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A GEOSPATIAL RETROSPECTIVE STUDY OF AFRICAN SWINE FEVER SPREAD IN SERBIA (2019–2025) IN DOMESTIC PIG AND WILD BOAR POPULATION

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Abstract

African Swine Fever (ASF) has been posing a significant threat to pig production and wildlife management in the Republic of Serbia since its first detection in 2019. The goal of this study is to provide a comprehensive overview of the spatial and temporal spread of ASF in both domestic and wild pig populations in Serbia from the initial outbreak in the summer of 2019 through May 2025. Additionally, the study aimed to map the risk of ASF in the domestic pig sector to identify the areas where enhanced biosecurity measures could improve future disease control efforts.

The analysis is based on official data obtained from the Serbian Veterinary Directorate, specifically from issued decisions related to all affected settlements and hunting grounds where ASF was confirmed. The data on the number of disease outbreaks used in the study are official records submitted to the European Commission through the Animal Diseases Information System for the 2019– 2025 period.

Between 2019 and 2025, the spread of ASF in Serbia followed a distinct trajectory, moving from the southeastern regions toward the northwest, ultimately reaching the West Bačka District in Vojvodina in 2025. The disease demonstrates distinct seasonal dynamics: in domestic pigs, the majority of outbreaks occur during summer months, with an additional peak observed

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in November and December, while in wild boars the number of cases increases during the winter period. ASF is showing signs of becoming endemic in Serbia.

The implemented control strategies have largely been reactive rather than preventive, demonstrating limited effectiveness in altering the overall trajectory of the epidemic. The principal risk factors contributing to the spread of ASF in Serbia are linked to the continued circulation of the virus between domestic pig and wild boar populations, predominantly driven by human activities associated with both animal groups.

Keywords: African swine fever, Serbia, Geographical information systems, Analysis, Epidemiology, Spatial distribution, Retrospective studies**.**

GEOPROSTORNA RETROSPEKTIVNA STUDIJA ŠIRENJA AFRIČKE KUGE SVINJA U SRBIJI (2019–2025) KOD DOMAĆIH I DIVLJIH SVINJA

Vladimir Polaček[1], Milena Samojlović[2] , Diana Lupulović[3], Milica Živkov-Baloš[1], Jelena Petrović[1], Danka Polaček[4] and Jasna Prodanov-Radulović[1*]

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Kratak sadržaj

Afrička kuga svinja predstavlja značajnu pretnju za proizvodnju svinja i lovstvo u Republici Srbiji. Cilj ovog istraživanja je da pruži sveobuhvatan pregled prostornog i vremenskog širenja afričke kuge svinja u populacijama domaćih i divljih svinja u Srbiji, počev od inicijalne pojave bolesti 2019. godine pa do maja 2025. godine. Pored toga, cilj studije bio je mapiranje rizika od pojave afričke kuge svinja u proizvodnom sektoru domaćih svinja, kako bi se identifikovale oblasti u kojima bi unapređene mere biosigurnosti mogle doprineti efikasnijoj kontroli bolesti u budućnosti.

Analiza se zasniva na zvaničnim podacima Uprave za veterinu Republike Srbije, a posebno na rešenjima izdatim za sva zaražena naseljena mesta i lovišta u kojima je potvrđena afrička kuga svinja. Podaci o broju izbijanja bolesti koji su korišćeni u ovom istraživanju predstavljaju zvanične podatke dostavljene Evropskoj komisiji putem informacionog sistema za o bolestima životinja za period od 2019. do 2025. godine.

Tokom navedenog perioda, širenje afričke kuge svinja u Srbiji pratilo je specifičnu putanju – od jugoistočnih delova zemlje ka severozapadu, da bi u 2025. godini zahvatila i Zapadnobački okrug u Vojvodini. Bolest pokazuje izraženu sezonsku dinamiku: kod domaćih svinja, najveći broj žarišta registruje se tokom letnjih meseci kao i pojavi velikog broja slučajeva u novembru i decembru, dok se kod divljih svinja broj slučajeva povećava u zimskom periodu.

Rezultati ukazuju da afrička kuga svinja poprima karakteristike endemske infekcije u populaciji domaćih i divljih svinja u Srbiji. Dosadašnje strategije kontrole bolesti u najvećoj meri su bile reaktivne, a ne preventivne, sa ograničenom efikasnosti u promeni ukupnog toka enzootije. Ključni faktori rizika za širenje afričke kuge svinja u Srbiji povezani su sa kontinuiranom cirkulacijom virusa između populacija domaćih i divljih svinja, pri čemu ljudske aktivnosti povezane sa obe populacijske grupe životinja predstavljaju dominantni mehanizam širenja.

Ključne reči: Afrička kuga svinja, Srbija, Geografsko informacioni sistemi, Analiza, Epidemiologija, Prostorna distribucija, Retrospektivne studije.

INTRODUCTION

African swine fever (ASF) is a significant infectious hemorrhagic disease affecting domestic and wild pigs, with a case fatality rate that can reach up to 100%. It is caused by the African swine fever virus (ASFV), the sole member of the *Asfarviridae* family, which affects only members of the Suidae family (domestic pigs and wild boars) (Calò et al., 2023; Guinat et al., 2016; Lazov et al., 2025). Although initially identified only on the African continent, several instances of the virus spreading outside of Africa have been reported (Chenais et al., 2019; Lazov et al., 2025). A genotype II virus entered Georgia in 2007 and subsequently spread across Europe, into Asia, and more recently into the Americas, affecting a total of 62 countries globally since 1 January 2022 (WOAH, 2024). Nowadays, the disease is present worldwide and represents a global challenge for the pig production sector (Sánchez-Cordón et al., 2018; Whitaker et al., 2024).

In Europe, ASF is primarily maintained within wild boar populations, with frequent spillover events into domestic pig herds (Calò et al., 2023; Gervasi et al., 2020; Martinez Aviles et al., 2023) through direct contact with infected animals and via various indirect routes, including contaminated fomites and feed, which enable long-distance spread often driven by human activity (Boklund et al., 2020; Chenais et al., 2019; Guinat et al., 2016; Lazov et al., 2025; Whitaker et al., 2024). Since its introduction into the European Union in 2014, the virus has become endemic in wild boar populations in several member states, posing a continuous threat to domestic pig farming (Stahl et al., 2025). Since there is currently no effective vaccine available, control efforts depend on prevention, early detection, and the enforcement of strict biosecurity protocols (Calò et al., 2023; Plut et al., 2023). To date, biosecurity remains the most important tool in preventing ASF spread and combating the disease (Plut et al., 2023; Prodanov-Radulovic et al., 2022). While ASF does not pose a zoonotic threat, its economic and socio-political consequences are profound. Trade restrictions and depopulation measures associated with outbreaks cause significant disruptions on both local and international markets (Calò et al., 2023; Whitaker et al., 2024).

One of the most important differences between pig production in Western Balkan countries and EU member states lies in the structure of the pig production sector (Plut et al., 2023). The Serbian pig sector is largely made up of backyard farms, where pig rearing provides both a source of meat and plays a role in preserving the traditional rural lifestyle (Prodanov-Radulovic et al., 2022). Notably, over 60% of pig holdings in Serbia are backyards and small-scale operations with fewer than ten pigs and minimal or no biosecurity measures (Plut et al., 2023; Prodanov-Radulovic et al., 2022). According to the Statistical Office of the Republic of Serbia, pig farming accounted for the largest share of livestock production, comprising approximately 52% in 2023 (Stat. Office-Serbia, 2024). Pig production sector plays a vital role in Serbia's agricultural economy, with pork being the second most consumed animal protein after poultry (Kureljušić et al., 2024; Prodanov-Radulovic et al., 2022). Intensive commercial pig farms are mainly situated in the northern part of the country, particularly in the Vojvodina Province, where pigs are raised indoors under strict biosecurity measures (Prodanov-Radulovic et al., 2022). However, surrounding these commercial operations are numerous smallholdings and backyards that operate under traditional, semi-extensive, or extensive systems (Kureljušić et al., 2025). These farms are especially susceptible to disease outbreaks due to the absence of effective biosecurity measures. Since its emergence in 2019, ASF has severely affected small-scale and backyard farms, which remain a significant source of risk for virus spillover into large commercial operations (Nešković et al., 2021; Plut et al., 2023; Polaček et al., 2021). Furthermore, various anthropogenic factors - such as activities of farm workers, veterinarians, household pig keepers in villages and hunters - can act as bridges for ASFV transmission.

The transboundary nature of ASFV transmission had become evident even before its first detection in Serbia. Neighbouring countries - Romania (2017) and Bulgaria (2018) - reported ASF outbreaks near the Serbian border shortly after the initial cases were confirmed within their territories (Gao et al., 2023; Gervasi et al., 2020; Nielsen et al., 2019). Serbian border in this region is not fenced, enabling the natural migration of wild boars across national boundaries. Moreover, disease control strategies implemented in neighbouring countries, which included intensified hunting of wild boars, likely contributed to the transboundary spread of the virus (BFSA 2018).

However, the first confirmed ASF outbreaks in Serbia were not reported in border areas but rather in central municipalities - Mladenovac and Smederevska Palanka - in July 2019 (Milićević et al., 2019). In response, emergency control measures were promptly implemented. These included stamping out, preventive depopulation, disinfection, bans on restocking and swill feeding, movement restrictions, and the suspension of live animal markets (2019). By September 2019, an isolated outbreak was recorded in the northern province of Vojvodina, near the Romanian border.

Since 2020, ASF has been detected annually in both domestic pigs and wild boars across Serbia (Nešković et al., 2021; Polaček et al., 2021; Prodanov-Radulovic et al., 2023).

From 2022 onward, the disease has also been detected in several neighbouring countries. ASF was confirmed in North Macedonia (2022); in Croatia, Bosnia and Herzegovina, and Kosovo (2023); and in Albania and Montenegro (2024) - with the latter reporting cases only in wild boars. As of 5 July 2025, during the current year, ASF remains present in both domestic pigs and wild boar populations in Serbia, Croatia, and Bosnia and Herzegovina, in North Macedonia (ADIS, 2019, 2020, 2021, 2022, 2023, 2024, 2025).

Since 2020, both active and passive surveillance programs have been implemented in the domestic pig sector in Serbia. Based on our direct involvement in the organization, coordination, and laboratory diagnostics, as well as feedback from field veterinarians, we assume that the effectiveness of active surveillance has been limited. This limitation appears to result from weak local response, insufficient cooperation at the field level, and various logistical challenges. Consequently, the majority of positive ASF cases have been detected through passive surveillance. A specific feature of the program is its adaptation to backyard holdings, which also includes the requirement for ante-mortem health checks and laboratory testing to be conducted at least 24 hours prior to home slaughter.

The program requires the submission of samples in suspected ASF cases, regardless of farm size and production type. In other words, every pig holding must participate in the program of active and passive surveillance, while the number and type of samples collected vary depending on herd size and production type (farrow-to-wean, farrow-to-finish, or fattening only).

In the existing production sector, with a large number of different farm types concentrated in a relatively small area, spatial tools such as GIS-based mapping and cluster detection techniques are valuable for visualising disease hotspots, while temporal analyses help identify seasonal or cyclical patterns. When combined with descriptive epidemiology, these tools can reveal key risk factors, support targeted intervention planning, and inform the development of tailored biosecurity programs for small-scale systems.

The aim of this study was to investigate the spatiotemporal patterns of ASF spread in Serbia from the first confirmed case in 2019 through the end of May 2025 and to make ASF epidemiological patterns more visible and accessible for interpretation. Additionally, the study aimed to map the risk of ASFV in the domestic pig sector in order to identify areas where enhanced biosecurity measures could improve future disease control efforts.

MATERIAL AND METHODS

In this study, official data from the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia – Veterinary Directorate (2023–2025) were utilised, available at the following link: https://www.vet.minpolj.gov.rs/zarazne-bolesti-zivotinja/africka-kuga-svinja-aktuelne-informacije.

Publicly accessible data consists of official decisions (resolutions) issued for all settlements and hunting grounds where ASF was confirmed. For the 2019–2022 period, additional detailed data were obtained directly from the Veterinary Directorate upon formal request. These data included all official resolutions issued for each settlement and hunting ground where ASF was diagnosed in the form of MS Excel spreadsheets.

In order to determine the total number of confirmed outbreaks during the 2019- 2025 period, publicly available official data submitted by the Republic of Serbia to the Animal Diseases Information System (ADIS) of the European Commission were used (ADIS, 2019, 2020, 2021, 2022, 2023, 2024, 2025). These data are accessible on the following link: https://webgate.ec.europa.eu/tracesnt/adis/public/notification

Geospatial visualisation and analysis were conducted using QGIS software, version 3.4. Administrative boundary polygons within the Republic of

Serbia were obtained from OpenStreetMap datasets provided by NextGIS in GeoPackage format. Hunting ground boundaries were obtained from the official website of the Forest Directorate of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia, available on the links below:

https://upravazasume.gov.rs/wp-content/uploads/2025/01/Granica_lovista_RS.zip, in ESRI shapefile format.

In order to visualize the occurrence of ASF over the 2019–2025 period, the Dynamic Temporal Control option in QGIS was used, based on the official data on the issuance of veterinary authority decisions. The selected time frame for displaying cases included July 2019 - May 2025 period, with the event duration set to one month. Additionally, the accumulation over time option was enabled.

For exporting individual frames, the Temporal Controller was configured with a display step of one month. The exported frames were subsequently combined into GIF and MP4 formats using free online tools available at https://ezgif.com.

Data processing for visualisation, as well as analyses were carried out using Microsoft Excel 365.

RESULTS

In 2019, during the July–August period, a total of 18 outbreaks of ASF were reported in domestic pigs in the city municipality of Mladenovac, Belgrade (villages of Rabrovac and Velika Krsna), Podunavski district municipality of Smederevska Palanka (village of Kusadak) and Žitiste municipality (Srpski Itebej). No cases were detected in wild boar populations during this year (Figure 1).

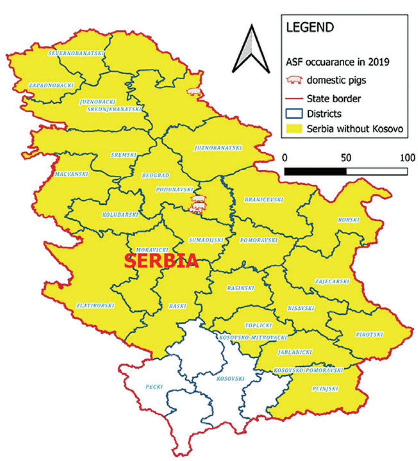


Figure 1. ASF occurrence in 2019 in Serbia

In 2020, ASF was confirmed in 16 domestic pig outbreaks across the municipalities of Bela Palanka, Negotin, and Pirot, and in 69 outbreaks in wild boars across the Borski, Zaječarski, and Pirotski districts (Figure 2).

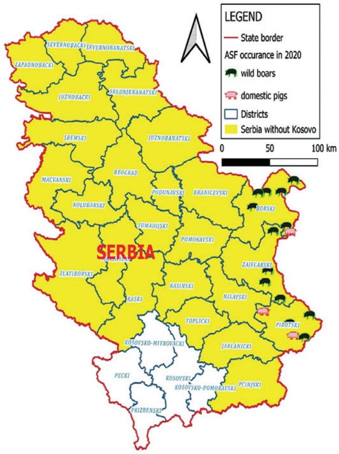


Figure 2. ASF occurrence in 2020 in Serbia

In 2021, a total of 32 outbreaks in domestic pigs were reported across several districts, including South Banat, Braničevski, Zaječarski, Nišavski, Pirotski, and Pomoravski (Paraćin municipality), as well as in the Rasinski district (Kruševac and Aleksandrovac municipalities) and the Borski district. A total of 43 wild boar outbreaks were registered in the South Banatski, Braničevski, Zaječarski, Nišavski, and Pirotski districts (Figure 3).

Figure 3. ASF occurrence in 2021 in Serbia

In 2022, 107 ASF outbreaks were reported in domestic pigs across the South Banatski, Braničevski, Pomoravski (Despotovac municipality), Zaječarski, Nišavski, Pirotski, and Pčinjski districts , and 146 outbreaks in wild boars across the South Banatski, Braničevski, Jablanički, Zaječarski, Nišavski, Pčinjski, and Zlatiborski districts (Figure 4).

Figure 4. ASF occurrence in 2022 in Serbia

The year 2023 marked the highest number of outbreaks, with 992 cases in domestic pigs occurring across the South Banatski, Sremski, City Municipality of Belgrade, Mačvanski, Braničevski, Podunavlski, Zaječarski, and Pčinjski districts. Additionally, 213 outbreaks in wild boars were recorded in the South Bački, South Banatski, Sremski, Mačvanski, City Municipality of Belgrade, Braničevski, Pomoravski, Jablanički, and Pčinjski districts (Figure 5).

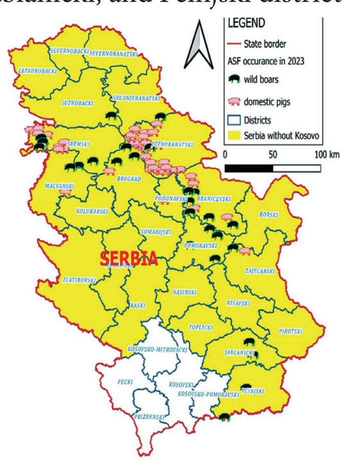


Figure 5. ASF occurrence in 2023 in Serbia

In 2024, 310 outbreaks in domestic pigs were reported in the Srednje Banatski district (Zrenjanin municipality), City Municipality of Belgrade, Sremski, Kolubarski (Ub municipality), Nišavski, Zaječarski, and Pčinjski districts, while 101 outbreaks in wild boars were detected in South Bački, Sremski, City Municipality of Belgrade, Pomoravski, Borski, Zaječarski, and Zlatiborski districts (Figure 6).

Figure 6. ASF occurrence in 2024 in Serbia

As of May 26, 2025, 18 outbreaks in domestic pigs were confirmed in the City Municipality of Belgrade (Obrenovac, Sopot), Kolubarski (Ub municipality) and Nišavski (Aleksinac municipality), and 28 outbreaks in wild boars occurred in the City Municipality of Belgrade (Obrenovac, Sopot, Kosmaj), South Bački, Western Bački (Kamarište), and the Podunavski district (Figure 7).

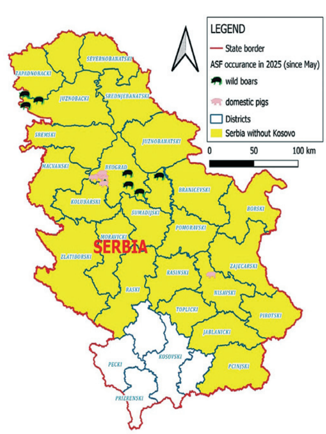


Figure 7. ASF occurrence in 2025 (since May) in Serbia

The occurrence of all ASF cases in Serbia during the observed period (2019–May 2025) is presented in Figure 8.

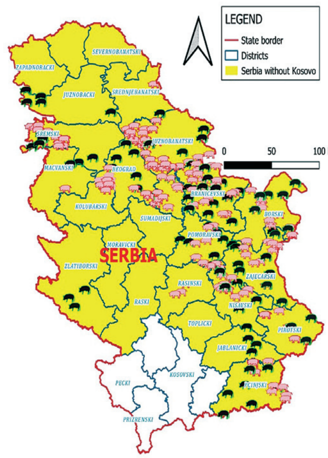


Figure 8. ASF occurrences 2019-2025 in Serbia

The total area of hunting grounds in which ASF has been confirmed in wild boars amounts to 23,776 km², representing 30.64% of the territory of the Republic of Serbia (excluding the territory of Kosovo) (Figure 9).

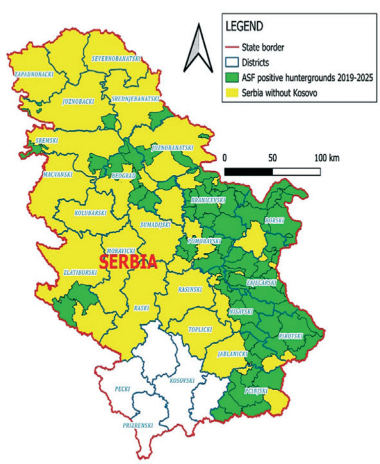


Figure 9. Affected hunter grounds with ASF 2019-2025 in Serbia

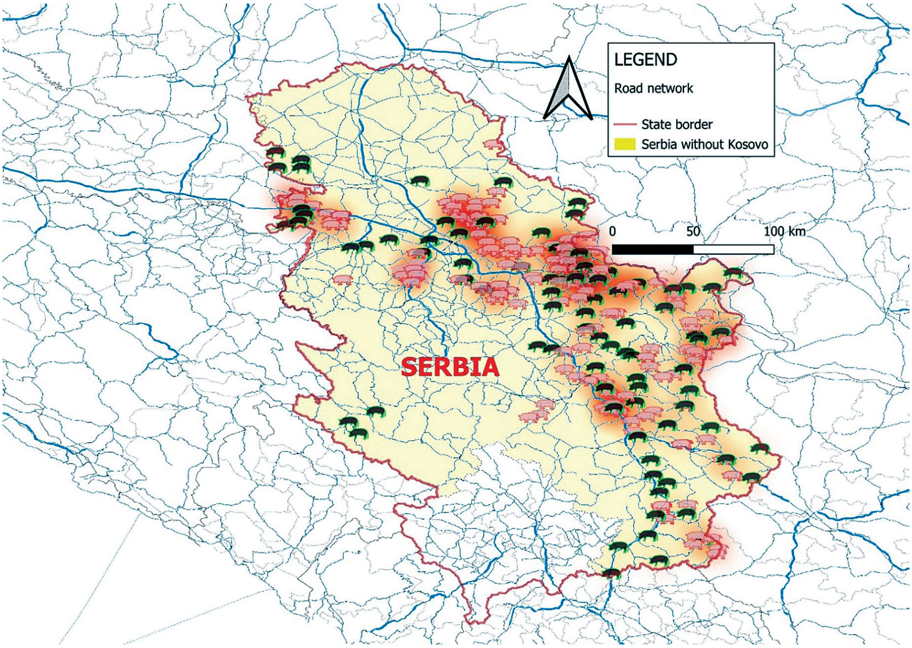
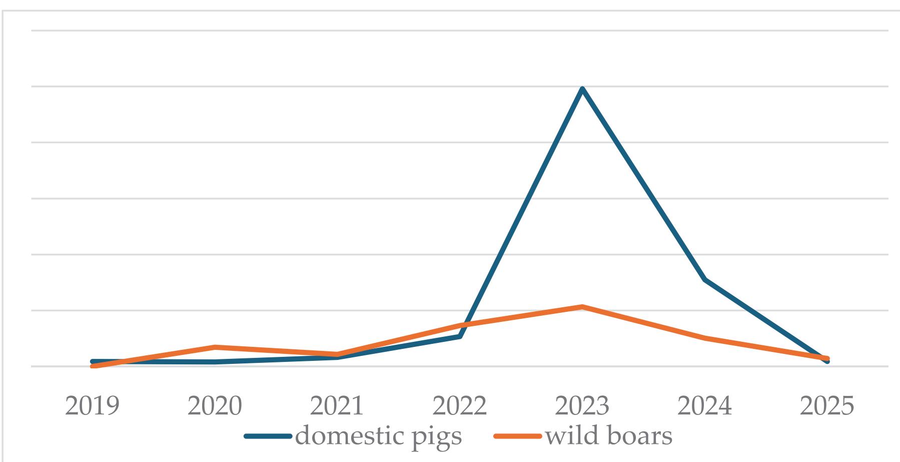


Figure 10. Highway network influence on ASF spread in Serbia

Figure 11. Number of ASF outbreaks in domestic pigs and wild boars by years (ADIS 2019-2025) in Serbia



From the detection of the first confirmed ASF case in 2019 through May 26, 2025, the highest number of reported cases occurred in 2023, predominantly affecting domestic pigs (Figure 11). Outbreaks in domestic pigs were most frequently detected during the summer months, whereas in wild boars, ASF was more commonly detected in winter (November -December) (Figure 12).

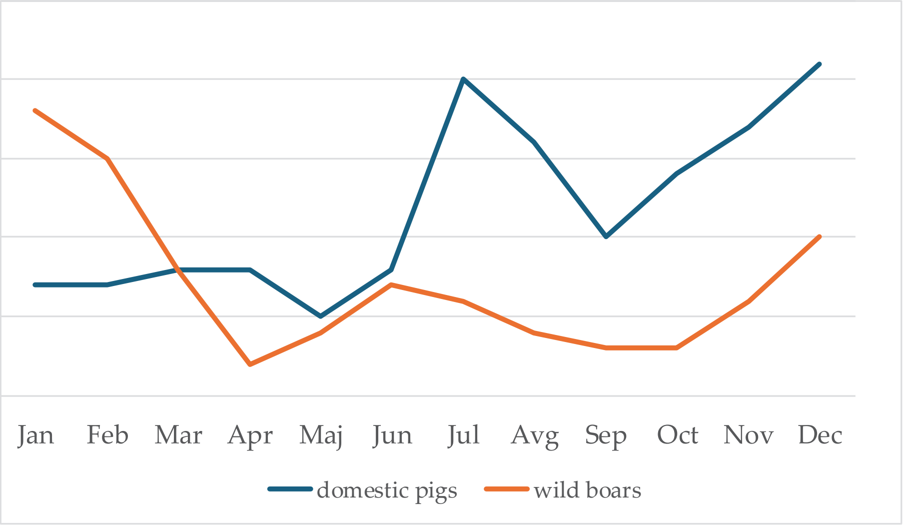


Figure 12. Seasonal dynamics of African Swine Fever (ASF) occurrence in domestic and wild pigs in Serbia, 2019–2025.

DISCUSSION

Today, six years after the first case was detected, ASF continues to spread throughout Serbia's entire pig production sector as well as the wild boar population. As observed in other parts of Europe (Calò et al., 2023; Rusinà et al., 2023), ASFV presents a significant threat to smallholder farms, which are especially susceptible to outbreaks due to limited biosecurity measures, proximity to wild boar populations, and farmer attitudes and behaviours related to disease risk and biosecurity practices. Human activities have already been identified as the main drivers of disease transmission (Nešković et al., 2021; Polaček et al., 2021; Prodanov-Radulovic et al., 2023). From the perspective of the Serbian economy, these small-scale farms do not contribute significantly to annual pork production (Polaček et al., 2021; Prodanov-Radulovic et al., 2022). However, as in other parts of Europe, these outdoor farming systems carry substantial socio-economic and cultural importance (Boklund et al., 2020). The loss of smallholder farming systems in rural Europe would represent an economic loss for farmers and local economies, as well as a loss of cultural and natural heritage (Stahl et al., 2025; Whitaker et al., 2024).

Studies on biosecurity in backyard and smallholder farms in Serbia have demonstrated a low level of biosecurity and a high risk of ASF spread from wild boar to domestic pigs in villages (Kureljušić et al., 2025; Kureljušić et al., 2024). It is well known that inadequate biosecurity on smallholder farms contributes to ASF outbreaks in domestic pigs (Stahl et al., 2025; Penrith et al., 2023; Whitaker et al., 2024). The proximity of wild boars to farms is also associated with increased ASF risk (Boklund et al., 2020; Prodanov-Radulovic et al., 2022). Wild boars can transmit the disease directly to domestic pigs, and domestic pigs can also transmit it to wild boar. Moreover, humans in contact with wild boars may inadvertently introduce the virus to farms (Nešković et al., 2021; Rusinà et al., 2023). Indeed, in different parts of the world human behaviour and socio-cultural factors are increasingly recognized as crucial in the spread and control of ASF (Chenais et al., 2019; Penrith et al., 2023; Whitaker et al., 2024). Understanding farmer decision-making and the socio-ecological context of small-scale farming is essential for developing national regulations that incorporate biosecurity measures (Biebaut et al., 2025; Whitaker et al., 2024).

During the 2018–2019 period, it was anticipated that ASF would first appear in the bordering regions between Serbia and Romania or Bulgaria, due to the high number of reported ASF cases in those countries, including areas close to Serbian borders (Boklund et al., 2020). However, the first ASF case in Serbia was detected in central Serbia, far away from the Romanian or Bulgarian border (Milićević et al., 2019).

