive deficit on the right front leg. In a study conducted by Lazzerini et al.[11] in 2017, which included 22 dogs diagnosed with meningoencephalocele or meningocele, lateralized proprioceptive deficits were observed in 9 dogs, while 7 dogs had no abnormal neurological findings reported. Although the pathological mechanism causing unilateral proprioceptive deficit is not fully understood, it is believed to be the result of the mechanical traction effect of the protruding tissue on the remaining brain tissue. In this case, it is observed that the rhinencephalon, which not only transmits the sense of smell but also conveys instinctive and emotional behaviors, is protruding. Despite the patient's young age, the aggressive behavioral pattern exhibited is also thought to be related to the protrusion of the rhinencephalon.

It has been observed that clinical and neurological examination findings are not sufficient for the diagnosis of this pathology, and advanced imaging methods must be included in the diagnostic process. Due to the presence of a calvarial defect in MEC cases, diagnosis can be made not only with MRI but also with CT.

The patient's T1-weighted post-contrast MR images show mild contrast enhancement in the meninges associated with the protruded rhinencephalon tissue. Such contrast enhancement is thought to be potentially due to focal meningitis or low cerebrospinal fluid pressure

and resulting intracranial hypotension. Although no pathology was detected in the CSF analysis of the case, it should be noted that CSF analysis findings may not always be consistent in cases of focal meningitis. It is thought that intracranial hypotension may have developed due to the disruption of intracranial volume balance caused by the protruding brain tissue.

Although the possibility of traumatic MEC formation cannot be ruled out since the medical history of the dog is unknown, the fact that the dog started showing clinical symptoms at a very young age increases the likelihood of this case having a primary neural tube defect etiology.

In human medicine, surgery is considered as first-line treatment for encephaloceles [13]. Surgery was recommended in this case; however, the dog was elected to be managed on anti-epileptic drugs. If refractory epilepsy or CSF rhinorrhea ever develop, surgical intervention is planned right after a follow-up MRI and CT scan.

In conclusion, neither medical nor surgical treatment for MECs in dogs is warranted. Although MECs are rare, it is important to include them as a potential diagnosis when assessing young dogs displaying neurological deficits, seizures, or changes in behavior. Even though surgical treatment is considered as the first-line treatment, no seizure activity was observed in 12 months following the diagnosis and the medical treatment was considered favorable for this patient.

Declarations

Availability of Data and Materials: The data that support the findings of this case report are available from the corresponding author (D. P. A.) upon reasonable request.

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

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Short communication manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but the abstract should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages in total. The page limit does not include tables and illustrations. Additionally, they should not contain more than 4 figures or tables.

Preliminary scientific reports are a short description of partially completed original research findings at an interpretable level. These should be prepared in the format of full-length original articles. The length of the text should be no longer than 4 pages in total.

Case reports describe rare significant findings encountered in the application, clinic, and laboratory of related fields. The title and abstract of these articles should be written in the format of full-length original articles (but the abstract should not exceed 100 words) and the remaining sections should be followed by the Introduction, Case History, Discussion and References. The reference numbers should not exceed 15 and the length of the text should be no longer than 4 pages in total. The page limit does not include tables and illustrations.

Letters to the editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases. The length of the text should be no longer than 3 pages in total. The page limit includes tables and illustrations.

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Narrative reviews describe current published information on a scientific topic. However, it does not include a specific methodological process.

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Meta-analysis is a method of evaluating the results of many studies on a subject with the methods defined in this category and statistical analysis of the obtained findings.

7- The necessary descriptive information (thesis, projects, financial supports, etc.) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of the title.

8- At least 30% of the references of any submitted manuscript (for all article categories) should include references published in the last five years.

References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

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DOI number should be added to the end of the reference.

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