Retrospective epidemiological investigations - including interviews with residents of affected holdings - indicated that official detection of ASF cases in 2019 did not reflect the actual time of virus introduction into Serbia. Timeline reconstruction suggested earlier virus entry in May, likely in the border municipality of Veliko Gradište, which lies on the Danube River, bordering Romania. The virus likely circulated undetected for several months and may have spread in multiple directions. Large rivers, like the Danube, do not serve as effective barriers to ASF spread; rather, they may facilitate it, especially through the movement of infected wild boar carcasses. The risk increases with improper disposal of domestic pig carcasses in the river. Water may even aid in viral persistence by shielding it from sunlight, UV radiation, and desiccation, while maintaining low temperatures (Carrau et al., 2023; Prodanov-Radulovic et al., 2023). Similarly, during the ASF outbreak in the Vršačke Planine hunting grounds of South Banat, a stream separating two areas was identified as the most likely route for ASFV transmission (Prodanov-Radulovic et al., 2023).

In 2020, ASF emerged in the Hrastovača hunting ground (Požarevac municipality) on the southern Danube bank. Shortly thereafter, the virus was detected on the northern bank in Deliblatska peščara (South Banat), suggesting that improper carcass disposal into the river may have facilitated ASFV transport via water to adjacent areas (Prodanov-Radulovic et al., 2023).

Human activity is now widely recognised as the primary contributor to ASF spread, followed by wild boar transmission (Glisic et al., 2023; Glišić et al., 2025; Polaček et al., 2021; Prodanov-Radulovic et al., 2022). This may explain why the initial ASF outbreak occurred in central Serbia, in domestic pigs, rather than near the borders or in wild boar populations. Hungary had constructed a double fence along its southern border with Serbia for migrant control, reducing the likelihood of ASF spreading through wild boar in that direction.

In contrast, Serbia's highway network appears to function as a physical barrier - or at least as a decelerating factor - in the spread of ASF, particularly among wild boars (Figure 10). Contrary to the findings of Glišić et al. (2025), our results suggest that fenced highways and railway corridors may effectively impede ASFV transmission by wild boars. The previous study likely assessed road proximity without differentiating between fenced and unfenced highways.

Serbia's major longitudinal highway, stretching from Horgoš (Hungary) to Preševo (North Macedonia), effectively divides the country along an east–west axis. The predominant direction of ASF spread follows a southeast-to-northwest pattern, with significantly fewer ASF cases reported west of this highway (Figure 10).

Though highways can present ASF risks due to discarded food waste, double-fencing and heavy traffic reduce wild boar movement. Unfenced roads near rest stops, scenic spots, and recreational areas may present a higher risk, particularly in locations where food is prepared or consumed. Improper waste management, especially when infected food waste is transported to unfenced landfills may further increase this risk.

Intensive hunting in Serbia's border regions with Bulgaria may have pushed wild boars across the border, contributing to the initial detection of ASF in wild boar in Bulgaria in 2020. This movement correlates with the emergence of new ASF cases in eastern Serbia during 2021 and 2022. In Pomoravlje District, no link was found between ASF spread in wild boars and domestic pigs; transmission was attributed to transport (Glisic et al., 2023). In 2021, Serbia confirmed its first ASF outbreak on a large commercial farm near the Bulgarian border, resulting in the culling of 18,000 pigs (Nešković et al., 2021).

In 2023, the number of ASF cases peaked, with domestic pig outbreaks exceeding wild boar cases. The virus advanced northwest, reaching Vojvodina, Serbia's most pig-dense region. That summer, ASF was detected on several large industrial farms in the Srem district (Stara Pazova, 21,000 pigs) and in

South Banat (Pančevo~14,000 pigs, Crepaja~10,000, and Straža, Vršac~7,000 heads). On all affected farms, the entire pig population was stamped out. Additionally, in same year, numerous smallholder farms were affected by ASFV nationwide.

In 2023–2024, outbreaks continued in Vojvodina's Srem, South Banat, and Central Banat districts. In 2025, the spread extended toward West Bačka, previously unaffected.

Unlike most of Europe, Serbia reported more ASF cases in domestic pigs than wild boars during the 2022–2024 period (Stahl et al., 2024; Stahl et al., 2025). The seasonal analysis showed that outbreaks in domestic pigs peaked in summer, while wild boar cases peaked in winter. Winter peaks likely relate to hunting season visibility, longer carcass persistence, reduced vegetation, and increased hunter activity. During summer, decreased hunting activity and quicker carcass decomposition hinder ASF detection, which is consistent with findings by Glišić et al. (2025). Seasonal wild boar activity may influence summer/autumn farm outbreaks. Wild boars reproduce in winter and farrow in spring, though milder winters have lessened this seasonality (Pascual-Rico et al., 2022). Human and wild boar activity increases during summer harvests, boosting spillover risk (Penrith et al., 2023). In Romania, 2022 ASF outbreaks on small farms peaked in summer and winter, while in other EU states, summer remained peak season (Stahl et al., 2024; Stahl et al., 2025). Similar seasonal patterns are observed globally, with summer/autumn peaks in Europe and Asia.

In many villages or municipalities, hunting grounds are managed by fee-based local hunting associations. In Serbia, only two large state-owned forestry enterprises, two hunting grounds are managed by the army, and national park areas may implement structured measures. (Prodanov-Radulovic et al., 2023). Since 2021, fenced hunting areas and grounds have experienced a complete loss of wild boars. Single perimeter fencing proved to be inadequate. These hunting grounds are all located in Vojvodina (Vršačke Planine, Dragićev Hat, Karađorđevo, Ristovača, Plavna, Kamarište, Karakuša, Morović, Bosutske šume, and Kučine). Between 2019–2025, ASF was confirmed in over 30% of hunting grounds in Serbia (Figure 10), a likely underestimate since urban zones are excluded.

As in some other European countries, it was established that the highest number of domestic pig cases occurred in summer, coinciding with peak insect activity (Olesen et al., 2020; Olesen et al., 2018a; Olesen et al., 2022; Olesen et al., 2018b). For example, in most villages in Vojvodina Province, smallholder farms are often located several tens of meters away from each other and mechanical transmission by insects such as houseflies is plausible (Vasic et al., 2024). However, the role of hematophagous insects outside Africa in summer is unclear and remains to be fully understood (Guinat et al., 2016).

It should be emphasised that studies on ASFV environmental persistence rely mainly on molecular detection of viral DNA, which does not confirm viral viability or infectivity (Bergmann et al., 2021; Carrau et al., 2023; Olesen et al., 2022; Vasic et al., 2024). Field experience in Serbia suggests that ASFV has limited environmental transmission under real-world conditions. Despite its high transmission potential, the virus spreads slowly. On some industrial farms, outbreaks took weeks to exceed the expected mortality rates. For instance, on one large farm in Zaječar, the High-Risk Period (HRP) ranged from 7–19 days (minimum) to 21–34 days (maximum), depending on the scenario (Nešković et al., 2021).

A significant increase in reported ASF cases in domestic pigs is also observed in the final months of the year (November and December). This may be attributed to the traditional home slaughter of pigs, which typically takes place during this time of year. Uncontrolled disposal of offal can certainly heighten the risk of ASF spreading. These activities typically involve relatives, neighbours or friends, groups of people who assist one another in the slaughter and processing of meat products, often using shared equipment and tools. Human population movement also increases during this time due to the celebration of Orthodox family patron saints' day (Slava), as well as Christmas and New Year holidays. During these family and religious holidays, pork products are frequently consumed or given as gifts, and kitchen waste, potentially ASF-contaminated, may end up in swill, even though swill feeding is legally prohibited in Serbia.

Certainly, municipalities that host both industrial and small-scale farms require heightened surveillance and rigorous enforcement of biosecurity measures. These two farm circuits are interconnected through shared transports, slaughterhouses and finally by personnel. The outlined potential transmission cycle allows infection to flow from wild boars to backyard pigs, and subsequently to industrial farms. Even small-scale or backyard ASF outbreaks can significantly impact the entire pig sector.

Overall, the measures implemented in Serbia between 2019 and 2025 appear to have been reactive rather than preventive. Although effective in the local control of infected premises, these measures had limited impact on controlling the spread of ASF on the national level and even less in preventing its transboundary dissemination. Future policies need to focus more on proactive strategies, early detection, and enhanced coordination with hunting associations, especially regarding carcass search and removal in affected ecosystems.

Above all, when it comes to the domestic pig production sector, it is necessary to legally define biosecurity measures in the future for each type of farm unit (e.g., backyards, small-scale family farms, intensive holdings). Moreover, it raises the important question of whether, and under what conditions, extensive production units should be allowed to participate in commercial or trade activities.

For a more comprehensive understanding of the dynamics of ASF spread in Serbia during the period 2019–2025, access to precise geolocations of each individual case, together with detailed data on the course and outcome of the disease, would have been highly valuable. In this study, however, the processed data were derived from the official records of the competent authorities, which identify only the settlement where an outbreak occurred. These records often encompass multiple cases within the same locality and lack information on the disease outcome, representing the main limitation of this study. Nevertheless, this limitation does not diminish the relevance of the findings or the validity of the conclusions drawn. Furthermore, the official data submitted by the Republic of Serbia to the ADIS system consist of aggregated annual figures on the number of outbreaks in domestic and wild boar populations. For future analyses, the consolidation of all available information at the level of individual cases, including the outcome of infection, would provide more accurate results and significantly improve the detection of spatiotemporal patterns in the spread of ASF in Serbia.

CONCLUSION

Since the summer of 2019, ASF has been reported annually in Serbia, affecting both the domestic and wild boar populations. It is important to emphasise that the virus predominantly circulates within the population of domestic pigs on rural backyards and small-scale family farms. However, at the beginning of 2021, virus transmission into industrial pig production was reported (Nešković et al., 2021), along with cases in fenced hunting grounds involved in wild boar breeding for commercial hunting purposes (Prodanov-Radulovic et al., 2022). Approximately 70,000 pigs have been culled on five industrial-type farms, four of which are located in the Vojvodina Province. In fenced hunting grounds, all located in the territory of Vojvodina, ASFV has completely devastated the wild boar population. Moreover, based on the established disease outbreak occurrence trends, it can be assumed that the disease has now become endemically present in the country. It can be concluded that its spread has followed a consistent southeast-to-northwest trajectory. In 2025, ASF reached its northernmost point in the country and expanded into the West Bačka District in the domestic pigs and wild boar population. The accuracy of the predictions made in this study is confirmed by the emergence of new ASF cases reported during the preparation of this conclusion in July 2025. Specifically, this month, the Veterinary Directorate reported ASF outbreaks in the Western Bačka District, in the villages of Bogojevo and Sonta. Additionally, the presence of ASF was confirmed in the wild boar population within the enclosed hunting ground Apatinski rit.

Given the current structure of the pig production sector, studying the livestock–wildlife interface and their interactions is highly complex and involves multiple factors to consider. Considering the heterogeneous epidemiological contexts and their potential impact on ASF seasonality, further research could help clarify the relative contributions of wild boar-to-farm/backyard and farm-to-farm transmission routes of ASFV, thereby informing targeted prevention and control strategies.

In Serbia, ASF appears to be spreading with partial autonomy from policy interventions. Control measures seem to have been largely reactive rather than preventive. The persistence of the virus in domestic and wild pig populations is most commonly sustained through various human activities, which may serve as the dominant link for virus transmission between these two populations.

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Author's Contribution:

VP conceived the study, developed the conceptual framework, and prepared the thematic maps. JPR contributed to the writing of the manuscript. MŽB and DL were involved in proofreading and final revisions. JP and MS participated in data preparation and analyses. DP translated the manuscript into English.

Competing interest

The authors declare no conflict of interest.

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Supplementary Data

The supplementary material (GIF file) associated with this article is available online at the following permanent link: **https://niv.ns.ac.rs/e-avm/index.php/e-avm/article/view/493/317**

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LARGE GAME MEAT PRODUCTION AND CONSUMPTION IN SERBIA

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Abstract

Game meat has always been a part of the human diet. It is rich in protein and low in fat, and its consumption is increasing across Europe. The main objective of this paper is to enhance knowledge and understanding of large game meat production and consumption in Serbia. This study uses the official data provided by the Statistical Office of the Republic of Serbia to calculate average hunting ground size, game population density and harvest rate. Game meat production data were obtained from four large game meat processing facilities and used for calculation of game meat yield and consumption rate. Serbia has a relatively small number of hunters, yet hunting grounds are extensive, covering nearly 92.6% of the country’s territory. Among large game species, roe deer constitute the most abundant population in Serbia. This population is characterized by medium size and density, with a relatively low harvest rate compared to other European countries. Wild boars are the most frequently hunted large game species in Serbia. Although their population density is low, it varies significantly across different hunting grounds, and the harvest rate remains high. The red deer population is comparable in size to that of neighboring countries, but has low density and a low harvest rate primarily due to ongoing population increase programs. The fallow deer population is steadily declining. Consumption rate, as well as game meat production Serbia is lower than in most European countries. A Serbian resident consumes as little as 0.120 kg of large game

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meat annually, accounting for just 0.3% of total meat consumption. These data represent the first quantitative data on game meat consumption in Serbia. Our findings suggest that there is significant potential to promote large game meat and boost its consumption in Serbia, especially considering the lower price of game meat – such as wild boars compared to domestic pork, and venison compared to beef.

Key words: venison, game meat, roe deer, red deer, wild boar, Serbia

PROIZVODNJA I KONZUMIRANJE MESA KRUPNE DIVLJAČI U SRBIJI

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Kratak sadržaj

Meso divljači je oduvek deo ishrane čoveka, bogato je proteinima, ima nizak sadržaj masti. U Evropi se zapaža trend porasta konzumiranja. Osnovni cilj ovog rada je unapređenje postojećeg znanja i razumevanja proizvodnje i konzumiranja mesa krupne divljači u Srbiji. U radu su korišćeni zvanični podaci Republičkog zavoda za statistiku Srbije za izra**čunavanje prosečne veličine lovišta,** gustine populacije divljači i odstrela. Podaci o proizvodji mesa divljači dobijeni iz **četiri** veća objekta za obradu mesa krupne divljači su korišćeni za izračunavanje prinosa mesa i konzumiranja mesa divljači. Srbiju odlikuje relativno mali broj lovaca, lovišta su velika i zauzimaju gotovo 92,6% teritorije države. Srne su najbrojnija populacija krupne divljači u Srbiji, populacija je srednje veličine i gustine u odnosu na ostale Evropske zemlje a udeo odstrela je nizak. Divlje svinje su najčešće lovljena vrsta krupne divljači u Srbiji, gustina populacije je niska ali značajno varira po lovištima dok je odstrel je visok. Veličina populacija običnog jelena je slična susednim zemljama, gustina je niska a zbog programa povećanja populacije odstrela je nizak. Populacija jelena lopatara stalno opada, u nekim delovima Srbije je istrebljen. U odnosu na druge zemlje Evrope, Srbija je jedan od najmanjih proizvođača mesa krupne divljači, takođe količina mesa divljači koja se konzumira po glavi stanovnika je niska. Prosečan stanovnik Srbije pojede 0,120 kg mesa krupne divljač, što čini svega 0,3% ukupne potrošnje mesa. Ovo su prvi kvantitativni podaci o konzumiranju mesa divljači u Srbji. Naši podaci ukazuju da postoji prostor za popularizaciju mesa krupne divljači i povećanje potrošnje u Srbiji, posebno treba uzeti u obizir *i nižu cenu mesa koje npr. imaju divlje svinje u odnosu na domaće svinje, kao i jelensko meso u odnosu na junetinu.*

Ključne reči: divljač, meso divljači, srna, jelen, divlja svinja, Srbija

INTRODUCTION

Hunting is among the oldest skills developed by humans, initially used as a means to obtain food. Today, hunting in Europe serves multiple purposes, including recreation, entertainment, trophy hunting, food supply, and the management of wild animal populations (Benko et al., 2020). Game meat has long been part of the human diet, once serving as a primary source of protein. Although its role in modern diets has diminished, there is a growing trend of increased game meat consumption in Europe. Game meat is high in quality protein, low in fat, and has optimal fatty acid composition (Harguess et al, 2020).

In Serbia, large game animals include wild boars, red deer, fallow deer, roe deer, chamois and European mouflon. These populations are managed through selective hunting (with age- and sex- specific quotes) significantly affected by population growth rates (Rivrud et al., 2014).

Wildlife management in Serbia follows a regal hunting system, in which game species are regarded as a natural resource owned by the state. This system is regarded as outdated in Europe (Lavadinović et al., 2017, Popović et al, 2023). The state allocates these resources to hunting ground users for management. In Serbia, hunting grounds users are primarily hunting associations and state forest enterprises, which account for 90% of users (Lavadinović et al., 2017). These users are required to develop a 10-year hunting management plan, which the state monitors to ensure proper implementation and control of hunting ground management (Popović et al., 2023). Game meat inspection is mandatory and carried out by veterinary officers or veterinarians. Due to the epidemiological situation in Serbia, the examination of wild boar meat for

Trichinella presence is also mandatory. Over the past twenty years, the quality of hunting grounds in Serbia has improved significantly, primarily due to advancements in infrastructure (Lavadinović et al., 2017). However, not all hunting grounds have game handling facilities for harvested animals – there are 110 facilities in total, with 60% located in Vojvodina. Game meat is most commonly purchased directly from hunters and is available in retail outlets through only a limited number of retail chains.

Although Serbia has a significant body of scientific research on hunting and game animal management, there is a noticeable gap in data regarding game meat production and consumption. Addressing this gap is one of the key objectives of this paper. Currently, there is no available data on the amount of game meat consumed by Serbian consumers. Quantitative data on game meat consumption would enable more precise calculations of chemical hazards intake from food, including pesticides and heavy metals – particularly lead from ammunition. This information is essential for assessing potential health risks for consumers, including both the general population and hunters, who typically consume the largest amounts of game meat.

Therefore, the primary aim of this paper is to provide comprehensive overview of large game meat production in Serbia, covering the entire chain from hunting grounds and hunters to game population indicators and production levels, ultimately concluding with the amount of meat consumed by general population.

MATERIAL AND METHODS

Official data were obtained from the website of the Statistical Office of the Republic of Serbia: the area and population of the Republic of Serbia, including additional data related to the northern province of Vojvodina (SORS, 2023), data on hunters, hunting grounds, large game population status and number of harvested game animals (SORS, 2022). Average hunting ground size, game population density and harvest rate were calculated from these official data.

In Serbia and most Southeast European countries, game animal populations are generally assessed in March (Tomašević et al., 1972). This count represents the number of individuals after the hunting season and before the arrival of new offspring. In contrast, Western European countries report game populations in September, reflecting the number of individuals before hunting and after the reproduction. This difference in reporting methods can lead to discrepancies in the estimated population size. To align the March count with September estimate, the number of piglets per sexually mature female is added, which can range from 1.5-3 piglets for wild boars (Tomašević et al., 1997).

Such variations can result in misinterpretations of game population densities and harvest rates.

The harvest rate was calculated by dividing the number of animals counted in March (before reproduction and after hunting) by the number of animals harvested during the hunting year.

Although Serbia has a total of 13 registered game meat processing facilities, only four have been operational for processing of large game in 2021/2022. Game meat processing facilities are located in the South Banat, South Backa, Zlatibor and Borski district (Figure 2.). The calculation of game meat production was based on data obtained from four game meat processing facilities. Game meat consumption was calculated from total skinless carcass production and population in Serbia/Vojvodina in 2021. Skinless carcass weight is weight of carcass without head, skin, blood, offal and distal portions of hind- and fore- legs.

The statistical analysis was conducted using Microsoft Office Excel 2007. The geographical distribution and density map was created using ESRI ArcGIS Pro version 3.0

Results

Hunting grounds in Serbia encompass 92.6% of the country's total territory, and in the Vojvodina region, they cover 99.4% of the land. There are 347 hunting grounds in Serbia, with an average size of 20,683 ha (13,773 ha in Vojvodina), though their size varies significantly.

Table 1. Hunting grounds and hunters in the Republic of Serbia in 2021

	Surface area (km^2)	Population	Hunters	HG	Surface area of hunting ground (1000ha)			
					Hunting area	Non-hunting area	Total	Forest**
Serbia*	77,474	6,797,105	87,567	347	6,072	1,105	7,177	1,813
Vojvodina	21,614	1,825,982	28,583	156	1,884	264	2,148	162

*-without Kosovo; HG- hunting grounds; ** Forest covered hunting ground

Among large game ungulates in Serbia, roe deer and wild boar exhibit the highest population densities. Density is slightly higher in the Vojvodina compared to the national average.

Table 2. Population of large game animals in 2021

	Red deer	Fallow deer	Roe deer	Cham-ois	Euro-pean mouflon	Wild boar
Serbia Population * Population density (ani-mals/km^2)	7,478 (0.10)	1,504 (0.02)	144,991 (2.02)	821 (0.01)	662 (<0.01)	23,856 (0.33)
Vojvodina Population Population density (ani-mals/km^2)	4,782 (0.22)	777 (0.04)	61.931 (2.88)	0 (0,00)	172 (<0,01)	8,531 (0.40)

* number of animals before reproduction and after the hunting season (March count)

Table 3. Hunted large game animals in 2021

	Red deer	Fallow deer	Roe deer	Chamois	Euro-pean mouflon	Wild boar
Serbia Hunted animals Harvest rate (%)	984 13.16%	188 12.50%	11.454 7.90%	50 6.09%	43 6.5%	15.228 63.83%
Vojvodina Hunted animals Harvest rate (%)	819 17.3%	143 18.40%	3 844 6.21%	0 (0,00)	11 6.40%	5.164 60.53%

The harvest rate is the highest for wild boar, followed by red deer, fallow deer, and roe deer.

Table 4. Average meat yield data from game meat processing facilities

	Red deer	Fallow deer	Roe deer	Wild boar

No. of animals	289	157	182	360
Average carcass weight (kg) ± SD	69.13±34.61	41.18±13.04	16.93±12.94	34.68±20.87
Skin (%) /range	11.54 (7.5-18.8)	13.43 (10.5-18.1)	14.00 (9.1-31.7)	33.29 (11.9-42.1)
Meat without bones (%)/range	48.51 (10.4-71.6)	45.54 (21.7-53.3)	44.19 (6.7-58.2)	32.36 (4.0-60.6)

Table 5. Large game meat production in hunting season 2021/2022

	Red deer	**Fallow deer**	**Roe deer**	**Cham-ois***	**Europ. mou-flon***	**Wild boar**	**Sum**
Serbia (animals)	984	188	11.454	50	43	15.228	**27.947**
Total skinless carcass (t)	68.02	7.74	193.92	1.00	1.29	528.11	800.08
Processed – Half carcass (t)	60.17	6.70	166.77	0.86	1.11	352.30	587.91
Meat without bones (t)	32.99	3.53	85.69	0.44	0.57	170.89	294.12
Vojvodina region (animals)	819	143	3.844	0	11	5.164	9.981
Total skinless carcass (t)	56.62	5.89	65.08	/	0.33	179.09	307.00
Processed – Half carcass (t)	50.08	5.10	55.97	/	0.28	119.47	230.90
Meat without bones (t)	27.46	2.68	28.76	/	0.15	57.95	117.00

* precise information about weights and yields are not available, total weight of mouflon and Chamois participates with 0,3% of annual production

Regarding total game meat production, wild boar contributes the most, followed by roe deer, red deer, and fallow deer. The estimated consumption rate of large game meat in Serbia is 0.120 kg per person per year with Vojvodina having higher rate of 0.176 kg per person per capita.

Figure 1. Average consumption (kg) of large game meat in general population in Serbia and Vojvodina (red and green bar) compared to other European countries presented by Sevillano Morales et al, 2018 (grey bars)

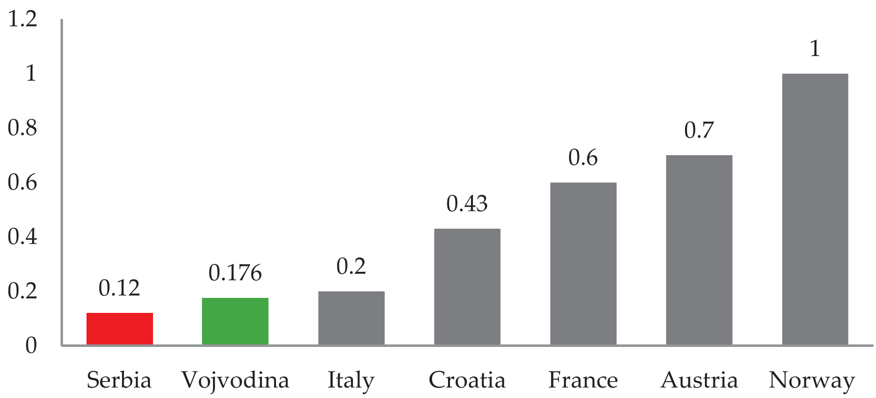
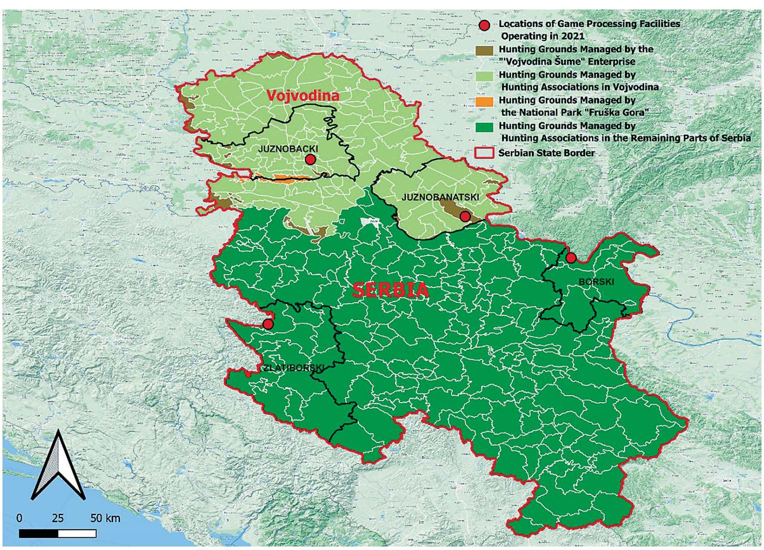


Figure 2. Map of hunting ground management organizations in Serbia and locations of game meat processing facilities operating in 2021 (red dots)



DISCUSSION

According to data from the European Federation of Associations for Hunting (FACE), there were 7 million registered hunters in Europe in 2021.

France had the largest number, totaling 1.3 million hunters, followed by Spain (980,000), the UK (800,000), and Italy (750,000) (Benko et al., 2020). In 2021, Serbia had 87,567 registered hunters, representing 1.2% of the total population (6,797,105). This percentage is slightly below the European average of 1.4%. However, in Vojvodina, which has a population of 1,825,982, the proportion of hunters is higher than the European average, standing at 1.6%. Studies suggest that hunter density is more closely linked to overall population density rather than prey density (Mysterud et al., 2020).

In Serbia, hunting grounds are managed primarily by hunting fellowships (nearly 80%), followed by state forest enterprises, national parks, universities, the army, and private hunting grounds (Lavadinović et al., 2017). Hunting grounds cover 92.6% of Serbia's total territory, with an even higher percentage (99.4%) in Vojvodina (Figure 2.). Hunting is allowed throughout most of the country, with the exception of settlements, industrial zones, transportation routes, and historical sites (Lavadinović et al., 2017). A significant proportion of hunting grounds (over 90%) were also found in Austria, Poland, Denmark, and Germany, in contrast to several other European countries (Šelmić et al., 2001).

The average size of hunting grounds in Serbia has been decreasing due to fragmentation. In 2001, the average hunting ground covered 27,500 hectares, but by 2021, this declined to 20,683 hectares. Compared to hunting grounds in Europe, those in Serbia are still significantly larger. For instance, the average size of hunting grounds in Austria is 75 hectares, in Finland 105 hectares, and in Belgium 300 hectares (Šelmić et al., 2001). Each hunting ground in Serbia includes a non-hunting area, which, on average, makes up 15.4% of the total area (12.3% in Vojvodina). The average non-hunting area covers 3,184 hectares, which is larger than the average hunting ground in Europe. This extensive size presents challenges for managing hunting grounds, game populations, and wildlife conservation efforts. The dominant land use within these hunting grounds is agricultural land, accounting for 45.5%, followed by forests and woodlands (28.7%), grasslands (18.8%), water surfaces (1.3%), and other uses (5.6%) (Lavadinović et al., 2017).

In Serbia, each hunting ground typically employs one wildlife professional and one to three gamekeepers, which remains insufficient for effective wildlife management. However, in recent years, infrastructure improvements have been noted, including an increase of number of hunting facilities, feeding stations, and salt licks. Despite these improvements, only about one-quarter of hunting grounds have cold storage facilities for venison (Lavadinović et al., 2017), as well as other necessary facilities for carcass processing, veterinary inspection, cleaning and disinfection. This largely complicates the safe production of game meat.

The roe deer (*Capreolus capreolus* L. 1758), red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*) are widely distributed game species across Europe, with population consistently increasing over last 50 years. Roe deer populations have been rising since the early 20th century, while wild boar populations have grown significantly since mid-20th century (Burbaitė and Csányi 2010; Burbaitė and Csányi 2009; Massei et al., 2015). The increase in certain game populations is believed to be a result of greater food availability in forested areas, reduced hunting pressure, and a decline in natural predators over the course of the twentieth century (Côté et al., 2004). This trend has also been observed in Serbia, where populations of these species have expanded (Burbaitė and Csányi, 2009; Burbaitė and Csányi, 2010; Lavadinović et al., 2020).

The roe deer, wild boar and red deer are autochthonous species and represent the most biologically and economically valuable large game species in Serbia. The allochthonous large game species include fallow deer (*Dama dama*) and mouflon (*Ovis aries musimon)*, while chamois *(Rupicapra rupicapra)* are considered autochthonous, but have limited economic value due to small population size. As deer and wild boar populations continue to grow, their harvest rates are also increasing (Burbaitė and Csányi, 2009; Burbaitė and Csányi, 2010; Escobar-González et al., 2024). Roe deer is the most abundant wild ungulate species in Serbia. In Europe, Germany has the largest roe deer population, estimated at 600,000 to 3,000,000 animals, followed by France, Poland, and Sweden (Burbaitė and Csányi, 2009). Serbia's roe deer population stands at 144,991, placing the country in the mid-range among European nations. Population size in Serbia is similar to that of Switzerland, Norway, and Romania, while also being the largest population among the former Yugoslav countries. According to official data, Serbia had a roe deer density of 2.02 animals/km^2 in 2021 (table 2). However, monitoring using advanced methods such as binoculars, thermal cameras, night vision monocular, and photo cameras has recorded significantly higher densities, ranging from 16 to 112 animals/km^2 (Popović et al., 2023). Roe deer density across Europe vary considerably. The highest densities are recorded in Denmark, Luxembourg, Austria, and Germany, where populations reach 8 to 9 animals/km^2. As of 2005, Serbia was classified as having a medium roe deer density, with an average of 1 to 3 animals per square kilometer (Burbaitė and Csányi, 2009). Countries with the largest roe deer populations also tend to have highest harvest rate, ranging from 34% to 44%. In contrast, Serbia's harvest rate is 7.9%, which is slightly higher than Romania's 5.1%, but lower than Croatia's 15.4% (Burbaitė and Csányi, 2009).

The data from Serbia are comparable to those of other Southeastern European countries, as the same calculation methods are used.

Wild boars are widespread across Serbia and play a significant role in local hunting, inhabiting 75% of the country's hunting grounds (Lavadinović et al., 2017). France and Germany have the largest populations of wild boars in Europe, each with around half a million animals, followed by Poland and Spain, with populations ranging between 200,000 and 300,000. The wild boar population in Serbia is similar to that in Croatia (FAO, 2018). According to the EFSA report (ENETWILD Consortium et al., 2022), the density of wild boars in Europe ranges from 0.35±0.24 to 15.25±2.41 animals/km^2. In Serbia, however, the density was found to be 0.33 animals/km^2 in 2021 (Table 2). Previous reports (SANCO, 2011) suggest that the density of wild boars in Serbia varies between 0.2 and 1.38 animals/km^2. The population density of wild animals, in general, varies significantly depending on the hunting grounds and the methods used for data collection. In contrast to deer, the harvest rate for wild boars in Serbia is statistically very high at 63.83%. This rate is influenced by the fact that the March population of wild boars is reported as the breeding stock in the official data from the Statistical Office of the Republic of Serbia. However, domestic studies that calculate harvest rates in relation to the parent population reported a harvest rate of 29-30% (Lavadinović et al., 2020). The wild boar is the most frequently hunted large game species in Serbia, not only due to the appeal of the hunt but also because of the significant damage these animals can cause to agriculture and the frequent conflicts with humans. (Lavadinović et al, 2020).

The UK, Spain, Poland, and Denmark have the largest populations of red deer in Europe, ranging from 150,000 to 300,000 animals. Serbia is among a group of countries, including Belgium, Turkey, and Croatia, with population of several thousand red deer (Burbaitė and Csányi, 2010). The population density of red deer varies across European countries and regions from 0.3 to 44.6 animals/km^2. In Serbia, the population density of red deer is slightly lower than the European average, at approximately 0.1 animals/km^2 (Table 2), which is similar to that of Croatia. The population density is influenced by the presence of large carnivores, but the most significant impact comes from human activities, particularly hunting and agriculture (Van Beeck Calkoen et al., 2023). The average harvest rate for red deer has remained consistent in Europe over the past few decades, at around 25% (Burbaitė and Csányi, 2010). In Serbia, however, the red deer harvest rate is considerably below the European average, standing at only 13.6%. This discrepancy is partly due to the breeding stock being reported based on the number of deer counted in March, which reflects a population increase program in the central mountainous regions of the country.

The fallow deer is one of the most common and widespread cervid species in Europe. Its population has increased fivefold since 1984, reaching nearly 650,000 animals by 2020 (Bijl and Csányi, 2022). Among cervid species, fallow deer are experiencing the fastest population growth in Europe, making them one of the most overabundant species. The increase in the fallow deer population is also influenced by hunters' preferences, as they often favor hunting roe and red deer (Bijl and Csányi, 2022). In Serbia, the population of fallow deer is smaller than that of roe and red deer and is in continuous decline. In 2021, the population density was just 0.02 animals/km^2 (Table 2). The harvest rate for fallow deer in Europe is 21.32% (Bijl and Csányi, 2022), whereas in Serbia, it is much lower at 12.5%, despite differences in how the breeding stock is reported and ongoing efforts to preserve the population.

The chamois inhabits the mountainous regions of central and southern Europe, with an estimated population of around half a million individuals. Most subspecies of chamois are considered endangered, primarily due to human activities. The European mouflon, originally native to Sardinia and Corsica, spread across much of Europe over the past century. At one point, its population declined due to expansion of sheep farming (Temerario et al., 2024). The population density of chamois was 0.01 animals/km^2, while that of mouflon was less than 0.01 animals/km^2 (Table 2). The harvest rate is very low for both mouflon and chamois due to efforts to preserve this small population in Serbia.

Game meat farming offers several significant benefits, including conservation of endangered species, economic advantages (such as meat production, sale of by products and live animals) and social benefits (such as employment, tourism, and potential reduction of poaching) (Lindsey et al., 2013; Urošević et al. 2016). The legal requirements for farmed game are outlined in Regulations 852/2004 and 853/2004, as well as national legislative of member states (Urošević et al. 2016). These regulations cover animal health, farm management and animal welfare. Serbia lacks a specific legislative framework that would enable the legal production of farmed game.

In Serbia, the production of domestic animal meat in 2021 totaled 587,000 tons (SORS, 2022). According to our data large game meat production reached 800.08 tons in the same year. The most commonly produced game meat was wild boar, totaling 528.11 tons, followed by roe deer at 193.92 tons and red deer at 68.02 tons, 7.74 tons of fallow deer and 1 tone each chamois and mouflon. (table 5). While global game meat production is on the rise, Europe has seen a slight decrease. However, import of game meat into Europe has been on the rise. Compared to other European countries, Serbia is one of the smallest producers of large game meat. Germany and France are the leading producers of wild boar meat, with annual outputs of 36,444 tons and 40,991 tons, respectively. Other countries, such as Czech Republic, Spain, and Poland, produce between 10,000 and 23,000 tons, while neighboring Croatia produces 1,800 tons (FAO, 2018). The largest producers of roe deer meat are Germany and France, with 22,791 tons and 11,021 tons, respectively, while most other European countries produce between 1,200 and 4,000 tons. When it comes to red deer meat, Spain, Poland, and Germany are the largest producers, with 20,881 tons, 13,376 tons, and 11,154 tons, while other countries produce between 1,000 and 8,000 tons. The largest producers of fallow deer meat are Germany, Sweden, and the Czech Republic, with between 1,000 and 3,000 tons, while most other European countries have production levels below 1,000 tons (FAO, 2018).

In Serbia, game meat is most commonly obtained during hunts or directly at hunting grounds following the hunt. It is also available in some restaurants and can be purchased in a limited number of retail outlets. In Eastern Europe, game meat is often obtained directly from hunters, either through purchase or as gifts (Tomasevic et al., 2018). This pattern is similar in other European countries and the USA (Corradini et al., 2022). Game meat is primarily procured for preparing game dishes or for the production of traditional meat products. Research on consumer attitudes in Eastern Europe (Tomasevic et al., 2018) found that game meat is consumed more frequently and preferred by men. It is also more commonly consumed by residents of rural areas compared to urban settings, and by adults more than children. When it comes to concerns about meat safety, consumers in Croatia have concerns regarding the presence of *Trichinella* spp. (Tolušić et al., 2006), which is likely the case in Serbia as well. Despite this, consumers generally believe that game meat is ecologically healthy to eat (Tolušić et al., 2006; Corradini et al., 2022). Some consumers avoid game meat due to its distinct taste and smell, while others prefer it and consider it tastier than domestic meat. This preference is particularly common among hunters (Marescotti et al., 2020; Hoffman et al., 2005). The highest levels of meat consumption worldwide are found in hunting families (Corradini et al., 2022).

The average resident of Serbia consumes 45.9 kg of meat from domestic animals annually (SORS, 2022). According to our survey, the average Serbian resident eats 0.120 kg of large game meat each year, with a slightly higher rate of 0.176 kg in Vojvodina. These amounts are 0.3% of total meat consumption in Serbia and 0.4% in Vojvodina. In Croatia, the average consumer eats about 0.55 kg of game meat annually, accounting for approximately 1% of total meat consumption (Tolušić et al., 2006). According to Sevillano Morales et al, (2018) who summarized the data from European countries, game meat consumption in the general population is relatively low: 0.1-0.3 kg in Italy, 0.6

kg in France, 0.7 kg in Austria, and 1.0 kg in Norway. In contrast, hunting families in Spain consume an average of 6.87 kg, while in Norway, the figure is 7 kg. Germany has the largest population of large game animals, a high game density, and leads in the production of game meat, reaching 18 kg per person in hunter families. Overall, the consumption of large game meat in Serbia is lower than in most European countries, which aligns with the fact that game meat production is also lower here.

The low production and consumption of large game meat suggests that there is potential for the popularization of game meat and an increase in its consumption. For example, the lower price of wild boar meat compared to pork, and venison compared to beef, could be important factors in encouraging greater consumption of game meat.

CONCLUSION

The number of hunters in Serbia is somewhat lower than the European average. The hunting grounds in Serbia cover nearly the entire country, and their average size is significantly larger than the average hunting grounds in Europe. This contributes to the relatively low population density of large game animals in Serbia. Among large game animals, only the roe deer has a medium population density, while other species have relatively low population densities compared to their counterparts in other European countries. Additionally, the fallow deer population in Serbia is declining, with some areas facing complete extermination of the species. The harvest rate of large game animals in Serbia is generally low, except for wild boars, and is influenced by the abundance or overabundance of certain species. Overall, the production and consumption of large game meat are quite low in the country. This data suggests there is potential for the popularization of large game meat and an increase in its consumption.

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Author's Contribution

JP carried out literature research, designed a study, analyzed the results and drafted the manuscript, JM designed a study, carried out data collection, survey of consumption, IL carried out survey of consumption, JK and JPR were involved in literature research, MU was involved in data analysis. JM revised the manuscript critically and together with JP prepared the final draft of the manuscript. All authors have read and approved the final manuscript.

Competing interest

All authors disclose any financial or personal relationships with individuals or organizations that might inappropriately affect their work.

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PREPARATION AND *IN VITRO* WOUND HEALING EFFECTS OF ANIMAL-BASED PLATELET-RICH PLASMA LYSATE

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Abstract

Wound healing is a vital field of study in both human and veterinary medicine, particularly in the treatment of chronic wounds. Platelet-rich plasma, known for its potential to enhance tissue regeneration due to its high concentration of growth factors, can be further processed into Platelet-rich plasma lysate. As an acellular product, Platelet-rich plasma lysate may exhibit reduced immunogenicity and can be stored frozen for future applications. Using heterologous Platelet-rich plasma lysate derived from larger animal species can be beneficial, particularly for veterinary patients who are unable to provide their own blood due to conditions such as low body weight, coagulopathy, or anemia. This study aims to evaluate and compare

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the *in vitro* wound-healing effects of Platelet-rich plasma lysate derived from equine, porcine, and caprine sources on dermal fibroblasts. Platelet-rich plasma lysate was prepared using a double centrifugation method and freeze-thaw protocol, and its effects were assessed through cell viability and *in vitro* wound healing assays. The cell viability assay revealed a positive dose-dependent effect of Platelet-rich plasma lysate from all tested animal species. Notably, 10% and 20% Platelet-rich plasma lysate from caprine and equine sources, as well as 20% caprine Platelet-rich plasma lysate, showed significantly higher cell viability compared to the negative control ($p < 0.05$). The *in vitro* wound healing assay showed that all Platelet-rich plasma lysate from the selected species had positive cell migration effects on dermal fibroblasts comparable to the positive control (10% Fetal Bovine Serum) at 5% concentration. However, a negative dose-dependent effect followed at higher concentrations. When compared to negative control, only positive control (10% Fetal Bovine Serum), 5% porcine Platelet-rich plasma lysate, as well as 5% and 10% caprine Platelet-rich plasma lysate were found to have significantly higher cell migration effect on dermal fibroblasts at 48-hour post-scratch. These findings highlight the potential of animal-derived Platelet-rich plasma lysate particularly of caprine origin, as an alternative treatment for wound healing, though further *in vivo* studies are needed to confirm its clinical applicability.

Key words: blood cells, fibroblasts, blood plasma, platelets, healing

PRIPREMA I *IN VITRO* EFEKTI LIZATA BOGATOG TROMBOCITIMA POREKLOM OD ŽIVOTINJA NA ZARASTANJE RANA

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Kratak sadržaj

Zarastanje rana predstavlja važno područje istraživanja kako u humanoj, tako u i veterinarskoj medicini, sa posebnim fokusom na lečenje hroničnih rana. Plazma bogata trombocitima, poznata po svom potencijalu da poboljša regeneraciju tkiva zahvaljujući visokoj koncentraciji faktora rasta, može se dalje preraditi u lizate plazme bogate trombocitima. Kao acelularni proizvod, lizat plazme bogate trombocitima može imati smanjenu imunogenost i može se čuvati zamrznut za buduću upotrebu. Korišćenje heterolognog lizata plazme bogate trombocitima dobijene od većih životinjskih vrsta može biti korisno, naročito kod veterinarskih pacijenata koji nisu u mogućnosti da daju sopstvenu krv zbog male telesne mase, koagulopatije ili anemije. Cilj ovog rada je da proceni i uporedi *in vitro* efekte zarastanja rana lizatom plazme bogate trombocitima dobijenog iz konjske, svinjske i kozje plazme na dermalne fibroblaste. Lizat plazme bogate trombocitima je pripremljen pomoću metode dvostruke centrifuge i protokola zamrzavanja i odmrzavanja, a njegovi efekti su procenjeni putem testova ćelijske održivosti i *in vitro* zarastanja rana. Test ćelijske održivosti je pokazao pozitivan, i od doze zavisan efekat lizata plazme bogate trombocitima kod svih ispitivanih životinjskih vrsta. Značajno je da su 10% i 20% lizata plazme bogate trombocitima poreklom od koza i konja, kao i 20% kozji lizat plazme bogate trombocitima, pokazali značajno višu ćelijsku održivost u poređenju sa negativnom kontrolnom grupom ($p < 0{,}05$). *In vitro* test zarastanja rana pokazao je da su svi lizati plazme bogate trombocitima iz odabranih životinjskih vrsta imali pozitivan efekat na migraciju dermalnih fibroblasta, uporediv sa pozitivnom kontrolom (10% fetalni goveđi serum) pri koncentraciji od 5%. Medjutim, pri višim koncentracijama zabeležen je negativan, od doze zavistan, efekat. U poređenju sa negativnom kontrolom, samo pozitivna kontrola (10% fetalni goveđi serum), 5% svinjski lizat plazme bogate trombocitima, kao i 5% i 10% kozji lizat plazme bogate trombocitima imali su značajno veći efekat na migraciju dermalnih fibroblasta 48 sati nakon nastanka ogrebotine. Ovi rezultati ističu potencijal lizata plazme bogate trombocitima životinjskog porekla, naročito onog kozjeg porekla, kao alternativnog tretmana za zarastanje rana, iako su dalja *in vivo* istraživanja neophodna kako bi se potvrdila njegova klinička primenljivost.

Ključne reči: krvne ćelije, fibroblasti, krvna plazma, trombociti, zarastanje

INTRODUCTION

Wound healing is extensively researched in both human and veterinary medicine because of its clinical importance, scientific relevance, and economic impact (Abegão et al., 2015; Farghali et al., 2017), despite the availability of numerous treatment options (Tanimu et al., 2022). In veterinary practice, skin wounds are among the most commonly encountered conditions (Kožár et al., 2018). These wounds can often progress to chronic conditions, caused by various factors like infection, certain medications, metabolic diseases, nutritional deficiencies and radiation therapy (Lux, 2022). Many complications can arise from having chronic wounds as it may not only lead to prolonged hospitalization and high financial burden, but could also lead to infection and worse, limb amputations (Järbrink et al., 2016). In wound healing management, if regeneration remains incomplete despite all efforts, adjunct therapies such as platelet-rich plasma (PRP) may be considered (Lux, 2022; Harries et al., 2016).

PRP is a plasma fraction enriched with platelets, obtained by separating plasma from whole blood and concentrating the platelets through single or double centrifugation (Chicharro-Alcántara et al., 2018). Research has shown that PRP's potential in tissue regeneration stems from its high platelet concentration, which acts as a reservoir for essential growth factors (Abegão et al., 2015). Clinically, PRP has been used to promote healing in soft tissues, orthopedics, dentistry, ophthalmology, neurology, and chronic skin ulcers (Chicharro-Alcántara et al., 2018; Notodihardjo et al., 2019). PRP can be further activated to release growth factors by adding substances such as thrombin, calcium salts, or collagen, transforming it into platelet gel (PG). Alternatively, it can undergo freeze-thaw cycles or sonication to become platelet lysate (PL) or platelet-rich plasma lysate (PRPL) (Bonferoni et al., 2019). Depending on its source, PRP can be autologous (derived from the same recipient), allogeneic (from a donor of the same species), or heterologous (from a donor of a different species) (Chicharro-Alcántara et al., 2018). As an acellular product, PRPL offers several advantages, including reduced immunogenicity, extended shelf life, and the ability to be stored frozen until use (Gilbertie et al., 2018).

Although limited data suggest that heterologous PRP is safe and does not cause adverse reactions, the use of heterologous platelet derivatives remains insufficiently explored. Among these data were the use of heterologous canine PRP in contaminated cutaneous wound in a feline case, and *in vivo* study on rabbit wound showing no adverse reactions while promoting tissue healing (Abegão et al., 2015; Gemignani et al., 2017). Heterologous PRPL can be beneficial in cases where patients are unable to donate their own blood, particularly those with small body size or conditions such as anemia or hypovolemic shock. Large animals like ruminants, porcine, or equine, could be selected as potential sources of heterologous PRPL as they have a higher total blood volume, and could be used as an option to treat smaller species of animal like cats and dogs in veterinary field, particularly in cases of delayed or chronic wounds.

This study aims to investigate and compare the *in vitro* wound healing effects using PRPL from equine, porcine, and caprine sources in order to provide an alternative for tissue regeneration. This research will focus on the characterization and effects of the animal-based PRPL dermal fibroblasts as one of the cells involved in skin wound healing. The detailed abbreviations and definitions used in the paper are listed in Table 1.

Table 1. List of abbreviations and definitions used in the paper

Abbreviation	Definition	Abbreviation	Definition
ACD-A	Acid citrate dextrose-A	NHDF	Normal human dermal fibroblasts
CBC	Complete blood count	NC	Negative control
CPPPL	Caprine platelet-rich plasma lysate	PC	Positive control
CPRPL	Caprine platelet-rich plasma lysate	PG	Platelet gel
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12	PL	Platelet lysate
DMSO	Dimethyl sulfoxide	PPP	Platelet-poor plasma
EPPPL	Equine platelet-rich plasma lysate	PPPL	Platelet-poor plasma lysate
EPRPL	Equine platelet-rich plasma lysate	PPPPL	Porcine platelet-rich plasma lysate
FBS	Fetal bovine serum	PPRPL	Porcine platelet-rich plasma lysate
IL-1β	Interleukin-1β	PRP	Platelet-rich plasma
PBS	Phosphate buffered saline	PRPL	Platelet-rich plasma lysate
LP-PPP	Leukocyte-poor platelet-rich plasma	RBC	Red blood cells
LR-PRP	Leukocyte-rich platelet-rich plasma	TNF- α	Tumor necrosis factor-α (TNF-α),
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide	WBC	White blood cells

MATERIAL AND METHODS

Blood collection and preparation of PRPL

PRPL was prepared using whole blood collected from three animal species: equine, porcine, and caprine. Blood samples were purchased from the Large Animal and Exotic Unit, University Veterinary Hospital, University Putra Malaysia from a 23-year-old castrated male horse, a 5-month-old intact female pig, and three 1- to 2-year-old intact female goats. Approximately 100 mL of whole blood, either single-source or pooled, was collected from each species. Ethical approval was not required, as the blood was collected during routine veterinary procedures by licensed personnel, following institutional animal welfare guidelines and ensuring that no unethical practices were involved.

The preparation of PRP followed a double-centrifugation protocol adapted and modified from Gilbertie et al. (2018). Blood samples were collected in tubes containing acid citrate dextrose-A (ACD-A) anticoagulant at a 1:9 ratio (anticoagulant: blood). A small aliquot of the anticoagulated whole blood was submitted for a complete blood count (CBC) analysis.

In order to separate plasma from red blood cells, the anticoagulated whole blood was transferred into 15 mL conical tubes and centrifuged (Eppendorf® Centrifuge 5702R, USA) under species-specific conditions: 300 × g at 25 °C for 15 minutes for equine and porcine samples, and 1000 × g at 25 °C for 30 minutes for caprine samples. Caprine samples required higher centrifugation speed and duration during the first spin because inadequate separation between plasma and red blood cells was observed. The plasma layer was carefully collected and transferred into 50 mL conical tubes, followed by a second centrifugation at 1500 × g at 25 °C for 15 minutes to concentrate the platelets. The resulting supernatant, designated as platelet-poor plasma (PPP), was transferred into a new conical tube, leaving approximately 10–15% of the original plasma volume to resuspend the platelet pellet, yielding the initial PRP.

A small aliquot of PRP and PPP were submitted for a haemogram to determine the initial platelet concentration. The PRP was then diluted with PPP accordingly to achieve a standardized platelet concentration of approximately 1×10^6/μL.

To produce PRPL, the PRP from all species were aliquoted into 1.5 mL centrifuge tubes and subjected to three consecutive freeze-thaw cycles. The samples were frozen at –80 °C for 24 hours, thawed in a 37 °C water bath for 5–10 minutes, and subsequently refrozen. After the third thaw, the samples were centrifuged (Eppendorf® Centrifuge 5417R, USA) at 20,000 × g for 20 minutes at 4 °C. The supernatant was collected and filtered through a 0.22 μm sterile syringe filters with polyethersulfone (PES) filter membranes (Microlab Scientific) to eliminate cellular debris and potential microbial contamination. Platelet-poor plasma lysate (PPPL) was also produced by the freeze-thawing PPP in the same manner as PRPL. PRPL and PPPL from the selected animal species were then aliquoted into new 1.5 - 2.0 mL microcentrifuge tubes (Figure 1) and stored at –80 °C until further use. The overall procedure is illustrated in Figure 2.

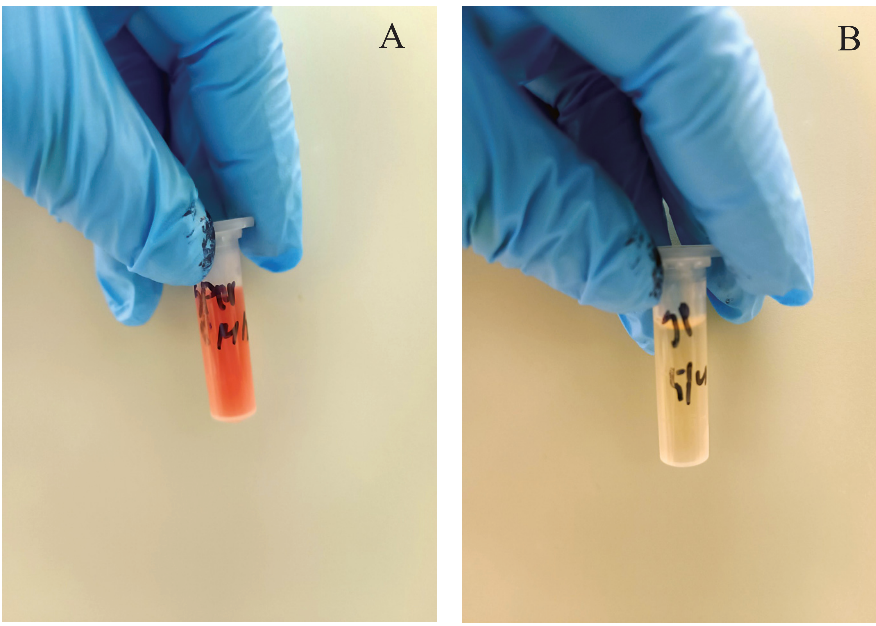


Figure 1. Images showing PRPL (A) and PPPL(B)

Haematological analysis

Haematological analysis was performed on whole blood, PRP, and PPP from the equine, porcine and caprine sources in order to compare red blood cell (RBC), white blood cell (WBC), and platelet concentrations prior to the freeze-thawing and filtration processes used to produce PRPL and PPPL.

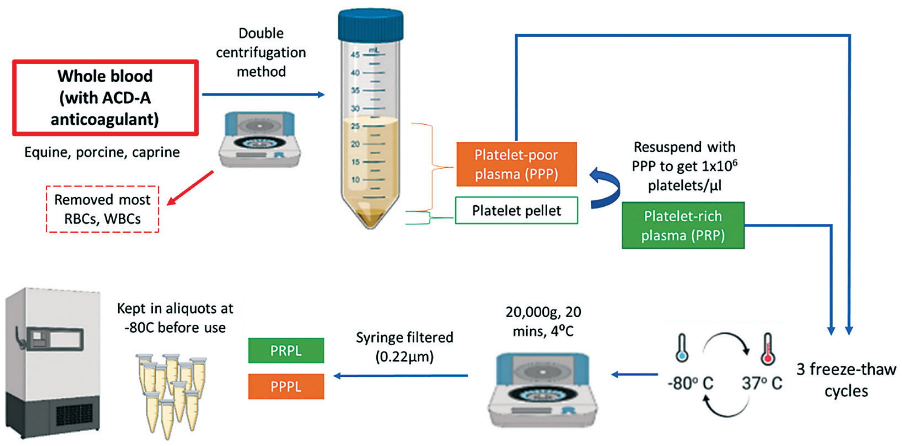


Figure 2. Schematic diagram showing the preparation of PRPL and PPPL.

Cell culture

Normal human dermal fibroblast (NHDF) cells were sourced from the Tissue Engineering Centre at Malaysia Medical Centre, Kebangsaan University, to serve as a representative cell type involved in skin wound healing for this study. The cells were cultured in flasks using Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, 1:1, 1×; Thermo Fisher Scientific, USA), supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, USA) and 1% Antibiotic-Antimycotic solution (100×; Thermo Fisher Scientific, USA). The cells were expanded until reaching confluence prior to use in in vitro experiments, with only passages 4 to 6 included throughout the study.

Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a colorimetric technique, was conducted to evaluate the effects of PRPL and PPPL derived from equine, porcine, and caprine sources on the metabolic activity of dermal fibroblasts, as an indicator of cell viability, proliferation, and cytotoxicity (Figure 3). Viably active cells can reduce MTT reagent to formazan. Fibroblasts were seeded at a density of 10,000 cells per well in complete media (DMEM/F12 supplemented with 10% FBS) in 96-well plates and incubated overnight at 37 °C. The following day, cells were treated with media containing 5%, 10%, and 20% PRPL or PPPL from each species. To prevent gel formation, heparinized saline was added to the treatment media at a final concentration of 2 IU/mL, as described by Naskou et al. (2018). Cells cultured in 10% FBS-supplemented media served as positive controls (PC), while those in serum-free media were used as negative controls (NC). After 24 hours of incubation at 37°C, the treatment media were removed, and 100 µL of serum-free DMEM/F12 along with 10 µL of MTT reagent (5 mg/mL in PBS; Thermo Fisher Scientific, USA) were added to each well. The cells were incubated for an additional 4 hours at 37 °C. Following incubation, the MTT-containing media were carefully aspirated, and 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The plates were placed on an orbital shaker set at 100 rpm for 20 minutes at room temperature. Absorbance was measured at 570 nm using a spectrophotometer, and cell viability was calculated as a percentage relative to the NC using the following equation:

Cell viability % = X 100%

Since the denominator in the formula is the mean absorbance of the NC, the calculated cell viability percentage for the NC will be 100%. Any value above or below 100% indicates higher or lower cell viability, respectively, compared to the NC.

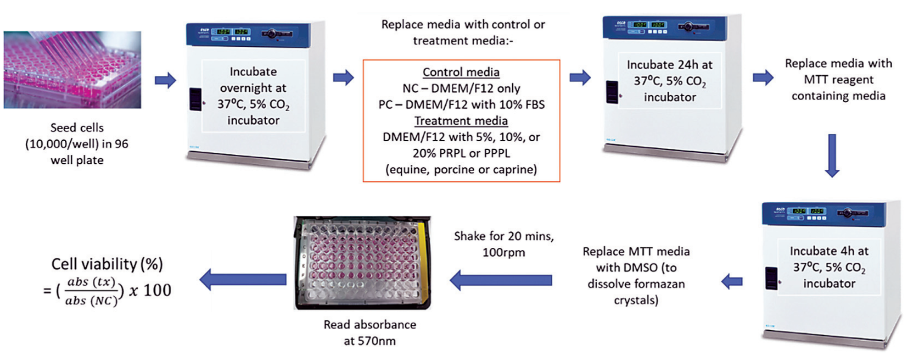


Figure 3. Schematic diagram showing the method of cell viability assay

In vitro wound healing assay

The scratch wound assay was conducted to assess the effects of PRPL and PPPL from different sources on dermal fibroblast migration (Figure 4). Dermal fibroblasts (5×10^4 cells) were seeded into 24-well plates with 500 µL of complete media (DMEM/F12 supplemented with 10% FBS) and cultured until confluence. Once confluent, a linear scratch was created at the centre of each well using an SPL Scar™ Scratcher. The old media were removed, and cells were washed with phosphate-buffered saline (PBS) to eliminate debris from damaged or dead cells.

Following the wash, 500 µL of treatment media containing 5%, 10%, or 20% PRPL/PPPL were added to the respective wells, alongside control media (10% FBS as a positive control and serum-free media as a negative control). To prevent gel formation, heparinized saline was added to the treatment media at a final concentration of 2 IU/mL, as described by Naskou et al. (2018). The plates were incubated at 37 °C for 72 hours.

Images of the scratch area were captured at 0, 24, 48, and 72 hours post-scratch using a digital camera are attached to an inverted microscope. The scratch areas were measured at each time point using ImageJ software, and the percentage of wound closure was calculated using the following formula:

Scratch closure % = = X 100%

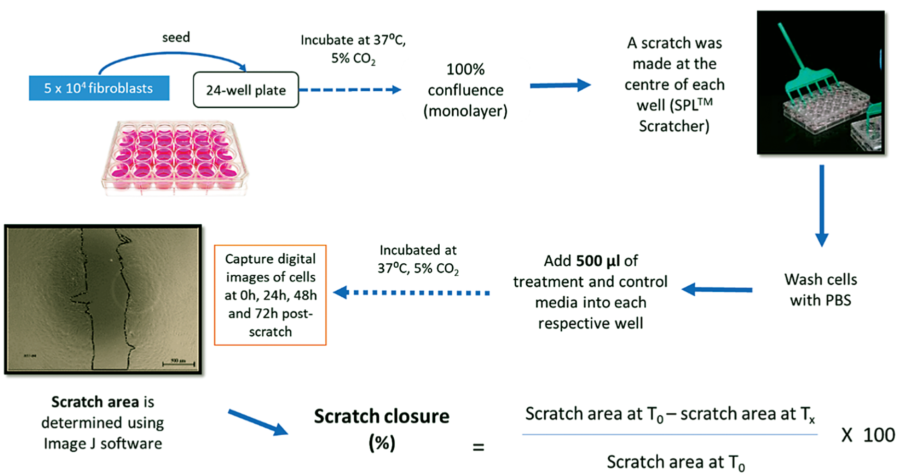


Figure 4. Schematic diagram showing the method of in vitro wound healing assay.

Statistical analysis

All experiments were performed in at least three replicates. Data analysis was conducted using GraphPad Prism (version 9.5.1, USA), employing the Kruskal-Wallis test followed by Dunn's multiple comparison analysis for the MTT assay and two-way ANOVA with Tukey's multiple comparison analysis for the scratch wound assay. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

The haematological results are presented in Table 2. The platelet concentration in the resultant PRP from each animal species was successfully maintained at approximately $1 \times 10^6/\mu l$, while the RBC and WBC concentrations were lower than those in whole blood. In contrast, PPP from each animal species contained lower concentrations of RBC, WBC, and platelets compared to their respective whole blood (WB) and PRP.

Table 2. Haematological profile of whole blood (WB), platelet-poor plasma (PPP) and platelet-rich plasma (PRP) from equine, porcine, and caprine sources.

Animals	Samples	RBC ($x10^6/\mu l$)	Reference values ($x10^6/\mu L$)*	WBC ($x10^3/\mu l$)	Reference values ($x10^3/\mu L$)*	Platelet ($x10^3/\mu L$)	Reference values ($x10^3/\mu L$)*
Equine	WB	5.87	6.00–10.40	8.20	5.60–12.10	109.00	117.00–256.00
	PPP	0.00	-	0.00	-	12.00	-
	PRP	0.01	-	1.50	-	1039.00	-
Porcine	WB	5.61	5.00–8.00	13.90	11.00–22.00	430.00	200.00–500.00
	PPP	0.00	-	0.00	-	26.00	-
	PRP	0.02	-	0.20	-	1111.00	-
Caprine	WB	19.00 ± 1.86	8.00–18.00	11.52 ± 3.92	4.00–13.00	423.40 ± 223.84	300.00–600.00
	PPP	0.01 ± 0.01	-	0.01 ± 0.01	-	88.67 ± 21.03	-
	PRP	0.80 ± 0.43	-	4.26 ± 1.46	-	1015.33 ± 80.75	-

*Note: The reference ranges in this table are adapted from "Hematology (Complete Blood Count) Reference Ranges," by MSD Veterinary Manual, n.d., https://www.msdvetmanual.com/multimedia/table/hematology-complete-blood-count-reference-ranges.

Figure 5. illustrates the cell viability percentages of dermal fibroblasts, as determined by the MTT assay, following treatment with PRPL and PPPL derived from equine, porcine, and caprine sources. A dose-dependent effect was observed, with increasing PRPL concentrations leading to higher cell viability percentages across all selected species. However, statistical analysis revealed that only 10% and 20% PRPL from caprine and equine sources, along with 20% caprine PPPL, demonstrated significantly higher cell viability ($p < 0.05$)

compared to the negative control. Among these, 20% caprine PRPL exhibited the highest cell viability, compared to PRPL from equine and porcine sources. Interestingly, caprine PPPL at 20% concentration demonstrated higher cell viability than equine and porcine PPPL at similar concentration.

A

B

C

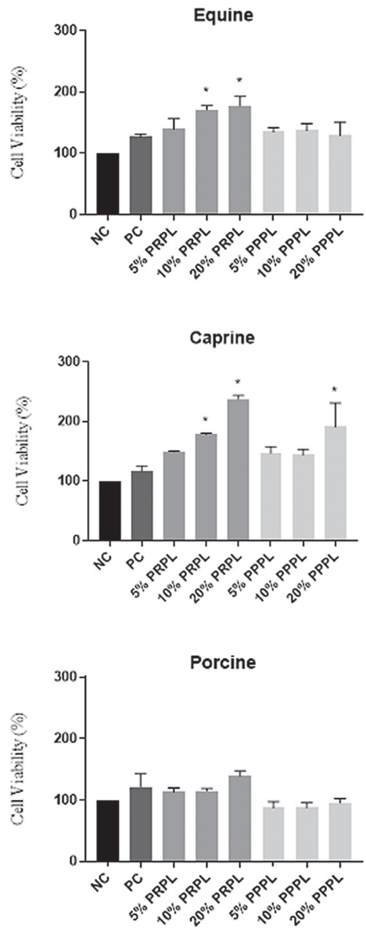
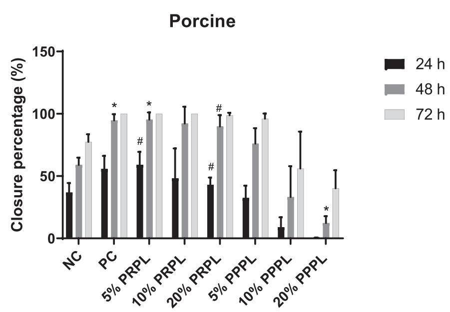


Figure 5. Dermal fibroblasts cell viability after 24 h incubation with control media (negative control (NC), DMEM/F12 only; positive control (PC), DMEM/F12 with 10% FBS) and treatment media (DMEM/F12 with 5%, 10% or 20% PRPL or PPPL from equine (A), porcine (B), or caprine (C) sources). Experiments were completed in at least triplicates, and the values were expressed as mean percentage cell viability ± SD. * $p < 0.05$ when compared with NC. DMEM/F12, Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 medium*;* FBS, fetal bovine serum; NC, negative control; PC, positive control; PRPL, platelet-rich plasma lysate; PPPL, platelet-poor plasma lysate.

Figure 6. illustrates the quantitative results of the *in vitro* wound healing assay, presenting the closure percentages, while Figure 7 provides representative micrographs of cell migration within the assay. Compared to the NC, only PC, 5% porcine PRPL, and both 5% and 10% caprine PRPL demonstrated a significantly enhanced cell migration effect on dermal fibroblasts at 48 hours post-scratch. A general negative dose-dependent effect on cell migration was observed as the concentration of PRPL from equine, porcine, and caprine sources increased from 5% to 20%. This trend was particularly evident at 24-hours post-scratch, with the optimal migratory effect consistently observed at the 5% PRPL concentration. A similar dose-dependent pattern was also noted for the corresponding PPPL groups. The highest concentration (20%) of equine PPPL resulted in significantly lower closure percentages than NC at both 24- and 48-hours ($p < 0.05$). A similar significant reduction ($p < 0.05$) was also found in the 20% porcine PPPL group when compared to NC at 48-hours post-scratch. When comparing PRPL to PPPL within the same species and at identical concentrations, the PRPL groups generally exhibited a higher wound closure percentage, especially at 24- and 48- hours post-scratch.

A



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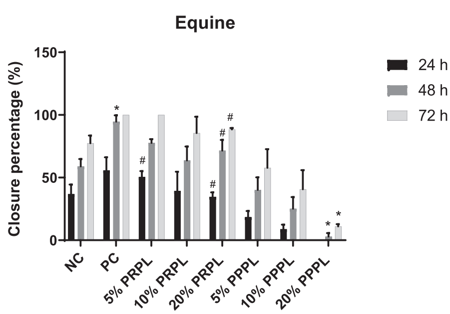

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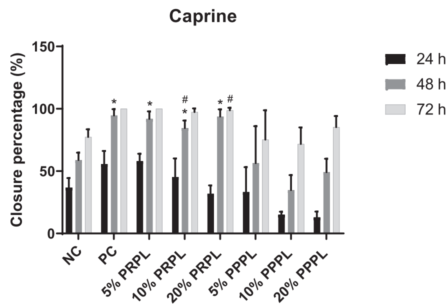
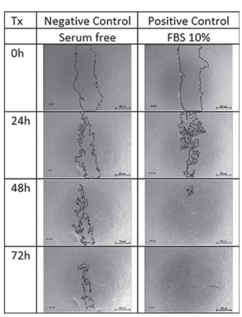


Figure 6. Closure percentage result of the *in vitro* wound healing assay. The graphs show the effect of control media (negative control (NC), DMEM/F12 only; positive control (PC), DMEM/F12 with 10% FBS) and treatment media (DMEM/F12 with 5%, 10% or 20% PRPL or PPPL from equine (A), porcine (B), or caprine (C) sources) at 24-, 48- and 72-hours post-scratch on the dermal fibroblasts' migration to close the wound area. Experiments were completed in at least triplicates, and the values were expressed as mean percentage cell viability ± SD. * $p < 0.05$ when compared with NC. $^{\#}p < 0.05$ when the same concentration PRPL is compared with PPPL at the specific time-point. DMEM/F12, Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 medium; FBS, fetal bovine serum; h, hour; NC, negative control; PC, positive control; PRPL, platelet-rich plasma lysate; PPPL, platelet-poor plasma lysate.

A



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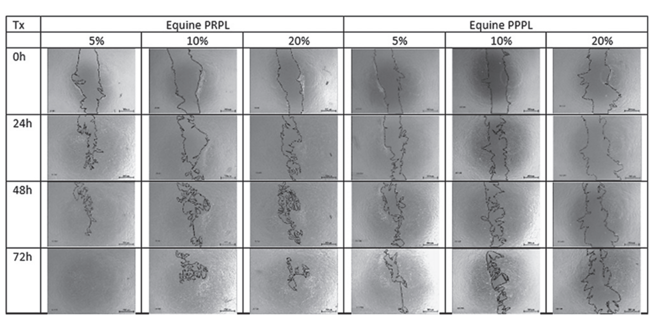

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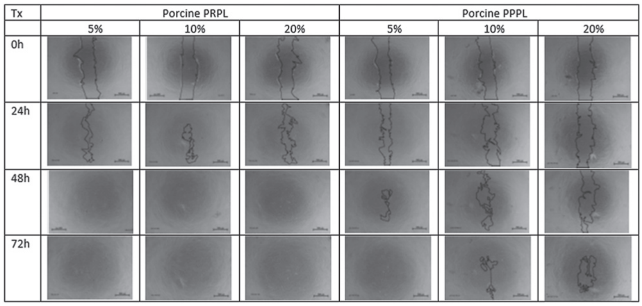

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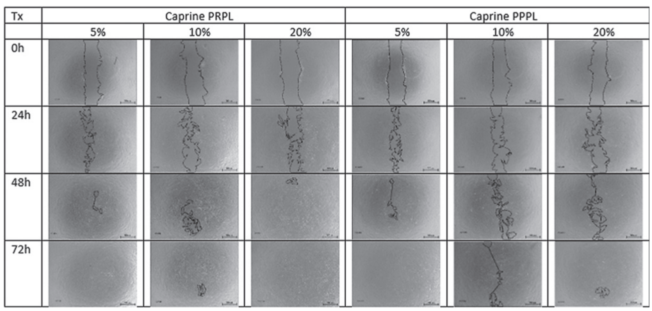

Figure 7: Micrographs of migrating cells in scratch wound assay. The micrographs show the effect of controls (A) (negative control (NC) and positive control (PC)) and different concentrations (5%, 10%, and 20%) of PRPL and PPPL from equine (B), porcine (C), and caprine (D) sources at 0-, 24-, 48- and 72-hours post-scratch on scratch closure of dermal fibroblasts. CPPPL, caprine platelet-poor plasma lysate; CPRPL, caprine platelet-rich plasma lysate; EPPPL, equine platelet poor plasma lysate, EPRPL, equine platelet-rich plasma lysate; h, hour; NC, negative control; PC, positive control; PPPL, platelet-poor plasma lysate; PRPL, platelet-rich plasma lysate; porcine platelet-poor plasma lysate; PPRPL, porcine platelet-rich plasma lysate.

DISCUSSION

Equine, porcine, and caprine sources were selected for this study because they are domestic animals with larger body sizes compared to smaller animals like cats and dogs. Their larger blood volume makes them more suitable for blood collection for the production of heterologous PRPL. Acid citrate dextrose-A (ACD-A) was used as the anticoagulant, as it is commonly employed in the PRP processing and is known to effectively preserve platelet integrity while preventing spontaneous activation, outperforming anticoagulants like heparin (Lei et al., 2009). To produce PRPL and PPPL lysates, three freeze-thaw cycles were used, based on findings by Strandberg et al. (2017), which suggest that three to five cycles are sufficient to release an optimal level of growth factors, with additional cycles offering minimal clinical benefit. For sterilization, 0.22 μm sterile syringe filters with polyethersulfone (PES) filter membranes (Microlab Scientific) were used to remove cellular debris (Gilbertie et al., 2018) and eliminate potential microbial contaminants from the lysates.

PRP is generally characterized by a platelet concentration approximately 3 to 4 times higher than the baseline (Kaneps, 2023). Although the minimum acceptable platelet concentration in PRP is said to be 200,000 platelets/μl (Kaneps, 2023), achieving a platelet concentration of $1x10^6$/μl may be necessary to elicit the desired regenerative effect (Conde-Montero et al., 2017; Tambella et al. 2018). In the study, PRP prepared from equine, porcine and caprine sources following the established protocol, consistently yielded a platelet count of approximately 1×10^6/μL (Table 2). Furthermore, the concentrations of RBCs and WBCs in all PRP samples were found to be at lower than their respective concentrations in whole blood. Although caprine PRP in this study exhibited relatively higher WBC and RBC counts compared to equine and porcine PRP, these counts remained below the baseline whole blood levels. The elevated WBC and RBC content observed in caprine PRP is likely attributable to challenges in achieving distinct separation between plasma and the red blood cell layer, even when employing higher centrifugation speed and duration (1000 g, 30 mins) during the first centrifugation of the whole blood. This is contrast to equine and porcine whole blood that had easier separation of plasma and RBCs at a lower centrifugation speed and duration (300 g, 15 mins). This suggests that during the initial transfer of caprine plasma into a new conical tube after the first centrifugation step, a greater proportion of WBCs and RBCs may have been inadvertently included before the subsequent spin designed to further concentrate the cellular components for PRP production.

Regarding the leukocyte content, PRP can be broadly classified as either leukocyte- rich PRP (LR-PRP) or leukocyte-poor PRP (LP-PRP). This classification is based on whether the leukocyte count in the PRP is higher or lower than the baseline level found in whole blood (Chun et al. 2020). Since all PRP samples in this study, including the caprine PRP, exhibited WBC and RBC counts below baseline levels, they are classified as LP-PRP. This classification applies before any freeze-thaw cycles, which would subsequently convert them into PRPL.

The impact of leukocytes within PRP on tissue healing is a subject of an ongoing debate among researchers. Some authors advocate for including leukocytes in PRP, citing their potential immunological and antibacterial benefits that may enhance healing (Tambella et al., 2018). Conversely, others avoid leukocyte-rich PRP (L-PRP) due to its content of pro-inflammatory and catabolic factors, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), which may provoke unwanted inflammation and impair tissue regeneration (Chun et al. 2020). Similarly, RBC levels were kept low, as RBCs contain molecules such as plasma-free haemoglobin, iron, and hemin, which have been associated with cytotoxicity, oxidative stress, and inflammatory responses (Gupta et al., 2023).

The increase in cell viability observed with increasing PRPL concentrations in this study aligns with findings by Russell and Koch (2016), which demonstrated a strong cell viability effect of equine PRPL on equine cord blood mesenchymal stromal cells. However, cell viability declined at concentrations exceeding 30% equine PRPL, whereas the response to FBS continued to increase (Russell and Koch, 2016). Interestingly, caprine PRPL demonstrated superior cell viability effect compared to both equine and porcine PRPL. Similarly, caprine PPPL also outperformed its equine and porcine counterparts. This may suggest that caprine PRPL and PPPL may contain a better composition of bioactive components that contribute to the enhanced healing effects on cells. However, this finding requires further validation, as individual variability in PRPL and PPPL batches may also contribute to the observed differences.

The *in vitro* wound healing assay showed that lower concentration of PRPL (5%) increased cellular migration of dermal fibroblasts than higher concentrations (20%). The adverse effect of higher PRPL concentrations on the cell migration is consistent with the findings by Xian et al. (2015), which reported a significant reduction in fibroblast migration rates at 20% PRP compared to 10% PRP at 48- and 72-hours post-scratch. While the exact mechanism remains unclear, it has been suggested that excessive growth factor concentrations in PRP may downregulate cell receptors via a negative feedback loop (Berndt et al., 2019).

To the authors' knowledge, direct comparative studies on PRPL across different animal species are limited, as most existing research focuses on individual species. Equine PRPL have been extensively investigated for their therapeutic applications, particularly in the context of osteoarthritis in horses (Gilbertie et al., 2018; Perrone et al., 2020), and for the expansion of equine mesenchymal stem cell (Russell and Koch, 2016). Porcine PRPL has shown promise in studies related to wound regeneration (Low et al., 2025) and osteoarthritis (Xiao et al., 2024) in rat models. Furthermore, porcine PRPL has been successfully used as a growth supplement for various animal cell cultures, including Vero (African green monkey kidney epithelial cells), Chinese hamster ovary (CHO) cells, and hybridoma cells, demonstrating efficacy comparable to that of fetal bovine serum (FBS) (Aldén et al., 2007). Information regarding the use of caprine PRPL is relatively scarce, with existing literature primarily focusing on other forms of platelet derivatives. For instance, caprine PRP has been utilized in osteoarthritis studies involving goat models (Wang et al., 2018), while caprine platelet-rich plasma gel has been applied in wound healing research (Khalaf and Salih, 2018) and bone healing in goats (Ferdousy et al., 2014). Given that PRPL offers distinct advantages over other platelet derivatives - such as its acellular nature and suitability for prolonged frozen storage (Gilbertie et al., 2018) - there is considerable potential for further investigation into the use of caprine PRPL in wound regeneration.

CONCLUSION

Based on the study results, PRPL derived from equine, porcine, and caprine sources demonstrated wound-healing potential *in vitro* on dermal fibroblasts. Among them, caprine PRPL appeared to have a superior healing effect compared to equine and porcine PRPL. However, since *in vitro* studies do not fully replicate the complex conditions of the skin, further *in vivo* research is required in order to evaluate the efficacy of animal-derived PRPL in wound healing.

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Author's Contribution:

ARR and SNB contributed to the conception and design of the study, data acquisition, and manuscript revision. CHC and RR were involved in the conception and design. MFWC contributed to data acquisition. AMJ, PJT, and MA participated in the study conception and design and provided final approval of the manuscript.

Competing interest

The author(s) declare that they have no competing interests regarding the publication of this paper.

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GENOMIC DIVERSITY OF THE *CLOSTRIDIUM PERFRINGENS* ISOLATES FROM HEALTHY AND ENTEROTOXEMIA AFFECTED LIVESTOCK USING MLVA METHOD

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Abstract

Clostridium perfringens is a component of the normal gut microbiota in humans and animals. However, it can become pathogenic under certain conditions, causing serious intestinal and systemic diseases. Enterotoxaemia is one of these diseases causing significant mortality in livestock annually. Analysis of genetic diversity or relatedness of isolates with molecular typing methods can be helpful for understanding the epidemiology of these infections and observing population structures of these bacteria. The aim of this study was to perform molecular typing of *Clostridium perfringens* isolates obtained from both healthy and clinically suspected enterotoxemic livestock in Fars Province using Multiple Locus Variable Tandem Repeat Analysis (MLVA) method. Collection of previously isolated and preserved *Clostridium perfringens* along with new collected isolates from healthy and diseased livestock were evaluated for toxin type and some virulence genes including cpe, tpeL, netB, and β2. Genetic typing of isolates was done using specific primers for amplification of eight Variable Number Tandem Repeat (VNTR) loci. PCR was done and electrophoresis banding patterns of fragments were analyzed by BioNumerics software. The results of typ-

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ing revealed 71 distinct MLVA profiles from 74 strains. The strains analyzed included 27 clinical isolates, 43 non-clinical isolates, three vaccine strains, and the reference strain 13 obtained from the GenBank database. *Clostridium perfringens* strain 13 sequence was used as a reference strain and was evaluated for comparison of sequences for each locus. Clustering of isolates according to the minimum spanning tree showed 5 different clusters, excluding three orphan isolates. Clinical and non-clinical isolates were scattered in all clusters and no defined cluster related to health status, date of isolation, kind of host and place of isolation was detected. The total diversity index of our typing method was estimated to be 0.99. Overall MLVA molecular typing method shows high genetic diversity among *Clostridium perfringens* isolates.

Key words: *Clostridium perfringens*, sequence analysis, bacterial genomes, livestock, MLVA

UTVRĐIVANJE GENETIČKE RAZNOVRSNOSTI IZOLATA *CLOSTRIDIUM PERFRINGENS* KOD ZDRAVE I OBOLELE STOKE OD ENTEROTOKSEMIJE PUTEM MLVA METODE

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Kratak sadržaj

Clostridium perfringens je sastavni deo normalne crevne mikroflore kod ljudi i životinja. Međutim, pod određenim okolnostima može postati patogena i izazvati ozbiljne crevne i sistemske bolesti. Jedna od tih bolesti je i enterotoksemija. Ona svake godine izaziva značajan mortalitet kod stoke. Analiza genetske raznovrsnosti ili srodnosti izolata pomoću metode molekularnog tipiziranja može biti od velikog značaja za razumevanje epidemiologije infekcija i proučavanje populacione strukture ovih bakterija. Cilj ovog rada je molekularno tipiziranje izolata vrste *Clostridium perfringens*, dobijenih od zdrave stoke i stoke sa kliničkom sumnjom na enterotoksemiju u provinciji Fars, korišćenjem "Multiple Locus Variable Tandem Repeat Analysis" (MLVA) Grupa ranije izolovanih i očuvanih sojeva *Clostridium perfringens*, zajedno sa novim izolatima prikupljenim od zdrave i obolele stoke, evaluirana je u pogledu tipa toksina i prisustva određenih gena virulencije, uključujući *cpe*, *tpeL*, *netB* i *β2*. Genetsko tipiziranje izolata izvršeno je korišćenjem specifičnih prajmera za amplifikaciju osam regija "*Variable Number Tandem Repeat"* (VNTR). Izvršena je PCR analiza kao i eletroforeza pojavljivanja bendova fragmenata koji su analizirani putem BioNumerics softvera. Rezultati tipiziranja pokazali su da postoji 71 različit profil od 74 soja. Analizirani sojevi su obuhvatali 27 kliničkih izolata, 43 neklinička izolata, tri vakcinalna soja i referentni soj 13 dobijen iz GenBank baze podataka. Sekvenca soja *Clostridium perfringens* 13 korišćena je kao referentni soj i evaluirana je za poređenje sekvenci na svakom lokusu. Grupisanje izolata prema *"minimum spanning tree*" analizi pokazalo je pet različitih klastera, isključujući tri izolata. Klinički i neklinički izolati su bili raspoređeni po svim klasterima, pri čemu nije uočen nijedan klaster koji bi bio povezan sa zdravstvenim statusom, datumom izolacije, vrstom domaćina ili mestom izolacije. Procenjeno je da je indeks ukupne raznovrsnosti metode tipiziranja 0.99. Metoda MLVA metoda molekularnog tipiziranja pokazuje visoku genetsku raznovrsnost među izolatima *C. perfringens*.

Ključne reči: *Clostridium perfringens*, analiza sekvenci, bakterijski genomi, stoka, MLVA

INTRODUCTION

Clostridium perfringens is a causative agent of economically significant enteritis, enterotoxemia, and other major diseases affecting both livestock and humans. This spore-forming anaerobic bacterium is also considered a component of the intestinal microbiome (Sting, 2009; Rood et al., 2018). There are no distinct defined criteria to distinguish the pathogenic strains from normal flora of these bacteria. It is believed that the virulence of *C. perfringens* is related to production of toxins, and other factors, some of which have not been detected or well defined so far (Li et al., 2013; Freedman et al., 2015; Rood et al., 2018).

Many toxins are produced by *C. perfringens* which have major role in virulence, and most are plasmid born. Four major toxins, namely alpha, beta, epsilon and iota have been used for toxin typing of this bacterium. Recently two other toxins - enterotoxin and beta2 toxin - have been included in this typing. According to this new typing scheme, two toxin types of F and G were added to five previous types of A, B, C, D, and E. (Chan et al., 2012; Rood et al., 2018).

Molecular typing of pathogenic isolates can be helpful for epidemiological purposes and the study of ecology, evolution and phylogenetic relationship between different isolates from clinical and non-clinical sources (Lukinamaa et al., 2004; Van Belkum, 2007; Hassan et al., 2020). Some molecular typing techniques are introduced and applied for *C. perfringens* such as Restriction Fragment Length Polymorphism (RFLP), Pulsed Field Gel Electrophoresis (PFGE), Ribotyping, Repetitive Element PCR (REP), Random Amplified Polymorphic DNA (RAPD), Multilocus Sequence Typing (MLST) and Multiple-locus variable-number tandem-repeat analysis (MLVA), from which MLVA is considered as a low cost, rapid, reproducible with high discriminatory power and easy molecular typing method (Lindstedt et al., 2005; Johansson et al., 2006; Gelfand et al., 2007; Afshari et al., 2016; Wang et al., 2021).

This study was designed to apply the MLVA method for genetic subtyping of *C. perfringens* isolates in order to assess the phylogenetic relationships among strains collected from both healthy and enterotoxemic hosts in Fars Province, Iran.

MATERIAL AND METHODS

Bacterial isolation and characterization

Samples of intestinal contents, plural and peritoneal fluids from 15 cases of sudden death due to enterotoxemia and 30 samples of intestinal contents of healthy goats, and sheep from slaughterhouses were collected between 2019 and 2022. The samples were cultured for isolation of *C. perfringens.* Eight *C. perfringens* strains which were isolated from enterotoxemia cases were also provided by Veterinary Office of Fars province (seven sheep and one jebeer). In addition, about 37 more isolates preserved in our laboratory archive which were collected over a period of 10 years (2009-2018), seven of which were associated with confirmed cases of enterotoxemia (two sheep, four goats and one cattle) and the rest were from healthy animal hosts (16 sheep, seven goats, seven cattle). These isolates were also defrosted and included in this study along with the vaccine strains of type B, C and D (the data of all isolates are presented in supplementary Table S1). Neomycin-supplemented blood agar

(40 μg/mL) was used for the primary culture and isolation of the bacteria. Suspected colonies of *C. perfringens* were purified and confirmed by biochemical and PCR tests for 16SrsRNA (Meer and Songer, 1997; MacFaddin et al., 2000). Toxin typing was done using related specific primers for alpha, beta, epsilon, iota, netB, enterotoxin, and the presence of other virulence factors including beta2, tpeL and tcpH for detection of conjugative plasmids were also evaluated (Table 1).

Table 1. Primers used for confirmation and evaluation of typing toxins and virulence genes of *Clostridium perfringens*

gene		primer sequence	Fragment size	Annealing temperature	references
16SrRNA	F	AAAGATGGCATCATCATTCAAC	279	55	Meer&Songer, 1997
	R	TACCGTCATTATCTTCCCCAAA			
cpa (*alpha toxin*)	F	GCTAATGTTACTGCCGTTGA	324	55	Meer&Songer, 1997
	R	CCTCTGATACATCGTGTAAG			
***cpb* (β-toxin)**	F	GCGAATATGCTGAATCATCTA	196	55	Meer&Songer, 1997
	R	GCAGGAACATTAGTATATCTTC			
***etx* (ε-toxin)**	F	TGGGAACTTCGATACAAGCA	376	55	Van Asten et al., 2010
	R	AACTGCACTATAATTTCCTTTTCC			
***iap* (ι-toxin)**	F	AATGGTCCTTTAAATAATCC	272	55	Meer&Songer, 1997
	R	TTAGCAAATGCACTCATATT			
***cpe* (enterotoxin)**	F	TTCAGTTGGATTTACTTCTG	486	56	Van Asten et al., 2010
	R	TGTCCAGTAGCTGTAATTGT			
***cpb2* (β2-toxin)**	F	CAATACCCTTCACCAAATACTC	567	55	Bueschel et al., 2003
	R	AGATTTTAAATATGATCCTAACC			
tcpH	F	CATTAACTGTTTTCTAAAGGC	931	55	Park et al., 2016
	R	GCGGAATAGTAATGATTAGTT			

gene		primer sequence	Fragment size	Annealing temperature	references
netB	F	CGCTTCACATAAAGGTTGGAAGGC	316	60	Bailey et al., 2013
	R	TCCAGCACCAGCAGTTTTTCCT			
tpeL	F	ATATAGAGTCAAGCAGTGGAG	466	59	Keyburn et al., 2008
	R	GGAATACCACTTGATATACCTG			

Data analysis

The Chi-square (χ^2) test was performed to analyze significant differences between the studied virulence genes in two groups using SPSS software (ver. 27.0.1).

MLVA typing

For MLVA analysis, eight VNTR (07, 17, 24d, O1e, cp6, cp42, cp13, cp19) primer pairs were used (Table 2). The PCR reaction was performed in a total volume of 20 µL per reaction containing 10 µL of 2× PCR master mix (Amplicon, Denmark), 1 µL (20 pmol) of each forward and reverse primer (Metabion International AG®, Germany), 6 µL ddH2O, and 2 µL of genomic DNA as a template. PCR was conducted (Eppendorf thermocycler, Germany) with the following amplification conditions: Initial denaturation at 95 °C for 4 min, followed by 30 cycles at 94 °C for 1 minute, annealing at 58 °C for 45 s, extension at 72 °C for 2 min, and final extension at 72 °C for 5 min.

Table 2. Primer pairs used for MLVA typing

VNTR locus		Primer sequence	reference
Cp6	F	GTAAAGATGATTGCTATTTAGAGATAA	Sawires and Songer, 2005
	R	TAAGGTATCATCAAAATCCACTCCAGG	
Cp13	F	AAGGAAGATGCTACTCAAGATG	Sawires and Songer, 2005
	R	GAAGCCATCATTACTACTCCTA	

VNTR locus		Primer sequence	reference
CP19	F	CTCAATCCAACAATATGTGCTGACTA	Sawires and Songer, 2005
	R	GTAGCAGCAATAAAACCAACCTAAA	
CP42	F	GATGGCCCAAGAAACAGAAC	Sawires and Songer, 2005
	R	GCTGGGAATAAAGGGTTTGA	
07	F	TTCAAAGCCAGGTACATTCAA	Chalmers et al.,2008
	R	TCTTCTGTTTCAAAATTAGTTTCAT	
17	F	TTAATGTCCATATTCCGCCA	Chalmers et al.,2008
	R	TTGTGGACAATCTGTAGGAGGA	
O1e	F	ATGGGGTAAGGGGATCCAA	Chalmers et al.,2008
	R	GGGGTTAAACTTGATTTTCTGTG	
24d	F	TATAGTGTGGTTAAAAT-ATTAAATTATAGTT	Chalmers et al.,2008
	R	GAATTACTTTTCTACAATT-TATTTTCATAA	

The PCR products were analyzed by electrophoresis on 1.5% agarose gel, and the band sizes were recorded. Some PCR products as a representative of amplicon sizes in each VNTR were sequenced (Microsynth, Switzerland). The sequences were analyzed using NCBI BLAST search, and multiple alignments were done using Clustal Omega program; https://www.ebi.ac.uk/jdispatcher/msa/clustalo (Sievers et al. 2020). The sequence of *C. perfringens* strain 13 was also used as a reference for comparison of strains. The tandem repeats were evaluated by tandem repeat finder program; TRF (Benson, 1999).

The resulting banding patterns were analyzed using the BioNumerics software, Version 6.7 (Applied Maths, USA). Network creation method was Minimum Spanning Tree. MST was obtained based on the shortest mathematically possible distance between isolates (nodes). The first priority rules were set as maximum number of N-locus variants (N=1) and Weight: 10000 and the second one as maximum number of N-locus variants (N=2) and Weight: 10. For partitioning, Nodes closer than 2 were put into the same partition. Every partition contained at least one entry and one node. To generate the dendrogram, the unweighted pair group method with arithmetic mean (UPGMA) was employed, using the multistate categorical similarity coefficient (MCSC) with equal weighting for all loci.

The discriminatory power of individual VNTR was determined by calculating the Nei's diversity index (D) using the equation D = 1 - Σ (allele frequency)[2]. The allelic diversity (h) of the individual loci was calculated using the equation $h = n\ (1- \Sigma xi^{2)}/(n\text{-}1)$, where n is the number of strains and xi is the frequency of the i^{th} allele at the related locus. The discriminatory power (Diversity Index = DI) of this typing system using combined eight loci was calculated according to Hunter and Gaston formula both individually and using online tool (http://insilico.ehu.es/mini_tools/discriminatory_power). The formula is as follows:

$$DI = 1 - 1/\ N\ (N - 1)\ s\ \sum j = 1\ Nj\ (Nj - 1)$$

where N in this equation is the total number of strains, s is the total number of genotypes, and Nj is the number of strains belonging to the j^{th} profile.

RESULTS

Culture, isolation and confirmation

Thirteen *C. perfringens* isolates were recovered from 30 intestinal content samples of healthy animals (three goats, 10 sheep) and 12 isolates from 15 intestinal content samples of livestock with suspected field cases of enterotoxemia (one goat and 11 sheep).

A total of 70 purified *C. perfringens* isolates showing a double zone of hemolysis - 43 from healthy animals (26 sheep, 10 goats, seven cattle) and 27 from clinical cases (20 sheep, five goats, one cattle and one jebeer) were evaluated and confirmed by biochemical tests including positive tests of sucrose, nitrate, gelatinase, lecithinase, lipase and negative tests of catalase, indole, motility, and lactose.

The results of PCR tests using 16SrRNA (figure 1-A) also showed 279 bp fragments indicating positive results for all of these confirmed colonies of *C. perfringens*.

Toxinotyping and other virulence genes

The results of toxin typing by PCR showed bands of 324, 196, 376 and 272 bp for alpha, beta, epsilon and iota toxins respectively (Figure 1). PCR results corresponding to identification of *cpe* and *netB* (487bp and 316bp) are shown in Figure 2. The number of different toxin types and related data are presented in Tables 3 and 4. Most of the isolates overall belonged to type A (53 isolates), followed by type D (14 isolates). In the healthy group, all isolates belonged to type A, except one, which was type D. In the clinical group, most of the isolates were type D (13 isolates), followed by type A (11 isolates), two type G, and one type C. *C. perfringens* Type B, Type E, and type F were not present in our isolates except for a vaccine strain of type B. PCR results for screening the presence of *beta2, tpeL* and *tcph* (567bp, 466bp and 931 bp respectively) are also shown in Figure 2.

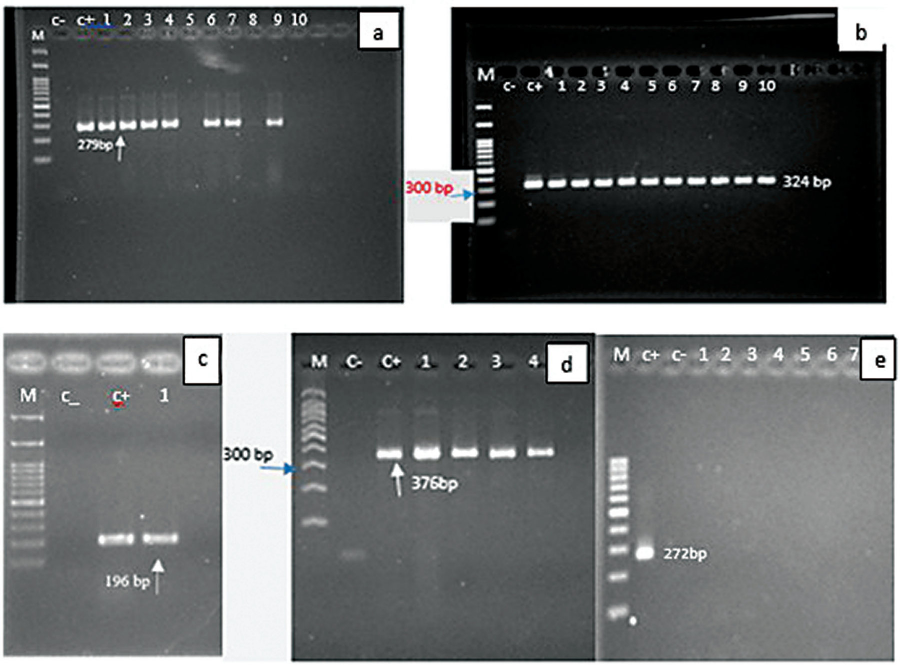

Figure 1. Agarose gel electrophoresis images of PCR products with specific primers for 16s rRNA (279 bp) [A]; *cpa* (324 bp) [B]; *cpb* (196 bp) [C]; *etx* (376 bp) [D]; and *iap* (272 bp) [E] genes. Lane 1 in each image is M: DNA marker 100 bp (SinaClon, Iran), c-: negative control, c+: positive control (type B vaccine strain for [A-D] and type E strain CN1241 [E]).

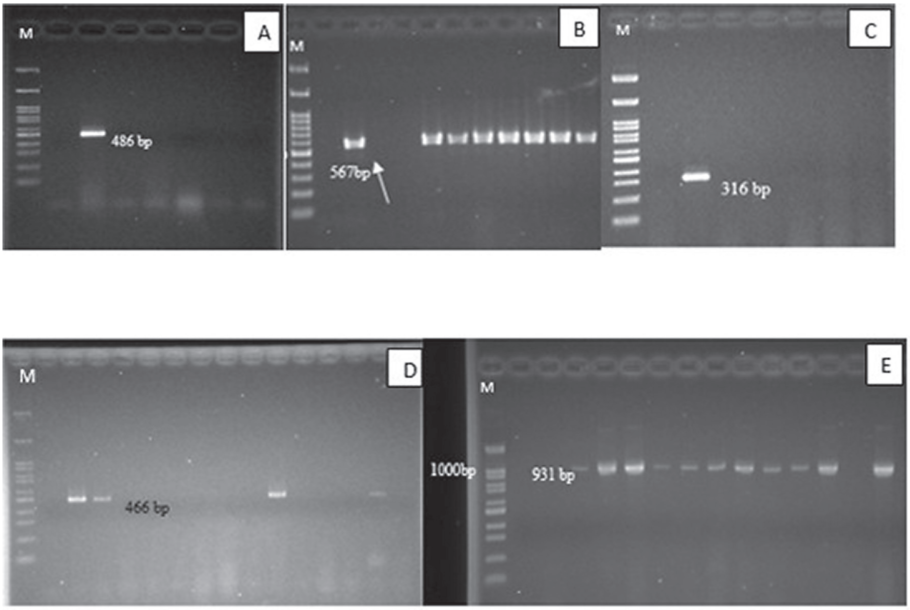

Figure 2. Agarose gel electrophoresis images of PCR for identification of *cpe* gene (487bp) [A]; *cpb2* (567bp) [B]; *netB* (316bp) [C]; *tpeL* (466bp) [D]; and *tcpH* locus (931bp) [E]. Lane 1 in each image is M: DNA marker 100 bp plus (SinaClon, Iran), Lane 2: negative control, Lane 3: positive control (strain Ba1/p, accession: OR725467 [A]; type B vaccine strain [B]; RTCC No: IRCPF-59 accession: PQ594181 [C]; type C vaccine strain [D]; type B vaccine strain [E]); Lane 4 and other lanes are different isolates.

The frequency of virulence genes in two groups is presented in Table 5. Statistical analysis of differences between the studied virulence genes in two groups using χ^2 test showed no significant difference except for beta2 which were higher among clinical group (92.5%) in comparison to non-clinical group (58%), ($p < 0.05$).

Table 3. Distribution of different toxin types in two groups of *C. perfringens* isolates

Toxin type	Healthy group		Clinical group		Total percent
	frequency	percent	frequency	percent	
A	42	97.67	11	40.74	75.71
B	0	0	0	0	0
C	0	0	1	3.70	1.43

Toxin type	Healthy group		Clinical group		Total percent
	frequency	percent	frequency	percent	
D	1	2.32	13	48.14	20
E	0	0	0	0	0
G	0	0	7.40	2	2.86
total	43	100	27	100	100

Table 4. Distribution of different toxin types of *C. perfringens* according to host and health status

Host source	Number in healthy group			Number in clinical group			
	Type D	Type C	Type A	Type D	type C	Type A	Type G
sheep	1	-	25	7	1	12	2
goats	-	-	10	5	-	-	0
cattle	-	-	7	1	-	-	0
Jebeer gazelle	-	-	-	-	-	1	0
total	1	-	42	13	1	13	2

Table 5. Frequency of virulence genes in *C. perfringens* in two clinical and non-clinical groups

Virulence genes	Non-clinical group			Clinical group					total	Total per-cent
	total	typeD	typeA	total	typeG	typeD	typeC	typeA		
beta2	25	1	24	25	2	12	1	10	50	71.42%
cpe	1	-	-	1	-	1	-	-	1	1.42%
tpeL	5	-	4	1	-	-	1	-	5	7.14%
tcpH	34	1	33	27	2	13	1	11	61	87.14%

MLVA typing

PCR using specific primers for eight VNTR loci produced 42 distinct banding patterns in total, excluding null alleles, which were observed at all loci. These results are shown in Table 6. A representative agarose gel is shown in Figure 3. For all loci, selected PCR amplicons of varying sizes from different samples were submitted for sequencing (Bioneer Co., South Korea). The resulting sequences were compared using the online BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/).

Table 6. VNTR characteristics obtained in this study.

Locus	Amplified fragment size	Number of different alleles	The most frequent alleles in the related locus
Cp6	503-656	8	630
Cp13	225-296	5	296
Cp19	636-1600	9	1500
Cp42	220-1345	8	220
07	121	2	121
O1e	350-463	7	437
17	134-176	4	155
24d	144-194	5	159

C. perfringens strain 13 sequence was also used as a reference strain and was evaluated for comparison of sequences for each locus.

Analysis of sequences using tandem repeat finder program online (https://tandem.bu.edu/trf/) (Benson, 1999) showed that in most loci tandem repeats were polymorphic and not conserved. Observation of some various repeat units in different strains, presence of more than one TR and overlapping repeat sequences were common in these loci. For instance, the results of tandem repeats for locus cp6 in only one strain is shown in supplementary Table S2. Therefore, the MLVA scheme was based on the length of the resulting amplicons, measured in base pairs (bp).

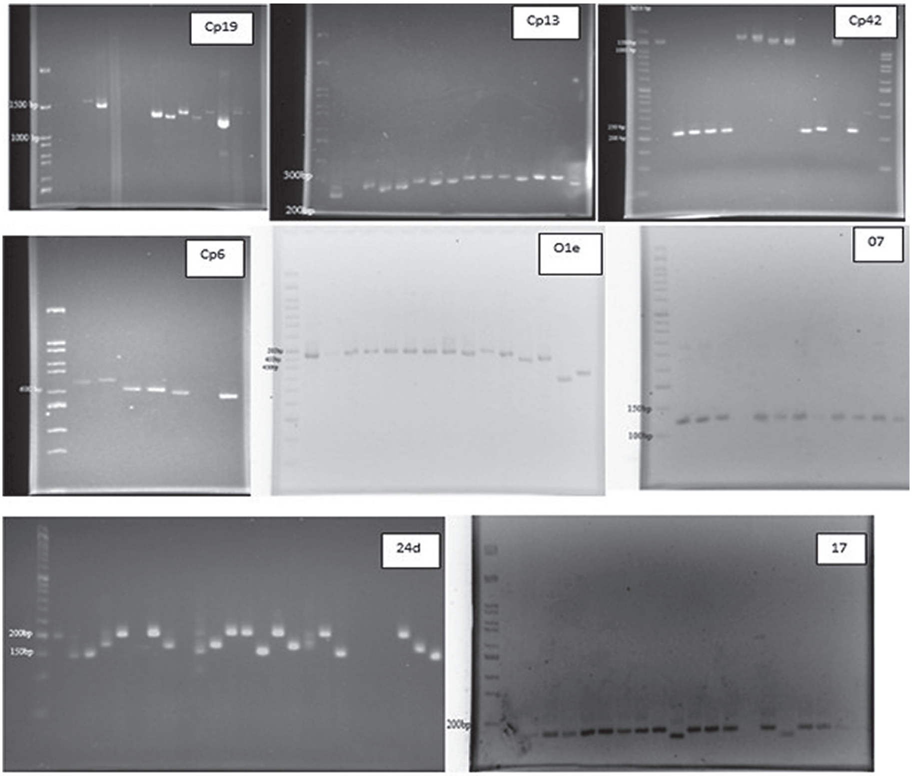

Figure 3. Agarose gel electrophoresis images of PCR products for eight loci.

The MLVA typing system used in this study classified the 74 *C. perfringens* strains into 71 MLVA distinct profiles. Figure 4. shows the minimum spanning tree (MST), created by Bionumerics software. As shown in MST, different clusters of strains are observed and the isolates have high genetic diversity. Six isolates exhibited identical profiles in pairs. Among them, two isolates shared the same profile despite differing in source (region and host), toxin type, and year of isolation. However, one pair - strains 54 and 55 - was isolated from enterotoxemic goats in the same region on the same date. Overall, five clusters (clonal complexes) and three singletons were identified.

Each cluster contained different toxin types, various hosts and isolation dates. Clustering is not related to toxin types i.e., type C isolate is more divergent from the vaccine strain of type C. There was no genetic relationship that separated the clinical isolates from the non-clinical ones.

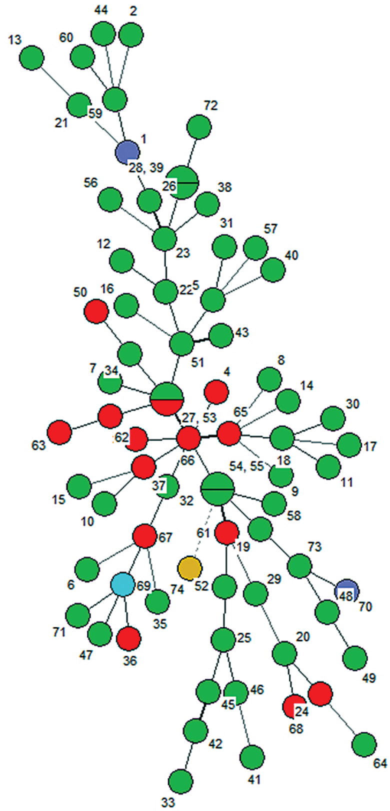

Figure 4. MST analysis based on 8 VNTR loci genotyping profiles of 73 *C. perfringens* strains plus strain 13 from GenBank as a reference strain. Circles represent genotypes, with the numbers beside them indicating the corresponding isolates. The color of each circle corresponds to a different toxin type: green for type A, red for type D, dark blue for type C, light (turquoise) blue for type B, and yellow for strain 13. Isolates numbered 59 and 60 are type G.

The discriminatory power of individual VNTR loci D (Nei's diversity index) was 0.09, 0.76, 0.25, 0.82, 0.79, 0.85, 0.8 and 0.84 for loci 7, 24d, 17, O1e, cp42, cp13, cp6 and cp19 respectively. Locus cp13 displayed the highest and locus 7 the lowest allelic diversity. The overall Diversity Index (DI) was calculated to be 0.99 for all eight loci.

The dendrogram based on the MLVA results is also shown in Figure 5. According to this dendrogram, eight clusters or clades are obtained. None of the clusters were associated with a specific characterization. *C. perfringens* strain 13 formed a closely related cluster, showing greater similarity to the type C vaccine strain and 10 strains isolated from both healthy and clinical cases between 2015 and 2022. Nine isolates from cattle hosts were included in four cluster along with other strains. One cluster included merely 3 isolates from goats and the rest of 12 isolates from goats are included in 3 other clades with other strains. The isolates from sheep hosts are located on seven clusters.

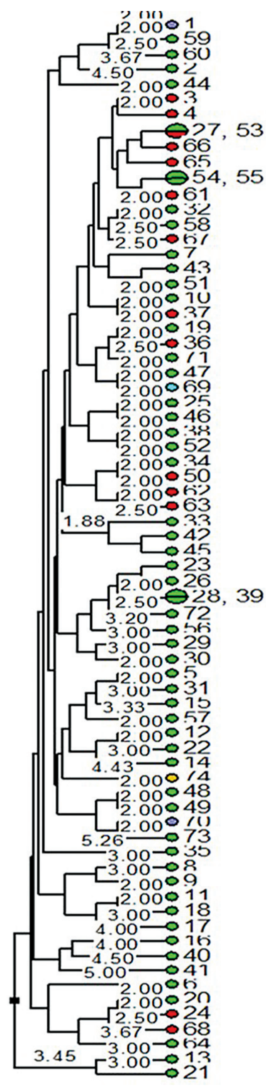

Figure 5. Dendrogram of the MLVA results for the *C. perfringens* strains in this study. The dendrogram was constructed by BioNumerics software using UPGMA and the Dice algorithm based on the band sizes on the electrophoresis gel.

DISCUSSION

The present study intended to determine the strain diversity of clinical and non-clinical *Clostridium perfringens* isolates, based on MLVA typing. *C. perfringens* isolates from different parts of Fars province of Iran isolated between 2009 and 2022 were used in this study. A total of 43 *C. perfringens* isolates were obtained from intestinal contents of healthy goats, sheep and cattle from slaughterhouses. In contrast, 27 isolates were recovered from confirmed enterotoxemia cases, either through field sampling or provided by the provincial veterinary office.

In addition to identifying the toxin types of our isolates, the presence of other virulence factors was also assessed. A majority of the isolates belonged to type A and D respectively, in both groups. According to the recent reclassification of *C. perfringens* by Rood et al., none of our type A strains carried the *cpe* gene to be classified as type F; however, one type D strain from the clinical group tested positive for this gene. The role of *CPE*⊠ *C. perfringens* isolates in the pathogenesis of enterotoxemia in livestock has not been precisely determined, although their contribution to enteritis and diarrhea in both animal hosts and humans is well established (Songer, 2010; Freedman et al., 2015; Suzuki et al., 2021; Alimolaei et al., 2023).

The other new reclassified type is type G, representing the presence of *netB* gene in type A strains. Although this virulence gene is associated with necrotic enteritis in poultry (Smyth et al., 2022), it was also found in two type A strains from the clinical group isolated from sheep, which were later reclassified as the new type G.

The majority of isolates in the two groups (a total of 87%) were positive for conjugative plasmids, as confirmed by amplification of the *tcph* gene. Among the virulence genes *cpe, tpel, β2* and *netB,* evaluated in this study, beta2 gene was the only one showing statistically significant different result in two groups. The presence of this gene was 92% in clinical and 58% in non-clinical isolates.

This result aligns with previous findings (Manteca et al., 2002) but contradicts both our earlier study and those of other researchers (Sting et al., 2009; Razmyar et al., 2014; Hayati et al., 2021). The discrepancy between the two studies may be explained by the loss of viability in some archived C. perfringens strains, which prevented their inclusion in the present analysis.

The role of this gene in the pathogenesis of *C. perfringens* in livestock remains unclear, although the isolation of type A strains harboring the beta2 toxin from enterotoxemic calves has been documented (Manteca et al., 2002). It is well established that beta2 toxin exerts cytotoxic effects on CHO cells and induces hemorrhagic necrosis in the guinea pig intestine, as well as enterocolitis and diarrheic disease in foals, piglets, dogs, and several other domestic, wild, and even human hosts (Fisher et al., 2005; Wasinski, 2007; Van Asten et al., 2010). The phylogenetic relationship between these *C. perfringens* isolates, obtained from different sources (healthy and enterotoxemic cases) was also assessed using MLVA method, as this method is considered a potential technique for genotyping a wide range of bacteria, including multiple *Clostridium* species (Lindstedt et al., 2005; Park et al., 2016)

In this study, MLVA analysis was performed using primer pairs targeting eight VNTR loci: 07, 17, 24d, O1e, cp6, cp42, cp13, and cp19. According to the investigations by Sawires and Songer (2005) and Chalmers (2008) on tandem repeats in the *C. perfringens* genome, five and six loci, respectively, were identified as high-priority targets among other TR sequences for use in MLVA typing of this species. They reported high typeability, reproducibility and Discrimination (DI = 0.99 and 0.97) of this technique for genotyping of their *C. perfringens* isolates (Sawires and Songer, 2005; Chalmers et al., 2008). Our results showed high Discrimination (DI = 0.99) as well. According to the criteria proposed by Hunter and Gaston (1988), a discriminatory index greater than 0.90 is considered desirable.

Considering allele frequency and tandem repeat length - and due to the absence of capillary electrophoresis - eight primers out of the eleven loci were selected to conduct this MLVA study. Four of these loci are located on protein coding sites on chromosome and the others on non-coding regions. Locus cp6 is located on collagen like protein sequence. The most frequent allele length recorded in the gene bank is 570 bp, whereas for our isolates from Fars province, Iran, it was 610 bp. The locus CP13 is located within the *nagK* gene, which encodes hyaluronidase. The most frequent allele in our study was 296 in two healthy and clinical groups. In the clinical group, 21 out of 27 isolates (77.7%) and in the healthy group, 20 out of 43 isolates (46.5%) exhibited a 296 bp band size for this locus, whereas the most frequent allele reported in Sawires and Songer' study was 282 bp. It has been suggested that variation in the hyaluronidase gene within a population may be linked to environmental adaptation, although further investigation is needed. Locus CP19 is related to a part of Iron transport protein B which has shown to have more null allele in both this and previous studies. Sequencing revealed that the polymorphism at this locus did not fully correspond to the number of VNTR repeat units. Locus CP42 is located partly on a hypothetical protein and partly on beta subunit of riboflavin synthase protein regions (Sawires and Songer, 2005). The most frequent allele of this locus was 220 bp in both clinical and nonclinical groups.

Other loci including O1e, 17, 07 and 24d were located on noncoding regions (Chalmers et al., 2008). The most frequent alleles of loci O1e, 17, 07 and 24d were 437 bp, 155 bp, 121 bp and (144 and 159 bp) respectively in both groups.

Forty-eight alleles with the average of six alleles per locus were detected using these eight loci in this study. Combining loci increased the discriminatory power for genotyping isolates; however, it failed to differentiate the clinical group from the non-clinical group, and clustering occurred independently of toxin type, health status, isolation date, region, or host. In addition, no single VNTR locus showed the specificity to reveal the adaptation of *C. perfringens* strains to the environment and virulence.

The extensive genetic diversity observed among our *C. perfringens* isolates is consistent with findings reported in other studies. This may be attributed to the high heterogeneity and variation of *C. perfringens* genome (Myers et al., 2006; Feng et al., 2020; Camargo et al., 2022). Although Johansson et al. (2006) showed a high genetic similarity among epidemiologically related isolates of *C. perfringens* and a wide genetic diversity among the isolates from different sources by PFGE.

The virulence of *C. perfringens* is primarily associated with its toxin production and the process of horizontal gene transfer (Van Asten et al., 2010; Gulliver et al., 2023). In addition, many of these toxin genes (major toxins related to typing and the rest ones) are present in *C. perfringens* isolates of intestinal microflora from healthy livestock (Freedman et al., 2015). It is believed that certain changes in the host and environment can trigger toxin production and contribute to disease development (Sawires and Songer, 2006; Uzal et al., 2014). Nevertheless, it is important to determine whether genetic differences exist between clinical and nonclinical isolates. Although phylogenetic analysis may not always effectively group these isolates, it remains an essential tool for tracking strains during an outbreak.

CONCLUSION

The VNTRs used for MLVA typing in this study revealed considerable genomic diversity among *C. perfringens* strains but did not demonstrate the expected close phylogenetic relationships among strains from the same source category or with similar virulence. It can be concluded that the limited number of VNTR loci examined (eight primers) and the lack of assessment of additional VNTRs across the genome may limit the representativeness of the results regarding overall genetic diversity and relatedness. Therefore, complementing MLVA with other phylogenetic methods is recommended for a more comprehensive analysis.

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Author's Contribution

S.S. collected the samples. M.M. and A.P. contributed to bacterial isolation. K.T. carried out the MLVA analysis using BioNumerics software. R.S., a colleague from the veterinary office, provided enterotoxemia-related isolates. Y.T. revised the manuscript. M.H. was involved in all aspects of the project, including experimental design, bacterial isolation, PCR, molecular analysis, data collection, and manuscript drafting. All authors read and approved the final version of the manuscript.

Competing interest

The authors declare that there are no commercial or financial relationships that could be seen as a potential conflict of interest.

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Supplementary Data

The supplementary material (Table S1) associated with this article is available online at the following permanent link:
https://niv.ns.ac.rs/e-avm/index.php/e-avm/article/view/475/321

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STUDY OF BACTERIAL CARRIAGE IN DARKLING BEETLES (*ALPHITOBIUS DIAPERINUS*) COLLECTED FROM INDUSTRIAL BROILER FLOCKS IN NORTH-EAST OF TUNISIA

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Abstract

Alphitobius diaperinus is regarded as a highly resilient pest in poultry production systems that use litter-based rearing . It is also known as a potential reservoir of several avian pathogens. The present study aimed to determine darkling beetle carriage of bacterial pathogens in Tunisian poultry houses. A total of 25 industrial broiler flocks naturally infested by darkling beetles and located in the North-East of Tunisia were sampled. A pooled sample of adult beetles (5 g) was collected from five distinct locations within each broiler house. Insects were placed in sterile containers and transported to the laboratory under cooled conditions. All samples were examined for entomological identification. Bacteriological analysis was performed to detect external and internal bacterial carriage, according to the standard protocols. Antimicrobial susceptibility for all *Escherichia coli* isolates was tested by the disk diffusion method on Mueller–Hinton agar using commercial disks. Antimicrobial susceptibility testing was not performed for the remaining bacterial isolates. Entomological identification showed that all sampled beetles belong to *Alphitobius diaperinus* species. A total of 108 isolates were detected from the surface (57 isolates) and the

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interior (51 isolates) of adult insects. Gram-negative (63.89%) (*Escherichia coli*, *Enterobacter* spp., *Klebsiella pneumoniae*, *Proteus vulgarus*, *Citrobacter* spp., *Pseudomonas* spp., *Pseudomonas aeruginosa*) and coagulase-negative staphylococci (36.11%) (Gram-positive) bacteria were isolated, with the global predominance of coagulase-negative staphylococci, followed by *E. coli* (23.15%), *Pseudomonas* spp. (13.89%) and *Pseudomonas aeruginosa* (10.19%). No *Salmonella* spp. was detected. Antibiotic resistance profile showed that all *Escherichia coli* isolates were multi-resistant. High resistance was observed to doxycycline (25/25; 100%), amoxicillin (24/25; 96%), cephalothin (23/25; 92%), streptomycin (22/25; 88%), tetracycline (22/25; 88%), enrofloxacin (22/25; 88%). A high level of resistance was observed to trimethoprim-sulfamethoxazole (13 out of 25 isolates; 52%) and gentamicin (9/25; 36%). However, a low level of resistance was observed to florfenicol (7/25; 28%) and colistin (3/25; 12%). Our results confirm the potential vector role of beetles in the spread of bacteria pathogens and antibiotic resistance.

Key words: Alphitobius diaperinus, broiler chickens, *Escherichia coli*, resistance to antibiotics, Tunisia

ISPITIVANJE PRENOŠENJA BAKTERIJA KOD BRAŠNENIH CRVA (*ALPHITOBIUS DIAPERINUS*) IZ JATA BROJLERA GAJENIH U INDUSTRIJSKIM USLOVIMA SA SEVEROISTOKA TUNISA

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Kratak sadržaj

Alphitobius diaperinus se smatra veoma otpornim štetočinama u živinarskoj proizvodnji na prostirci. Takođe je poznat kao potencijalni rezervoar brojnih ptičjih patogena. Cilj ovog rada je da se utvrdi prisustvo bakterijskih patogena kod brašnenih crva u živinarskim objektima u Tunisu. Ukupno 25 industrijskih jata brojlera, prirodno zaraženih brašnenim crvima sa severoistoka Tunisa uzorkovano je za potrebe istraživanja. U svakom objektu za uzgajanje brojlera prikupljeni su objedinjeni uzorci odraslih jedinki brašnenih crva (po 5 g) sa pet različitih mesta. Insekti su stavljeni u sterilne bočice i transportovani do laboratorije u uslovima hladnog laca. Svi uzorci su ispitani u cilju entomološke identifikacije. Bakteriološka analiza je sprovedena kako bi se otkrilo eventualno prisustvo spoljašnjih i untutrašnjih bakterija, u skladu sa standardnim protokolima. Antimikrobna osetljivost svih izolata *Escherichia coli* ispitana je metodom difuzije diska na Mueller–Hinton agaru, korišćenjem komercijalnih diskova. Antimikrobna osetljivost nije ispitivana za ostale bakterije. Entomološka identifikacija je pokazala da svi uzorkovani insekti pripadaju vrsti *Alphitobius diaperinus*. Ukupno je izolovano 108 sojeva - 57 sa površine i 51 iz unutrašnjosti odraslih jedinki. Gram-negativne (63.89%) (*Escherichia coli*, *Enterobacter* spp., *Klebsiella pneumoniae*, *Proteus vulgarus*, *Citrobacter* spp., *Pseudomonas* spp., *Pseudomonas aeruginosa*) koagulaza-negativne stafilokoke (36,11%) (Gram-pozitivne), sa ukupnom dominacijom koagulaza-negativnih stafilokoka, zatim *Escherichia coli* (23,15%), *Pseudomonas* spp. (13,89%) i *Pseudomonas aeruginosa* (10,19%). Nije utvrđeno prisustvo *Salmonella* spp. Profil rezistencije na antibiotike pokazao je da su svi izolati *Escherichia coli* višestruko rezistentni. Visoka rezistencija je zabeležena na doksiciklin (25/25; 100%), amoksicilin (24/25; 96%), cefalotin (23/25; 92%), streptomicin (22/25; 88%), tetraciklin (22/25; 88%) i enrofloxacin (22/25; 88%). Jaka rezistencija je zabeležena i na trimetoprim-sulfametoksazol (13/25; 52%) i gentamicin (9/25; 36%). Međutim, niska rezistencija je detektovana za florfenikol (7/25; 28%) i kolistin (3/25; 12%). Rezltati ove studije potvrđuju potencijalnu ulogu brašnastih crva kao vektora u širenju bakterijskih patogena i rezistencije na antibiotike.

Ključne reči: Alphitobius diaperinus, brojleri, *Escherichia coli*, rezistentnost na antibiotike, Tunis

INTRODUCTION

The confinement systems used in industrial poultry farming have created an ideal environment for the proliferation of the mealworm (*Alphito-*

bius diaperinus), turning it into a global issue. In intensive chicken farming, these insects cause sanitary and economic problems, affecting the health and performance of the birds (Skewes and Monroe, 1991). *Alphitobius diaperinus* (Panzer) is a beetle from the Phylum Arthropoda, Insecta Class, Coleoptera Order and Tenebrionidae Family (Krinsky, 2002). Lesser mealworms are omnivorous scavengers that feed on manure, spilled chicken feed, cracked eggs, chicken carcasses, house fly maggots, and detritus (Rueda and Axtell, 1997). They are considered major contributors to the transmission of bacteria among the fauna within poultry house environments and have been confirmed as vectors of various disease agents, including pathogenic bacteria (*Escherichia coli*, *Salmonella* spp., *Clostridium* spp., *Staphylococcus* spp. etc) (McAllister et al., 1995, 1996; Goodwin and Waltman, 1996; Watson et al., 2000; Terra et al., 2023). Indeed, both larval and adult forms have been shown to harbor viable pathogens (*Salmonella* spp., *E. coli*, *Staphylococcus* spp.) on their exoskeleton and in their digestive tract (Donoso et al., 2020; Tamburro et al., 2022).

The darkling beetles can carry pathogenic strains of *E. coli* long enough to infect chicks, as adult insects excrete the bacteria in their feces for up to six days, and larvae for up to ten days. Additionally, *E. coli* was detected both on external surface and internal content (internal organs, intestinal content) in adult and larval stages persisting for as up to 12 days (McAllister et al., 1996). The ability of the living or dead insect to carry *Salmonella* Typhimurium and *E. coli* inside is also evident (Skov et al., 2004; Roche et al., 2009; Smith et al., 2021). Additionally, darkling beetles can act as reservoirs, sustaining *Salmonella* spp. within poultry farms both during and between production cycles (Roche et al., 2009; Barua et al., 2023). Moreover, it has been found that the darkling beetles (larvae and adults) can naturally acquire pathogenic bacteria such as *Staphylococcus* spp. (Tamburro et al., 2022). Similarly, it has been confirmed that *Aphitobius diaperinus* can harbor *Pseudomonas* spp. in the digestive tract of both larvae and adult insects (Cuccini et al., 2020).

Notably, the larvae of these insects have received approval for human consumption, further emphasizing their significance. On 4 July 2022, EFSA confirmed the safety of frozen and freeze-dried *Alphitobius diaperinus* larvae (EFSA, 2022).

The objective of this study is to investigate, for the first time in Tunisia, the external and internal bacterial carriage of adult mealworms collected from industrial broiler houses in the northeast of the country, with the aim of highlighting the role of this pest as a potential vector of poultry pathogens in these farms.

MATERIAL AND METHODS

Study site

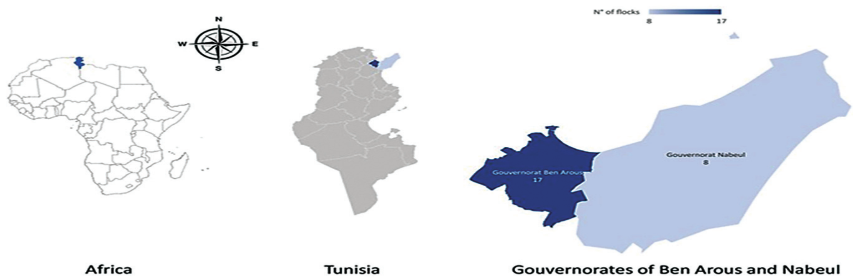

Figure 1. Location of sampled broiler flock

Our study was conducted on 25 poultry flocks located in the region of Nabeul (8 flocks) and Ben Arous (17 flocks) in the North-East of Tunisia (Figure 1), over the period of 6 months, between September 2018 and February 2019.

Nabeul (Coordinates: 36°27’15"N 10°44’5"E) is a coastal town located in northeastern Tunisia, on the south coast of the Cape Bon. It is known for its moderate climate. In January, the temperature reaches 8.4 °C on the minimum average, with the maximum average attaining 15.8 °C. Maximum temperature is 30,6 °C during August. In September, the minimum temperature is 20.9 °C while the maximum is 28.7 °C. Ben Arous (Coordinates: 36°44’50"N 10°20’0"E) is a city in north-eastern Tunisia, part the agglomeration of Tunis, also called "Grand Tunis". It is located south of Tunis city center. The climate is characterized by minimum temperatures recorded in January (7.2 °C) and maximum temperatures reaching up to 32.7 °C in August. In September, the minimum temperature is 19 °C while the maximum is 29.7 °C. These regions were classified as high concentrated poultry production zones (GIPAC, 2010 http://www.gipac.tn/en/).

Sample collection

Adult insects were collected from 25 broiler flocks that were naturally infested. In each poultry house, 5 g of adult darkling beetles were collected from the litter (wood shavings) in five different locations (1 g from each point) then pooled and conditioned in sterile wide mouth bottles and frozen. All the samples were collected using sterile equipment (gloves and anatomical forceps).

Entomological identification

All collected insects were identified entomologically based on morphological criteria described by Schawaller and Grimm (2014) and Nguyen et al. (2019).

Bacteriological analysis

All samples were transported to the laboratory under refrigerated conditions (coolerbox) (+4 °C). Bacteriological analysis was performed immediately. A sample of 1 g of darkling beetles per flock was prepared for bacterial detection. Both Gram-positive and Gram-negative bacteria were isolated from the external surface and internal parts of the insects (organs, digestive tract content) of the darkling beetles. The sampling from surface and interior of adult insects for bacteriological analysis was performed according to experimental design described by Crippen and Sheffield (2006). Five media were used for isolation of bacteria: Mac Conkey agar (Biokar-diagnostics; Beauvais, France) for *E. coli*, mannitol salt agar (Biokar-diagnostics; Beauvais, France) for *Staphylococcus* spp., Cetrimide selective agar (Biokar-diagnostics; Beauvais, France) for *Pseudomonas* spp., and Rappaport-Vassiliadis broth ((Biokar-diagnostics; Beauvais, France) and Xylose Lysine Deoxycholate agar (XLD) (Biokar-diagnostics; Beauvais, France) for *Salmonella* spp. and the classical biochemical gallery for identification (oxidase test, Kligler-Hajna agar, Mannitol-Motility agar, Simmons Citrate agar and urea indole medium) (Biokar-diagnostics; Beauvais, France). In case of identification difficulties, API 20E et API20 NE galleries (BioMérieux, Craponne, France) were used. The antimicrobial susceptibility test was performed using the disk method on Mueller–Hinton agar (Bio-Rad; Marnes-la Coquette, France).

Detection of bacteria from the surface of adult darkling beetles

For external carriage, 1 g of adult insects were sampled, placed in 10 mL of sterile buffered peptone water (BPW) and vortexed for 30 seconds to obtain the wash solution. The darkling beetles were removed with sterile anatomical forceps and placed in sterile bottle. Washing solution was incubated for 24 h at 37 °C for later bacterial isolation. For the isolation of *Salmonella* spp., 1 mL of washing solution was added to 9 mL of Rappaport-Vassiliadis enrichment broth incubated for 18-24 h at 37 °C. This enrichment broth was then inoculated on XLD agar. MacConkey agar was inoculated directly from the washing solution (0.1 mL) for the isolation of *E. coli*. For the *Staphylococcus* spp. isolation, mannitol salt agar was similarly inoculated (0.1 mL), while cetrimide agar was used for the detection of *Pseudomonas* spp. All these inoculated media were incubated for 18-24 h at 37 °C.

Detection of bacteria from the interior of adult darkling beetles

The same 1 g of adult insects used in the previous analysis was separately washed three times in 15 mL of sterile distilled water, then superficially disinfected by serial treatment with 95% ethanol (Manufacture) and hydrogen peroxide as described by Crippen and Sheffield (2006), after that they had been rinsed three times in 15 mL of sterile distilled water to remove any traces of disinfectants. Each sample was macerated and homogenized with a hand blender in 10 mL of sterile BPW. Dilutions from these main macerating solutions were generated as described previously. Bacterial isolation was then performed using previously described procedures for the external surface of the insects.

Bacterial identification

Bacterial colonies were identified using traditional Gram staining and biochemical techniques. To distinguish between coagulase-positive and coagulase-negative staphylococci, free coagulase detection was performed by mixing three to four staphylococcal colonies with rabbit plasma in a test tube (Bio-Rad; Marnes-la Coquette, France) (0.5 mL) and incubated for 4 h at 37 °C. These isolates are coagulase-positive staphylococci if the liquid solidifies and in case of non-coagulation, the tube is kept at room temperature overnight to avoid negative result.

Antibiotic resistance

Antimicrobial susceptibility was tested by the disk diffusion method on Mueller–Hinton agar using commercial disks for *E. coli* isolates. The plates were incubated for 24 h at 37°C, and inhibition zones were measured and interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2015). The following antimicrobials were used: amoxicillin 25 µg (AMX), cephalothin 30 µg (KF), gentamicin 10 µg (GEN), streptomycin 10 µg (STR), neomycin 30 µg (NEO), tetracycline 30 µg (TET), doxycycline 30 µg (DOX), florfenicol 30 µg (FFC), colistin 50 µg (CT), trimethoprim-sulfamethoxazole 1.25/23.75 µg (TMP-SMZ) and enrofloxacin 5 µg (ENF).

RESULTS

Entomological identification

Entomological identification was performed on all sampled adult darkling beetles. Morphological key characters of the head, thorax, abdomen and legs were described according to Schawaller and Grimm (2014) and Nguyen et al. (2019) (roughly body 5.5 to 6 mm long and widely oval in shape, black with reddish brown elytra, body surface is dotted with puncture-like impressions, antennae paler at the tips and covered in tiny, yellowish hairs, elytra with shallow longitudinal grooves). The results showed that all examined adult insects collected from broiler houses were compatible with *Alphitobius diaperinus* species.

Bacterial isolation

The present study showed that all sampled darkling beetles (from 25 broiler flocks) carried at least one type of bacteria on the body surface. In addition, 24 samples were positive for at least one type of bacteria detected in interior compartments of adult insects (Table 1). The results of identified pathogens showed the presence of a total of 108 isolates (Gram-positive: 39 isolates; Gram-negative: 69 isolates) detected from the surface and the interior of examined insects. A total of 57 isolates (52.78%) and 51 isolates (47.22%) were detected from the external parts and interior compartments of adult darkling beetles, respectively. The number of isolates per sample varied from one to three bacteria.

Table 1. Prevalence, type and number of bacteria isolated from the surface and the interior of darkling beetles collected from industrial broiler houses (n=25)

Site	Positive	Negative	Gram +	Gram -	1 bacterium	2 bacteria	3 bacteria
Surface	25	0	22	25	3	12	10
Interior	24	1	17	24	4	13	7

The identification of isolated bacteria revealed the presence of eight different species on the external surfaces of the whole samples, while seven distinct bacterial species were identified from the internal parts (organs and digestive tract contents) of the adult insects. Enterobacteriaceae were the most frequently isolated bacteria, accounting for 43 isolates (39.81%) and including *E. coli*, *Klebsiella pneumoniae*, *Citrobacter* spp., *Enterobacter* spp., and *Proteus vulgaris*. This was followed by *Staphylococcus* spp. with 39 isolates (36.11%) and Pseudomonadaceae with 26 isolates (24.08%) (Table 2). Out of Enterobacteriaceae isolates, *E. coli* was the most frequently detected (23.15%), followed by *Enterobacter* spp. (8.33%), *Klebsiella pneumoniae* (3.7%), *Proteus vulgaris* (2.78%) and *Citrobacter* spp. (1.85%). All *Klebsiella pneumoniae* isolates were detected only from surface of analyzed adult insects. Among Gram-negative isolates, *Pseudomonas* spp. was identified in 15 examined samples (13.89%), while *Pseudomonas aeruginosa* was detected in 11 samples (10.19%). *Staphylococcus* spp. was the only Gram-positive isolate in this study (36.11%) and all isolates were coagulase negative.

Type	Family	Species	Surface	Interior	Total
Gram – (63.89%)	Enterobacteriaceae (39.81%)	*E. coli*	10 (9.26%)	15 (13.89%)	25 (23.15%)
		Klebsiella pneumoniae	4 (3.7%)	0	4 (3.7%)
		Citrobacter spp.	1 (0.93%)	1 (0.93%)	2 (1.85%)
		Enterobacter spp.	4 (3.7%)	5 (4.63%)	9 (8.33%)
		Proteus vulgaris	1 (0.93%)	2 (1.85%)	3 (2.78%)
	Pseudomonadaceae (24.08%)	*Pseudomonas* spp.	7 (6.48%)	8 (7.41%)	15 (13.89%)
		Pseudomonas aeruginosa	8 (7.41%)	3 (2.78%)	11 (10.19%)
Gram + (36.11%)	Staphylococcaceae (36.11%)	Coagulase-negative staphylococci	22 (20.37%)	17 (15.74%)	39 (36.11%)
Total			57 (52.78%)	51 (47.22%)	108 (100%)

Table 2. Bacteria species isolated from the surface and the interior of darkling beetles collected from industrial broiler houses (n=108)

Antibiotic resistance

The susceptibility of the whole *E. coli* isolates from the surface (n=10) and interior (n=15) was tested for eleven antibiotics (Figure 2). The results indicate that all *E. coli* isolates detected from the surface of the adult insects were susceptible to colistin (10/10; 100%). However, all isolates proved to be resistant to doxycycline (25/25; 100%). Strong resistance to amoxicillin (24/25; 96%), cephalothin (23/25; 92%), streptomycin (22/25; 88%), tetracycline (22/25; 88%), enrofloxacin (22/25; 88%), trimethoprim-sulfamethoxazole (13/25; 52%) and gentamicin (9/25; 36%) was also observed. The *E. coli* isolates showed low percentages of resistance to florfenicol (7/25; 28%) and colistin (3/25; 12%). Moreover, 100% of the isolates proved to be multiresistant, showing resistance to at least three different classes of antibiotics.

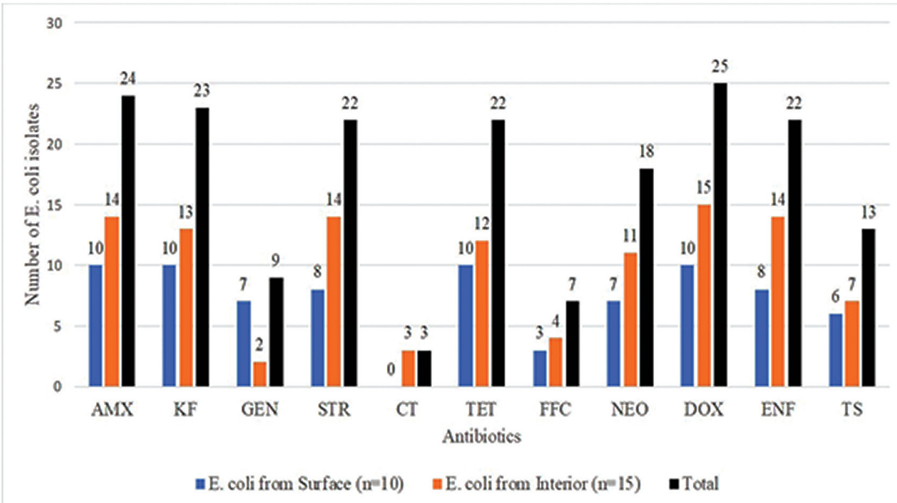

Figure 2. Antimicrobial resistance profiles of the *E. coli* isolates detected from the surface and the interior of the darkling beetles collected from broiler flocks (n=25)

DISCUSSION

Alphitobius diaperinus is a resilient pest commonly found in poultry houses. The field evidence suggests that this arthropod can survive cleaning and disinfection procedures, which represents a risk for its transmission between consecutive flocks (Crippen et al., 2018). Darkling beetles are regarded as either reservoir hosts or mechanical vectors for a variety of poultry pathogens (bacteria, viruses, fungi).

Routine biosecurity measures were implemented in poultry houses in Tunisia. Following insecticide treatment with deltamethrin and cypermethrin, manure and organic matter were removed. Sanitation procedures and disinfection protocols were carried out, and a downtime period of 7 to 14 days was observed. Despite these procedures, darkling beetles have not been eradicated and persist in consecutive flocks. Our findings are in agreement with other studies (Donoso et al., 2020).

All darkling beetles were collected from naturally infested broiler flocks. *Alphitobius diaperinus* was the only species identified in all sampled broiler flocks, which is consistent with findings from other studies conducted in Algeria (Agabou and Alloui, 2010) and Netherlands (Hazeleger et al., 2008). Several beetle species (*Carcinops pumilio*, *Alphitobius diaperinus*, *Gnathoncus* *nanus*, etc) have been reported to infest poultry houses, as mentioned in various reports (Pfeiffer and Axtell, 1980). Differences in findings can be explained by the studied geographical zone, the size of samples, the type of production, the type of used litter, the season, etc.

Both adult and larval stages of *Alphitobius diaperinus* serve as particularly effective reservoirs in broiler houses, as they inhabit the litter and manure where they feed on chicken feed, feces, and even carcasses - while also being consumed by the chickens themselves. Consequently, insects can be contaminated and/or spread pathogens. Darkling beetles have been identified as significant vectors for several pathogenic bacteria affecting poultry, including *E. coli*, *Campylobacter*, and *Salmonella* Enteritidis (Donoso et al., 2020; Barua et al., 2023). They can also be reservoirs for some viral diseases like leukosis, Marek's disease, infectious bursal disease, avian influenza, Newcastle disease etc. and also protozoa agents of coccidiosis such as *Eimeria* spp. (Goodwin and Waltman, 1996).

The aim of the surface disinfection after analyzing the external parts of the darkling beetles was to eliminate any surface flora, in order to differentiate bacteria carried externally or internally. The insect integument and presumably the fecal material adhering to the exoskeleton provide refuge to bacterial organisms (Crippen and Sheffield, 2006).

In the present study, 108 isolates were obtained from the surface and the interior of adult darkling beetles. The isolates were identified based on the macroscopic (colony) and microscopic characteristics and biochemical reactions. Microscopic examination of the isolates after Gram staining showed that Gram-negative (69 isolates; 63.89%) were predominant compared to Gram-positive (39 isolates; 36.11%).

Enterobacteriaceae (43 isolates; 39.81%) were the most prevalent isolates in our study, which is in agreement with similar reports (Segabinazi et al., 2005). Among these isolates, *E. coli* was detected in 25 samples (23.15%). In fact, *E. coli* is a ubiquitous and one of the most frequent pathogens in poultry. Several studies, including one by Tayh et al. (2024) conducted in Tunisia, have demonstrated that broiler chickens can act as reservoirs for *E. coli* strains pathogenic to humans, specifically Shiga toxin-producing *E. coli* (STEC), which are capable of causing severe and sometimes fatal hemolytic-uremic syndrome (HUS). Contrary to other reports (Hazeleger et al., 2008; Donoso et al., 2020), we did not isolate *Salmonella* spp. in our study. Lower frequency of other bacteria isolates in sampled beetles may be explained by a competitive exclusion phenomenon, that characterizes the transmission and colonization in poultry and inside the internal organs, in particularly, the gut of *Alphitobius diaperinus*

(Wynants et al., 2018) or by antimicrobial administration. Additionally, litter water activity and pH are considered key factors that promote the growth and multiplication of various important bacteria in poultry farming (Gehring et al., 2020).

Secondly, coagulase-negative staphylococci (36.11%) and *Pseudomonas* spp. (24.08%) were identified on both the surface and interior of adult insects . Beetles carrying bacteria on their exoskeleton or in their guts (Cuccini et al., 2020) can transmit those pathogens, especially to young chicks (McAllister et al., 1996). Furthermore, contaminated darkling beetles can preserve pathogens in their gut for more than five weeks (Crippen et al., 2022).

Pseudomonas aeruginosa (10.19%) is considered as an emerging pathogen in poultry. It is a leading cause of respiratory infection, sinusitis, endocarditis and septicemia (Badr et al., 2020) and it is frequently multiresistant to antibiotics (Atere and Atere, 2024).

Coagulase-negative staphylococci (36.11%) was the only Gram-positive bacterium isolated in the present study, consistent with the findings of Goodwin and Waltman (1996). This pathogen is known to cause osteoarticular infections in poultry (Andreasen, 2020).

In general, antibiotic susceptibility of *E. coli* isolated from darkling beetles showed high to very high resistance to most tested antibiotics (28% to 100%) (doxycycline, amoxicillin, cephalothin, streptomycin, tetracycline, enrofloxacin, trimethoprim-sulfamethoxazole, gentamicin, florfenicol). Several risk factors may contribute to development of antibiotic resistance. The inappropriate use of antimicrobials, whether for treatment or prevention of bacterial infections, is considered the most significant risk factor. Darkling beetles can ingest antibiotics by feeding on contaminated feces and manure (Rawat et al., 2023). Colistin resistance has been detected in three *E. coli* isolates (12%). Commonly used in poultry production in several countries, colistin (polymyxin E) is frequently used in Tunisian poultry sector for the treatment of infection caused by Enterobacteriaceae. Colistin resistance of *E. coli* detected in healthy and diseased chickens is on the rise in Tunisia (Grami et al., 2016; Maamar et al., 2018; Hassen et al., 2020; Di Francesco et al., 2023). Our results confirm the potential vector role of beetles in the spread of colistin resistance in poultry flocks in Tunisia.

Finally, it should be noted that all *E. coli* isolates were multidrug-resistant. Indeed, each bacterium isolate accumulated resistance to several antibiotics of different families, which can lead to therapeutic impasses in poultry and public health. In this way, darkling beetles can carry multidrug-resistant zoonotic bacteria, such as Salmonella enterica strains, complicating efforts to control infections in poultry environment and humans (Donoso et al., 2020).

It should be noted that the present study has certain limitations. All samples were collected exclusively from adult insects, although darkling beetle larvae are also recognized as potential vectors of poultry pathogens. In addition, *Campylobacter* spp., a major poultry public health pathogen, was not targeted in our study due to lack of specific media. A serotyping of *E. coli* isolates was not performed because of lack of antisera for the main pathogen serotypes.

CONCLUSION

In conclusion, *Alphitobius diaperinus* was the only darkling beetle species identified in selected industrial broiler houses. A total of 108 bacterial isolates were detected from the surface (57 isolates) and the interior (51 isolates) of adult insects. Gram-negative (Enterobacteriaceae and Pseudomonadaceae) and *Staphylococcus* spp. were isolated, with the predominance of coagulase-negative staphylococci, *E. coli* and *Pseudomonas aeruginosa*. The antibiotic resistance profile of *E. coli* isolates confirms the growing concern of this issue within the Tunisian poultry sector and further supports the potential role of darkling beetles as vectors in the dissemination of bacterial pathogens and antibiotic resistance. Consequently, more drastic measures must be taken in order to rationalize the use of antibiotics in chickens and limit the proliferation of mealworms in farms.

Abbreviations

AMX: amoxicillin; BPW: buffered peptone water; CLSI: Clinical and Laboratory Standards Institute guidelines; CT: colistin; DOX: doxycycline; ENF: enrofloxacin; FFC: florfenicol; GEN: gentamicin; KF: cephalothin; NEO: neomycin; STR: streptomycin; TET: tetracycline; TMP-SMZ: trimethoprim-sulfamethoxazole; XLD: Xylose Lysine Deoxycholate agar.

Author's Contribution

KK: Conceptualization, methodology, results interpretation, writing original draft; MG: sampling, bacteriological analysis, writing; CC: sampling; LM: writing-review, bacteriological analysis supervision and validation. All authors have read and approved the final version of the manuscript for publication.

Competing interest

The authors declare that they have no conflicts of interest.

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GLOBAL RESEARCH TRENDS IN FLORFENICOL: A FOUR-DECADE BIBLIOMETRIC ANALYSIS OF SCIENTIFIC EVOLUTION FROM VETERINARY THERAPEUTICS TO ENVIRONMENTAL AND RESISTANCE CONCERNS

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Abstract

Florfenicol, a broad-spectrum antibiotic extensively used in veterinary medicine, has gained significant attention in recent research because of growing concerns about antimicrobial resistance and its environmental impacts, particularly within the context of the One Health approach. A comprehensive bibliometric analysis of 972 florfenicol publications indexed in Scopus (1986-2025) was conducted using Bibliometrix R package and VOSviewer. Multiple indicators were assessed including annual production trends, journal distribution, author productivity, institutional affiliations, international collaborations, citation patterns, and thematic evolution through keyword analysis. Florfenicol research demonstrated remarkable growth with a 10.55% annual increase, evolving through four distinct phases from minimal activity (1986-1999) to exponential expansion (2018-2025). China dominated with 401 corresponding author publications (41.3%), followed by the USA (84 articles, 8.6%). Strong international collaboration was observed (17.6% multi-country publications), particularly between China and the USA (32 joint studies). Thematic evolution shifted from basic pharmacokinetics and residue studies to contemporary

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focus on antimicrobial resistance genes, impact on the environment, and advanced analytical methods. Highly cited studies emphasized resistance mechanisms and environmental dissemination. Florfenicol research has transformed from a niche veterinary topic into a multidisciplinary domain addressing global antimicrobial resistance challenges. The field demonstrates strong international collaboration but reveals geographic disparities requiring attention. Future research should prioritize prevention strategies over detection methods while establishing networks linking high-output countries with underrepresented regions for comprehensive global resistance surveillance.

Key words: Bibliometric analysis, antibiotics, resistance to antibiotics, veterinary medicine, florfenicol, research data

GLOBALNI TRENDOVI U ISPITIVANJU FLORFENIKOLA: BIBLIOMETRIJSKA ANALIZA NAUČNE EVOLUCIJE DUGE 4 DECENIJE- OD TERAPIJE U VETERINI DO ANTIMIKROBNE REZISTENCIJE

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Kratak sadržaj

Florfenikol, antibiotik širokog spektra koji se intenzivno koristi u veterinarskoj medicini, privukao je značajnu pažnju u novijim istraživanjima zbog sve veće zabrinutosti u vezi sa antimikrobnom rezistencijom i njegovim uticajem na životnu sredinu, naročito u kontekstu pristupa "Jedno zdravlje". Sprovedena je sveobuhvatna bibliometrijska analiza 972 publikacije o florfenikolu indeksirane u bazi Scopus (1986–2025), putem Bibliometrix-a u programu R i softvera VOSviewer. Procenjivani su brojni pokazatelji, uključujući godišnje trendove publikovanja, distribuciju časopisa, produktivnost autora, institucionalne afilijacije, međunarodne kolaboracije, obrasce citiranja i tematsku evoluciju putem analize ključnih reči. Istraživanja o florfenikolu pokazala su izuzetan rast sa prosečnim godišnjim povećanjem od 10,55%. Rast je tekao kroz četiri jasno definisane faze - od minimalne aktivnosti (1986–1999) do eksponencijalne ekspanzije (2018–2025). Kina je dominirala sa 401 publikacijom kao i autorima iz te zemlje (41,3%), a sledile su Sjedinjene Američke Države sa 84 rada (8,6%). Uočen je visok nivo međunarodne saradnje (17,6% publikacija sa više zemalja), naročito između Kine i SAD (32 zajednička istraživanja). U smislu tematskih promena, fokus se pomerio sa osnovnih studija farmakokinetike i ostataka leka ka genima antimikrobne rezistencije, uticaja na životnu sredinu i napredne analitičke metode. Najcitiranije studije isticale su mehanizme rezistencije i širenje u životnoj sredini. Istraživanja o florfenikolu promenila su kurs od usko specijalizovanih veterinarskih tema u multidisciplinarnu oblast koja se bavi globalnim izazovima antimikrobne rezistencije. U ovoj oblasti je prisutna izržena međunarodna saradnja, ali i geografske neujednačenosti kojima se treba baviti u budućnosti. Prioritet budućih istraživanja trebalo bi da budu strategije prevencije u odnosu na metode detekcije, uz uspostavljanje mreža koje povezuju zemlje sa visokim istraživačkim učinkom i nedovoljno zastupljene regione radi sveobuhvatnog globalnog praćenja antimikrobne rezistencije.

Ključne reči: Bibliometrička analiza, antibiotici, atpornost na antibiotike, veterinarska medicina, istraživački podaci

INTRODUCTION

Florfenicol is a broad-spectrum, synthetic antibiotic belonging to the amphenicol class, structurally related to chloramphenicol and thiamphenicol (Lunestad and Samuelsen, 2008, Trif et al., 2023a). Since its development in the late 20th century, it has been widely used in veterinary medicine (Trif et al., 2023a), particularly in livestock and aquaculture (Lunestad and Samuelsen, 2008), due to its effectiveness against Gram-positive and Gram-negative bacteria (Trif et al., 2023a), favorable pharmacokinetic properties (Huynh et al., 2025), and comparatively lower risk of resistance compared with other antimicrobials in the same class (Trif et al., 2023b). Its approval for use in food-producing animals was driven largely by the need for an alternative to chloramphenicol (Adesoji and Call, 2020, Dowling, 2013), which was banned in many countries owing to safety concerns in humans (Hanekamp and Bast, 2015), particularly for the risk of aplastic anemia in humans (Dowling, 2013). Florfenicol's relative safety profile, coupled with its strong antibacterial activity, made it an attractive therapeutic option in managing respiratory and enteric infections in cattle, swine, poultry, and fish (Trif et al., 2023a).

Despite its benefits, the use of florfenicol has also prompted significant scientific and public health concerns. The emergence of antimicrobial resistance (AMR) linked to florfenicol exposure has been increasingly reported in both clinical and environmental isolates, particularly in aquaculture systems where the drug is extensively applied (Miranda et al., 2013, Nüesch-Inderbinen et al., 2023). Genes such as *floR*, conferring florfenicol resistance, have been detected in diverse bacterial species and mobile genetic elements, heightening the risk of horizontal transfer and global dissemination (Lu et al., 2018). This has placed florfenicol at the intersection of veterinary practice, food safety, environmental microbiology, and the One Health framework. Consequently, the scientific literature on florfenicol has expanded to cover diverse themes, including its pharmacology (Selimov et al., 2023), resistance mechanisms (Müller et al., 2022), clinical applications (Watteyn et al., 2013), toxicology (Wang et al., 2021), environmental studies (Zhao et al., 2024).

Bibliometric analysis is a quantitative study of research publications in certain fields using mathematical and statistical methods. It provides valuable information about the research trends, evolving topics and quantitative analysis of citations, authors, institutes, countries contributions and thematic maps (Darvish, 2020, van Eck and Waltman, 2010, Kandeel and Mahmoud, 2025, Alsaleem et al., 2025). Bibliometric analysis is a valuable tool for mapping and evaluating scientific knowledge. Bibliometric analysis of florfenicol research can offer valuable insights into research growth, global contributions, and thematic shifts related to its clinical use, resistance, and regulatory challenges. Although bibliometric analyses have explored antibiotics, antimicrobial resistance (Sun et al., 2022), and veterinary pharmaceuticals in general (Arshad et al., 2020), a comprehensive assessment dedicated exclusively to florfenicol has not yet been conducted. Considering florfenicol's pivotal role in animal health and its significance within ongoing antibiotic stewardship discussions, undertaking such an assessment is both timely and recommended.

This study therefore conducts a bibliometric analysis of global florfenicol literature using data from Scopus database. The objectives are the following: (1) mapping research trends; (2) identifying leading authors, institutions, journals, and countries; and (3) exploring thematic evolution and emerging hotspots, particularly in relation to use, resistance, and One Health implications.

Through this approach, we aim to clarify how florfenicol research has developed, where it stands today, and potential directions for future investigation.

MATERIAL AND METHODS

Study Design

This study employed a bibliometric approach to deliver a comprehensive overview of global research on florfenicol.

Data Source and Search Strategy

The data were retrieved from the Scopus database, selected for its broad coverage of peer-reviewed journals across scientific disciplines and its compatibility with bibliometric tools. A topic search was conducted using the keyword "florfenicol". The search period extended from inception through August 2025, covering nearly four decades of research activity. To ensure data quality and relevance, only original research articles were included, while reviews, conference proceedings, editorials, and letters were excluded. Only articles published in English were included to minimize the risk of misinterpretation from other languages. The search query in Scopus included the following: (TITLE (Florfenicol) AND (LIMIT-TO (LANGUAGE,"English")) AND (LIMIT-TO (DOCTYPE,"ar")) AND (EXCLUDE (PREFNAMEAUID, "undefined"))).

Data Extraction and Processing

All bibliographic records were exported from Scopus in RIS and CSV formats with complete metadata fields, including author names, titles, abstracts, keywords, affiliations, journals, references, and citation counts. The dataset was then imported into the Bibliometrix R package (version 4.2.0) via the Biblioshiny interface for cleaning and processing (Darvish, 2020).

Bibliometric Indicators

Multiple bibliometric indicators were assessed to characterize the research landscape. Annual scientific production was examined to track trends in publication output. Journal-level analysis was conducted to identify the most influential publication sources. Author productivity and impact were evaluated through total publication counts and fractionalized authorship contributions.

Institutional and country-level productivity was analyzed based on corresponding author affiliations, distinguishing between single-country publications (SCP) and multi-country publications (MCP). Citation analysis identified the most influential studies, considering both total citations and normalized citation counts to account for differences in publication year.

Analytical Tools and Visualization

Bibliometric and network analyses were performed using Bibliometrix (Darvish, 2020) and VOSviewer (version 1.6.20) (van Eck and Waltman, 2010). These tools enabled the construction of co-authorship networks, international collaboration maps, and keyword co-occurrence clusters. Keyword analysis was applied to trace the chronological evolution of research focus and detect emerging topics. Visualization of results was carried out using Bibliometrix, VOSviewer, and Microsoft Excel, generating figures such as annual publication trends, institutional and journal distributions, international collaboration networks, and keyword evolution maps.

RESULTS

The bibliometric dataset comprised 972 publications on florfenicol indexed in Scopus between 1986 and 2025, originating from 367 different sources. Overall, scientific output demonstrated an annual growth rate of 10.55%, with documents averaging 8.87 years of age and 23.26 citations per article. The dataset included 31,854 references, 1,993 author keywords, and 6,748 Keywords Plus, reflecting broad thematic coverage. A total of 3,356 authors contributed, with a strong collaborative pattern averaging 6.14 co-authors per paper and an international co-authorship rate of 17.6%.

Annual Scientific Production and Trends

The bibliometric analysis revealed a remarkable evolution in florfenicol research over four decades, with scientific production growing from a single publication in 1986 to 94 articles in 2024. The distribution demonstrates four distinct phases of research development. The emergence phase (1986-1999) showed minimal activity. The steady growth phase (2000-2012) witnessed consistent expansion with 216 articles, marking the beginning of sustained research interest. The acceleration phase (2013-2017) produced 174 articles in just five years, indicating growing recognition of florfenicol's importance. The exponential expansion phase (2018-2025) generated 539 articles, representing more than half of total publications in merely eight years. A marked growth spurt occurred after 2018, with annual publications consistently surpassing 40 articles. The year 2024 marked the peak with 94 publications, while 2025 already shows 50 articles by mid-year (Figure 1).

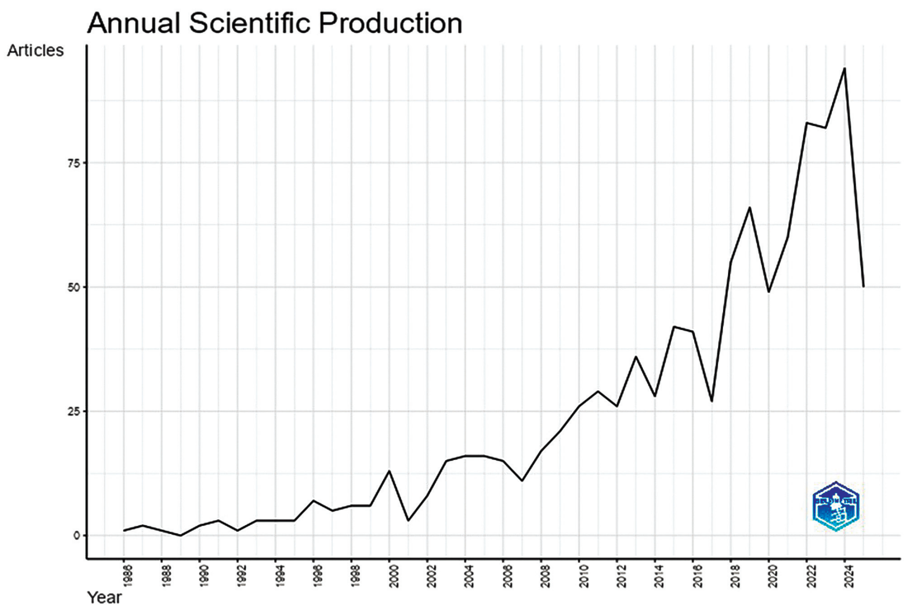

Figure 1. Annual scientific production of florfenicol research (1986–2025).

Journal Distribution and Publication Venues

Florfenicol research exhibits notable interdisciplinary scope, with the *Journal of Veterinary Pharmacology and Therapeutics* leading the field with 61 publications (6.3% of total output). Aquaculture journals contributed significantly with 49 articles (Aquaculture: 38, Aquaculture Research: 11), reflecting the compound's extensive use in fish farming. Environmental science publications appeared in Ecotoxicology and Environmental Safety (22), Science of the Total Environment (14), and Environmental Science and Pollution Research (10). Analytical chemistry studies were published in the Journal of Chromatography B (13), Journal of AOAC International (14), and Chemical Engineering Journal (14). Veterinary medicine research featured in the American Journal of Veterinary Research (11) and Frontiers in Veterinary Science (10). Microbiology and resistance studies were represented by Antimicrobial Agents and Chemotherapy (15) and Frontiers in Microbiology (11) (Table 1).

Table 1. Most relevant journals publishing florfenicol research

Sources	Articles
JOURNAL OF VETERINARY PHARMACOLOGY AND THERAPEUTICS	61
AQUACULTURE	38
ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY	22
JOURNAL OF FISH DISEASES	19
ANTIMICROBIAL AGENTS AND CHEMOTHERAPY	15
JOURNAL OF AQUATIC ANIMAL HEALTH	15
ANTIBIOTICS	14
CHEMICAL ENGINEERING JOURNAL	14
JOURNAL OF AOAC INTERNATIONAL	14
SCIENCE OF THE TOTAL ENVIRONMENT	14
JOURNAL OF CHROMATOGRAPHY B: ANALYTICAL TECHNOLOGIES IN THE BIOMEDICAL AND LIFE SCIENCES	13
POULTRY SCIENCE	12
AMERICAN JOURNAL OF VETERINARY RESEARCH	11
AQUACULTURE RESEARCH	11
FRONTIERS IN MICROBIOLOGY	11

Author Productivity and Collaboration Patterns

Author analysis reveals concentrated expertise among leading researchers. Li J and Liu Y emerged as the most productive authors with 33 articles each, though their fractionalized contributions differ significantly (5.54 and 4.16 respectively), indicating varying collaboration intensities. Wang X contributed 31 articles with 4.19 fractionalized publications, while Li X and Zhang Y each published 30 articles. Schwarz S stands out with the highest fractionalized contribution (6.43) from 23 total articles, demonstrating lower number of coauthors (Table 2).

Table 2. Top author productivity and impact analysis in florfenicol research

Authors	Articles	Articles Fractionalized
LI J	33	5.536544012
LIU Y	33	4.157669128
WANG X	31	4.189357864
LI X	30	4.023445019
ZHANG Y	30	3.898004202
WANG Y	26	3.399473158
LI Y	23	3.361446886
SCHWARZ S	23	6.431385281
ZHANG L	20	2.832178932

Institutional Landscape and Research Centers

Institutional analysis indicates pronounced geographical clustering, with Chinese universities leading the output of florfenicol research. China Agricultural University leads with 146 articles, followed by South China Agricultural University with 126 articles. Yangzhou University contributed 89 articles, and Wenzhou Medical University produced 84, reflecting a medical research perspective. In contrast, China Agricultural University (146), South China Agricultural University (126), Huazhong Agricultural University (78), Sichuan Agricultural University (70), Hebei Agricultural University (66), and Nanjing Agricultural University (63) highlight the compound's focus on agricultural and veterinary applications (Table 3).

Table 3. Top institutional affiliations and research centers in florfenicol research

Affiliation	Articles
CHINA AGRICULTURAL UNIVERSITY	146
SOUTH CHINA AGRICULTURAL UNIVERSITY	126
YANGZHOU UNIVERSITY	89
WENZHOU MEDICAL UNIVERSITY	84
HUAZHONG AGRICULTURAL UNIVERSITY	78
DONGGUAN UNIVERSITY OF TECHNOLOGY	73

Affiliation	**Articles**
SICHUAN AGRICULTURAL UNIVERSITY	70
HEBEI AGRICULTURAL UNIVERSITY	66
NATIONAL CHUNG HSING UNIVERSITY	66
GHENT UNIVERSITY	64
NANJING AGRICULTURAL UNIVERSITY	63
JILIN UNIVERSITY	59
UNIVERSIDAD DE CONCEPCIÓN	58

Three field plots also indicate that China clearly leads the field, with top institutions such as China Agricultural University, Wenzhou Medical University, and Sichuan University of China contributing most of publications. Their work is strongly tied to topics including florfenicol, pharmacokinetics, antimicrobial resistance, and oxidative stress (Figure 2A).

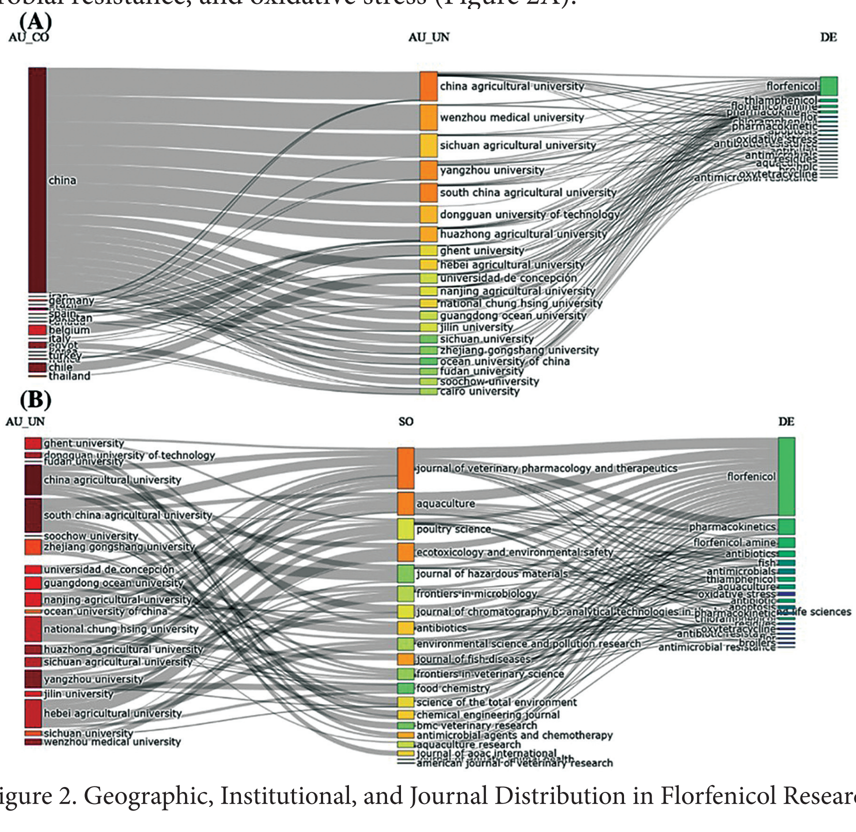

Figure 2. Geographic, Institutional, and Journal Distribution in Florfenicol Research.

Institutional output aligns closely with specific journals - particularly the Journal of Veterinary Pharmacology and Therapeutics, Aquaculture, Poultry Science and Ecotoxicology and Environmental Safety - reflecting a strong focus on veterinary and aquatic applications. The most frequent keywords, including florfenicol, pharmacokinetics, florfenicol amine, antibiotics, and tissue distribution, highlight the thematic emphasis on drug metabolism, resistance, and environmental safety (Figure 2B).

Global Research Distribution, Country Contributions, and Collaboration Map

Florfenicol research demonstrates a strong geographical concentration, with China leading as the corresponding author country, contributing 401 articles (41.3% of global output) (Figure 3). These findings confirm China's clear leadership in florfenicol research, with 345 single-country publications (86.0%) and 56 multi-country collaborations (14.0%). The United States ranks second, contributing 84 articles (8.6%) as corresponding author and demonstrating a greater inclination toward international collaboration, with 19.0% of its publications involving multiple countries. Chile follows in third with 32 articles (3.3%), closely followed by Egypt (29 articles, 2.9%) and Brazil (26 articles, 2.7%).

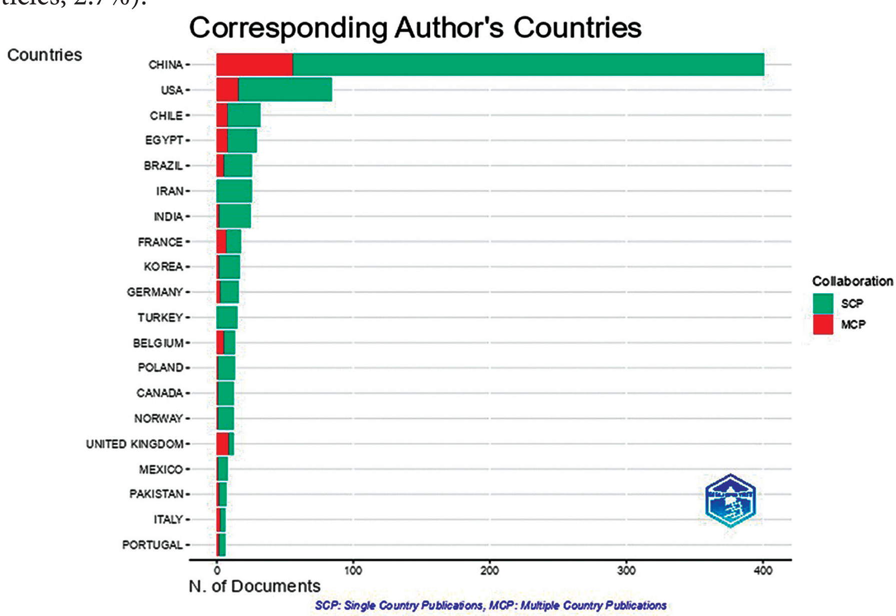

Figure 3. Corresponding author countries for florfenicol research (1986–2025).

The global research landscape on florfenicol demonstrates extensive international collaboration, with the United States and China emerging as the central hubs (Figure 4 and Table 4). China shows the strongest partnerships with the USA (32 studies), Thailand (9), Pakistan and Egypt (5 each), and Germany (4). Similarly, the USA maintains strong collaborations with Canada (8), the United Kingdom (10), and Norway (5).

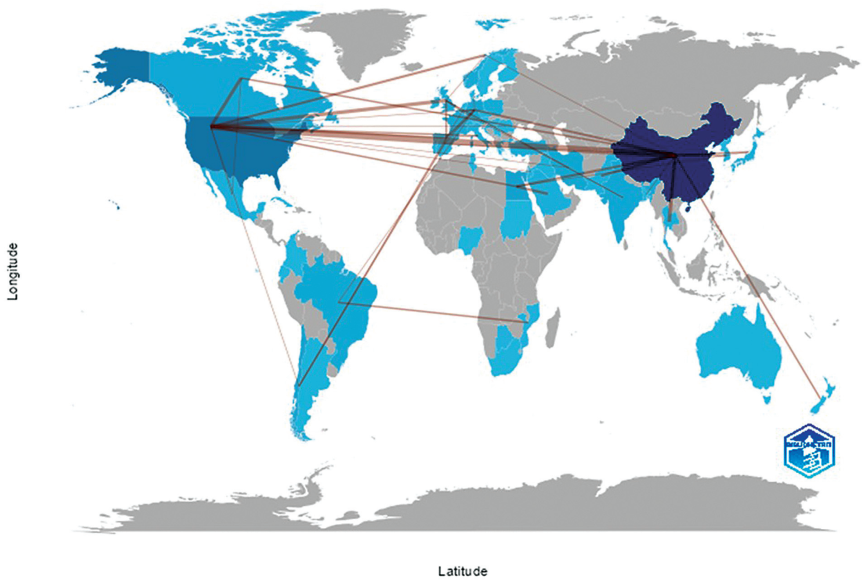

Figure 4. Country Scientific Production and Global Collaborations in Florfenicol Research.

Table 4. The top scores in international collaboration in florfenicol research

From	To	Articles
CHINA	USA	32
USA	UNITED KINGDOM	10
CHINA	THAILAND	9
USA	CANADA	8
EGYPT	SAUDI ARABIA	6
CHINA	EGYPT	5

From	To	Articles
CHINA	PAKISTAN	5
FRANCE	GERMANY	5
USA	NORWAY	5
CHINA	GERMANY	4
BELGIUM	GERMANY	3
BRAZIL	MOZAMBIQUE	3
CHILE	SPAIN	3
CHINA	CANADA	3
CHINA	JAPAN	3
CHINA	KOREA	3
CHINA	NEW ZEALAND	3
UNITED KINGDOM	BELGIUM	3
UNITED KINGDOM	FRANCE	3
USA	EGYPT	3

Most global cited studies

The distribution of florfenicol research by authors' nationality highlights a clear dominance of China, with 2,966 citations, surpassing other countries by far. The United States follows with 697 citations, while Chile (217), Egypt (170), and India (170) represent notable contributors from South America, Africa, and Asia. Brazil (152), South Korea (115), and Iran (108) also demonstrate strong regional outputs. European influence is reflected through the UK (98), Belgium (93), France (89), Germany (78), and Poland (77), underscoring diverse international participation (Figure 4).

The most highly cited studies on florfenicol span microbiology, antimicrobial resistance, and environmental impacts (Table 5).

Table 5. Most global cited studies in Florfenicol Research

Paper	DOI	Total Citations	TC per Year	Normalized TC
KEHRENBERG C, 2006, ANTIMICROBIAL AGENTS CHEMOTHER	10.1128/AAC.50.4.1156-1163.2006	368	18.4	6.773006135
SCHWARZ S, 2000, ANTIMICROBIAL AGENTS CHEMOTHER	10.1128/AAC.44.9.2530-2533.2000	302	11.61538462	3.508489723
CAO Z, 2017, ENVIRON SCI TECHNOL	10.1021/acs.est.7b02480	290	32.22222222	7.925101215
KEHRENBERG C, 2005, MOL MICROBIOL	10.1111/j.1365-2958.2005.04754.x	288	13.71428571	4.992416035
JIANG W-L, 2018, ENVIRON SCI TECHNOL	10.1021/acs.est.8b01894	240	30	8.499678042
GUILHERMINO L, 2018, SCI TOTAL ENVIRON	10.1016/j.scitotenv.2017.12.020	217	27.125	7.685125563
WHITE DG, 2000, J CLIN MICROBIOL	10.1128/jcm.38.12.4593-4598.2000	182	7	2.114387846
MITCHELL SM, 2013, BIORESOUR TECHNOL	10.1016/j.biortech.2013.09.048	173	13.30769231	6.12992126

Kehrenberg et al. 2006 (368 citations) investigated the genetic basis of florfenicol resistance in bacterial isolates (Kehrenberg and Schwarz, 2006). Schwarz et al. 2000 (302 citations) examined resistance mechanisms (Schwarz et al., 2000). Cao et al. 2017 (290 citations) explored the environmental dissemination of florfenicol residues and associated resistance genes (Cao et al., 2017). Kehrenberg et al. 2005 (288 citations) focused on molecular pathways underlying resistance development (Kehrenberg et al., 2005). Jiang et al. 2018 (240 citations) analyzed wastewater treatment as a potential source of resistance spread (Jiang et al., 2018). White et al. 2000 (182 citations) reported resistant bacteria in clinical samples (White et al., 2000). Mitchell et al. 2013 (173 citations) evaluated treatment technologies to decrease environmental contamination (Mitchell et al., 2013).

Research Evolution and Emerging Trends

Trend analysis reveals significant thematic evolution from basic compound characterization to complex multidisciplinary investigations (Figure 5). Early research (2006-2012) focused on fundamental aspects: floR gene studies (median year: 2007), residue depletion (2012), and analytical methods like ELISA (2014). The intermediate period (2013-2019) expanded to therapeutic applications with pharmacokinetic studies (median: 2018), species-specific investigations in rainbow trout (2015) and tilapia (2016), and resistance mechanisms. Contemporary research (2020-2025) emphasizes critical concerns: antibiotic resistance genes (median: 2023), environmental applications including adsorption studies (2024), and advanced materials like molecularly imprinted polymers (2023). Compound-specific terms show sustained growth: florfenicol (533 total occurrences, median: 2019), florfenicol amine (56 occurrences), and thiamphenicol (38 occurrences). Methodological evolution progresses from basic HPLC (median: 2016) to sophisticated LC-MS/MS techniques (2017). Recent emergence of terms like "inflammation" (2024), "egg" (2024), and "pig" (2023) indicates expanding therapeutic applications and food safety concerns.

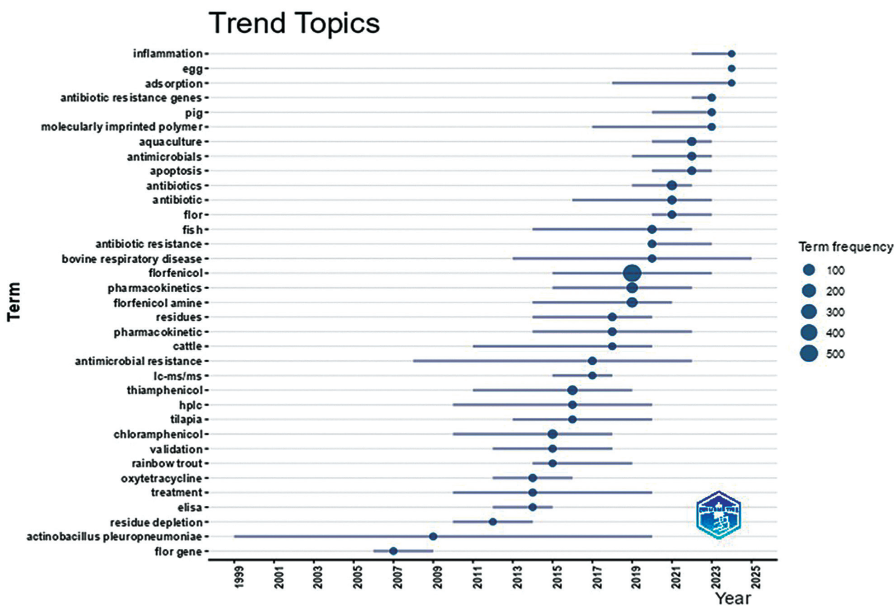

Figure 5. Trend Topics in Florfenicol Research.

Centrality analysis

Centrality analysis provides deeper insight into how research topics on florfenicol are interconnected within the scientific network. The "florfenicol" cluster stands out with the highest betweenness centrality (7632.9) and PageRank score (0.198), establishing it as the central hub connecting multiple thematic domains. Within this cluster, co-occurring terms such as *pharmacokinetics*, *florfenicol amine*, *thiamphenicol*, and *chloramphenicol* also show high centrality, reflecting their strong influence in bridging experimental, clinical, and residue-focused studies. The "antibiotics" cluster also exhibits substantial centrality, with *antibiotics* (2448.6) and *aquaculture* (1930.2) acting as major connectors, particularly in relation to fish and livestock health. Meanwhile, the "antimicrobial resistance" cluster contributes key bridging nodes - *antibiotic resistance* (826.5), *E. coli* (245.7), and *horizontal gene transfer* (410.4) - highlighting its pivotal role in connecting florfenicol research with broader public health concerns. (Supplementary file 1.).

Keyword map

The Keywords related to florfenicol research are presented in Figure 6A shows the overlay visualization, where keywords are displayed in gradient colours. Older research topics are represented in blue, whereas the most recent themes appear in yellow. Figure 6B shows clustering of these topics, where each group of related keywords in a certain field are displayed in the same colour. This bibliometric keyword map illustrates the temporal evolution of florfenicol research, with colors representing the average publication year linked to each keyword. Dark blue and green nodes denote topics predominant in earlier years (2014–2017), such as "solubility," "distribution," and "rainbow trout," highlighting foundational studies on drug properties and aquatic applications. Keywords shifting to green and yellow - such as *antibiotic resistance genes* and *nanoparticles* - are associated with studies published in more recent years (2020–2022), reflecting a shift toward emerging themes including antimicrobial resistance, advanced therapeutic strategies, and broader public health relevance.

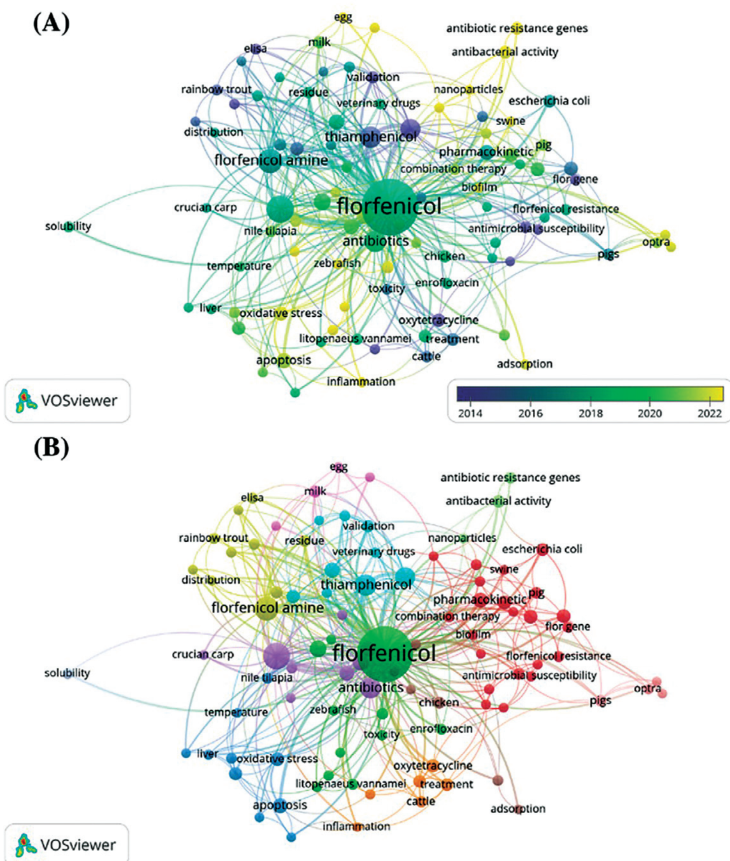

Figure 6. Keywords map in Florfenicol Research.

DISCUSSION

Our bibliometric analysis reveals that florfenicol research has transformed from a niche veterinary topic into a multifaceted scientific domain spanning four decades. The remarkable 10.55% annual growth rate, culminating in 94 publications in 2024, reflects growing global awareness of antimicrobial resistance challenges and the environmental footprint of veterinary antibiotics. This exponential expansion, particularly post-2018, coincides with international policy initiatives addressing antimicrobial resistance and the United Nations' sustainable development goals targeting responsible antibiotic use (Aslam et al., 2024).

China's dominance, contributing 401 articles as corresponding author country, arises from its position as the world's largest aquaculture producer and significant livestock industry (Zhao et al., 2021). Chinese institutions have invested heavily in veterinary pharmaceutical research, creating specialized research centers that bridge agricultural universities and medical institutions.

The interdisciplinary spread across 367 sources demonstrates florfenicol's relevance beyond veterinary medicine. Environmental science journals' strong representation (Science of the Total Environment, Ecotoxicology and Environmental Safety) signals recognition that veterinary antibiotics represent emerging environmental contaminants. Similarly, analytical chemistry journals' prominence reflects the technical challenges of detecting florfenicol residues at increasingly stringent regulatory limits. This disciplinary diversity, averaging 6.14 co-authors per paper, suggests that addressing florfenicol-related challenges requires collaborative expertise spanning microbiology, chemistry, ecology, and veterinary science.

The evolution of florfenicol research exemplifies the One Health concept, recognizing interconnections between animal health, human health, and environmental integrity (Pitt and Gunn, 2024). Initially developed as a safer alternative to chloramphenicol for veterinary use (Trif et al., 2023a), florfenicol now represents a microcosm of broader antimicrobial resistance challenges (Zhang et al., 2022).

The geographic concentration of research, though indicative of strong regional expertise, also highlights notable gaps in global representation. Limited contributions from Africa and parts of Asia, where florfenicol use may be increasing without adequate monitoring, suggest potential blind spots in global resistance surveillance (Vernet et al., 2014). The strong China-USA collaboration axis, while productive, may inadvertently create research paradigms that don't fully address challenges in resource-limited settings where regulatory oversight and analytical capabilities differ substantially.

The emergence of advanced analytical techniques (molecularly imprinted polymers, LC-MS/MS) in recent publications (Bardhi et al., 2023) indicates that detection and monitoring capabilities are evolving to meet regulatory demands. However, the persistent focus on detection rather than prevention suggests that research priorities may be reactive rather than proactive, addressing contamination after occurrence rather than preventing initial environmental release.

Review of similar related studies reveals that florfenicol research has been investigated from therapeutic, environmental, and bibliometric perspectives. Trif et al. (2023a) provided a systematic review of florfenicol in veterinary medicine, highlighting its ribosome-targeting antibacterial action, anti-inflammatory effects, and formulation challenges, while suggesting nanotechnology-based approaches to enhance efficacy and overcome solubility issues (Trif et al., 2023a). Subbiah et al. (2011) reported that unlike many antibiotics which degrade in soil, florfenicol and β-lactams remain bioactive, raising concerns about environmental persistence and resistance dissemination (Subbiah et al., 2011). Similarly, a global bibliometric analysis of antimicrobial resistance in the environment emphasized the growing but still underrepresented role of environmental research within the One Health framework (Sweileh and Moh'd Mansour, 2020). However, the absence of previous bibliometric studies dedicated solely to florfenicol highlights the originality and importance of the current research.

In special emphasis on resistance, the florfenicol resistance in human pathogens may be attributed to horizontal transfer from animal origin (Lu et al., 2018). In addition, the incidence of resistance of chloramphenicol and florfenicol is proved to be more than the expected levels among bovine bacterial isolates (White et al., 2000). This necessitates strict regulatory measures to the application of the drug in veterinary practices.

Strengths and Limitations

This study's comprehensive approach, analyzing 972 publications across four decades, provides unprecedented insight into florfenicol research evolution. The use of multiple bibliometric indicators - from simple publication counts to sophisticated network centrality measures - offers multidimensional understanding of the research landscape.

However, reliance on Scopus alone may underrepresent research from institutions or countries with limited database coverage. Regional journals publishing in languages other than English might contain valuable research not captured in our analysis. The exclusion of grey literature, conference proceedings, and regulatory documents potentially overlooks practical applications and policy-relevant findings. Additionally, bibliometric indicators measure quantity and formal impact but cannot assess research quality or practical implementation of findings.

CONCLUSION

This bibliometric analysis establishes florfenicol as a rapidly evolving research domain transitioning from veterinary therapeutic focus to environmental and resistance concerns. The field demonstrates strong international collaboration yet reveals geographic disparities requiring attention. Moving forward, we recommend establishing research networks linking high-output countries with underrepresented regions to ensure global coverage of resistance surveillance.

Future investigations should prioritize prevention strategies over detection methods, developing alternatives to florfenicol use and improving waste treatment technologies. Integration of bibliometric monitoring with resistance surveillance data could create early warning systems for emerging resistance patterns. Finally, interdisciplinary research teams should include social scientists and economists to address behavioral and economic drivers of antibiotic use, ensuring that technical solutions align with practical implementation possibilities.

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Author’s Contribution

Conceptualization (RN, MGES, SM, AA, MK); Data curation (RN, SM, MK); Formal analysis (RN, MGES); Funding acquisition (MGES); Methodology (RN, SM, MK); Project administration (MGES); Resources (MK); Software (SM, MK); Visualization (AA); Writing – original draft (SM, MK); Writing – review and editing. (RN, MGES, SM, AA, MK).

Competing interest

The authors declare no conflict of interest.

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Supplementary Data

The supplementary material (Supplementary file 1) associated with this article is available online at the following permanent link:
https://niv.ns.ac.rs/e-avm/index.php/e-avm/article/view/514/325

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THE EFFECT OF LIQUID SMOKE ON FOODBORNE PATHOGENS IN READY-TO-EAT MEAT PRODUCTS

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Abstract

Liquid smoke is increasingly used in ready-to-eat meat products as a natural preservative with antimicrobial and antioxidant properties. This study investigated the efficacy of a commercial liquid smoke preparation (Cloud S9, Kerry, USA) against *Listeria monocytogenes* ATCC 13932, *Escherichia coli* ATCC 25922, and *Salmonella Enteritidis* ATCC 13076 in chicken and beef ham. Meat products were inoculated with each bacterial strain and treated with liquid smoke at concentrations of 1%, 2.5%, and 5%, and untreated samples were used as controls. Bacterial counts were assessed after 2 hours, 7 days, and 14 days of refrigerated storage (4 °C). Liquid smoke significantly inhibited bacterial growth in a concentration-dependent manner. For *Listeria monocytogenes*, 5% liquid smoke achieved > 2 log cfu/g reductions after 14 days, while lower concentrations suppressed growth without elimination. *Escherichia coli* and *Salmonella Enteritidis* exhibited comparatively lower sensitivity, with most treatments producing bacteriostatic rather than bactericidal effects; however, 5% liquid smoke achieved a measurable reduction of *Escherichia coli* in chicken ham. These findings confirm the antimicrobial potential of liquid smoke as a complementary hurdle to enhance the microbial safety of ready-to-eat meat products, while also underscoring the ongoing necessity for stringent hygiene practices.

Key words: Liquid smoke, smoked meat, *Listeria monocytogenes, Escherichia coli, Salmonella* Enteritidis

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UTICAJ TEČNOG DIMA NA PATOGENE U PROIZVODIMA OD MESA SPREMNIM ZA KONZUMIRANJE

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Kratak sadržaj

Tečni dim se sve više koristi u proizvodima od mesa spremnim za konzumiranje kao prirodni konzervans koji ima antimikrobna i antioksidativna svojstva. Predmet ovog rada je efikasnost komercijalnog tečnog dima (Cloud S9, Kerry, USA) protiv bakterija *Listeria monocytogenes* ATCC 13932, *Escherichia coli* ATCC 25922, i *Salmonella* Enteritidis ATCC 13076 u pilećoj i goveđoj grubousitnjenom barenom kobasici. Proizvodima od mesa su dodati bakterijski sojevi i tretirani su tečnim dimom u koncentracijama od 1%, 2,5% i 5%, dok su netretirani uzorci korišćeni kao kontrolna grupa. Broj bakterija je beležen nakon 2 sata, 7 dana i 14 dana čuvanja u frižideru (4 °C). Tečni dim je značajno inhibirao rast bakterija u zavisnosti od koncentracije. Kada je u pitanju *Listeria monocytogenes*, tečni dim u koncentraciji od 5% doveo je smanjenja broja bakterija za više od 2 log cfu/g nakon 14 dana, dok su niže koncentracije inhibirale rast, ali bez potpune eliminacije. *Escherichia coli* i *Salmonella* Enteritidis pokazali su relativno manju osetljivost, pri čemu je većina tretmana imala bakteriostatski, a ne baktericidni efekat. Međutim, tečni dim u koncentraciji od 5% doveo je do znatnog smanjenja broja *Escherichia coli* u pilećoj grubousitnjenoj barenoj kobasici. Ovi rezultati potvrđuju antimikrobni potencijal tečnog dima kao komplementarnog faktora za poboljšanje mikrobiološke bezbednostiproizvoda od mesa spremnih za konzumiranje, ali istovremeno ukazuje na to da je neophodno strogo sprovođenje higijenskih mera.

Ključne reči: Tečni dm, suhomesnati proizvodi, *Listeria monocytogenes, Escherichia coli, Salmonella* Enteritidis

INTRODUCTION

Traditional smoking has long served as an effective food-preservation method, valued not only for imparting characteristic flavor, color, and aroma but also for providing important antioxidant and antimicrobial benefits (Suñen et al., 2001; Lingbeck et al., 2014; Maga, 2018). Modern food processing has increasingly adopted liquid smoke, an all-natural condensate of wood smoke components, which offers practical advantages over traditional methods, such as ease and consistency of application, precise concentration control, reduced processing time, and the significant removal of carcinogenic polycyclic aromatic hydrocarbons (PAHs) (Sofos et al., 1988; Guillen et al., 2000; Šimko, 2005; Lingbeck *et al.*, 2014). This transition is particularly relevant given the increasing consumer demand for "all-natural" and "clean label" products, free from synthetic additives, aligning liquid smoke with a natural extract appeal (Morey et al., 2012; Lingbeck *et al.*, 2014).

The multifaceted preservative effects of liquid smoke are primarily attributed to its complex composition, notably phenolic compounds, carbonyls, and organic acids, which are generated during the pyrolysis of wood (Morey et al., 2012; Lingbeck et al., 2014). These compounds exert their antimicrobial activity by damaging microbial cell walls and membranes, inactivating essential enzymes, inhibiting cellular metabolism, and lowering intracellular pH, thereby inducing bacteriostatic or bactericidal effects (Morey et al., 2012; Dien et al., 2022). Numerous studies have demonstrated liquid smoke's efficacy against various foodborne pathogens, including *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli*, and *Staphylococcus aureus*, as well as spoilage microorganisms like *Aeromonas hydrophila* and various molds (Suñen et al., 2001; Van Loo et al., 2012; Morey et al., 2012; Lingbeck et al., 2014; Soares et al., 2016; Deliephan et al., 2023). Beyond direct pathogen inhibition, its potent antioxidant properties effectively retard lipid oxidation, playing a critical role in extending the shelf life of food products (Milly et al., 2005; Soares et al., 2016). For example, liquid smoke has been shown to suppress *L. monocytogenes* growth in frankfurters for up to 130 days and significantly reduce pathogen and histamine levels in tuna loin sashimi (Lingbeck et al., 2014; Dien et al., 2022). The effectiveness of liquid smoke is influenced by its concentration, the specific chemical composition of its components, and the pH of the food matrix (Van Loo et al., 2012; Morey et al., 2012; Lingbeck et al., 2014). Antimicrobial effectiveness can also be aaffected by the composition of the food matrix (Hao et al., 1998). Currently in literature most of the studies on the antimicrobial effect of liquid smoke are focused on listeriocidical effects on salmon and salmon products (Messina et al., 1988; Suñen, 1998; Suñen et al., 2001; Van Loo et al., 2012). There are limited studies of ready-to-eat (RTE) meat sausages, and other foodborne pathogens like *Salmonella* and *Escherichia coli* (Lingbeck et al., 2014).

The aim of this study was to determine the effectiveness of topically applied commercial liquid smoke preparation (Zesty Smoke Cloud S9, Kerry, USA) in inhibiting the growth of major foodborne pathogens (*Listeria monocytogenes* ATCC 13932, *Escherichia coli* ATCC 25922, and *Salmonella* Enteritidis ATCC 13076) in RTE meat products (chicken and beef hams).

MATERIAL AND METHODS

RTE meat products

Two types of RTE meat products were used in this study: Chicken Ham - Coarsely Ground Sausage (CH) and Beef Ham - Coarsely Ground Sausage (BH). The CH formulation consisted of the following ingredients: chicken breasts min. 50%, water, salt, soy isolate, celery spice, carrageenan (E407), disodium diphosphate (E450), sodium tripolyphosphate (E451), disodium inosinate (E316), potassium nitrate (E252), sodium nitrate (E250), monosodium glutamate (E621). The BH consisted of the following ingredients: beef meat category I min. 50%, chicken meat 5%, water, starch, soya protein, salt, spice, E450, E451, E407, E252, E250, E316, E621. Both products were prepared from coarsely chopped meat, marinated, and stuffed into permeable cellulose casings. They were then steam-cooked at 80 °C until the internal temperature reached 74 °C. The composition of RTE meat products is shown in Table 1.

Table 1. Composition of RTE meat products

	Chicken Ham (CH) (per 100 g)	**Beef Ham (BH) (per 100 g)**
Total protein	14 g	14 g
Meat protein	12 g	12 g
Collagen	0.6 g	0.6 g
Carbohydrates	1.6 g	2.65 g
sugars	0.25 g	0.1 g
Total fat	0.8 g	1.5 g
saturated	0.24 g	0.6 g
Salt	2.9 g	2.9 g
Water	up to 80 g	up to 80 g

The sausages were sliced into 100 g portions, individually vacuum-packed, stored and transported to the laboratory at the temperature of 4 °C. For the purposes of this study, a total of 122 CH and 122 BH packages were used.

Bacterial strains

One strain of *Listeria monocytogenes* ATCC 13932, *Escherichia coli* ATCC 25922, and *Salmonella* Enteritidis ATCC 13076 were used in this study. All strains were obtained commercially (Microbiologics, USA).

The pellets were resuscitated according to the manufacturer's instructions. Each strain was inoculated in Brain Heart Infusion (BHI, Oxoid Ltd, UK) and incubated at 37 °C for 24 hours. After incubation each strain was stroked on a plate of Nutrient Agar (NA, Oxoid Ltd, UK) in a way to produce individual colonies and incubated at 37 °C for 24 hours. After incubation, individual colonies were suspended in maximum recovery diluent (MRD, Oxoid Ltd., UK) and adjusted to a turbidity up to 0.5 McF. Serial dilutions were made for every strain in order to obtain the required number of bacteria present in the suspensions. Through serial dilutions the number of every strain in the suspension was determined. 6.84 log cfu/mL for *L. monocytogenes*, 6.80 log cfu/mL for *E. coli* and 6.09 log cfu/mL for *S.* Enteritidis.

This suspension was used to contaminate the meat products.

Liquid smoke

Commercially available liquid smoke Zesty smoke Cloud S9 (Kerry, USA was used for this study. The specification of the product is that it has neutral taste and color, pH 5.5. liquid intended for spaying and dosing on meat products. The smoke was stored at room temperature until use. It was sprayed on the meat products using a handheld spray bottle. One spray shot contained 1.5 mL smoke suspension. Three different concentrations were used for this study: 1%, 2.5% and 5% concentration of the liquid smoke, diluted with sterile deionized water.

Bacteriological examination

Detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* in RTE meat samples without contamination and without liquid smoke applicatio*n* was carried out according to ISO 6579-1, ISO 11290-1, and ISO 16649-2 respectively.

ISO 11290-2 was used for the enumeration of *Listeria monocytogenes*, ISO 16649-2 for the enumeration of *E. coli* and for the enumeration of *Salmonella* spp. The same protocol used for Listeria monocytogenes was followed, with the exception of the agar employed for inoculation. According to the above-mentioned standards, the initial inoculation for all the samples was made in BPW (Biolife italiana S.r.l., Italy) and then from the initial suspension decade serial dilution was made in Maximum recovery diluent MRD (Biolife italiana S.r.l., Italy). From each of the serial dilutions, 1 ml was transferred on three plates selective agar and spread out with hockey stick. For Salmonella Enteritidis, XLD agar (Biolife Italiana S.r.l., Italy) was used; for Listeria monocytogenes, Ottaviani–Agosti agar (Biolife Italiana S.r.l., Italy) was employed, while XLD agar (Biolife Italiana S.r.l., Italy) was used for *E. coli*.

Experimental design

All manipulations were carried out in a BLU Space Class II Bio-safety Cabinet (PBI Internacional, Italy).

Before the contamination with *Listeria monocytogenes*, *Escherichia coli* and *Salmonella* Enteritidis, as well as the application of liquid smoke, five samples of each type of ham were tested for the absence of *Listeria monocytogenes*, *Escherichia coli* and *Salmonella* Enteritidis.

The vacuumed packages were cut with sterile scalpers and 3 mL liquid smoke was sprayed in each package. After that, 1 mL bacterial suspension was added. The packages were then vacuumed, re-sealed and stored at 4 °C until the analysis.

For each meat preparation / bacterial strain combination, 3 different concentrations of liquid smoke (1%, 2.5% and 5%) were applied. For every meat preparation / bacterial strain combination nine samples were made- three samples for each time interval of testing.

For positive control, only the bacterial strains were applied to each product, whereas for the negative control, three different concentrations of liquid smoke were tested on the meat products without the addition of any bacterial strain. The controls were also done in triplicate for each testing interval.

Bacterial counts were done in three intervals, at two hours, seven days and fourteen days after the meat product inoculation. Three samples from each combination of meat preparation/pathogen/Liquid smoke concentration were tested. In addition, three samples of each positive and negative controls were tested.

Enumeration of *L. monocytogenes* was performed according to ISO 11290-

2:2017, *E. coli* according to ISO 16649-2:2021, and for *Salmonella* spp., the same protocol as for *Listeria* was applied, differing only in the inoculation agar used. The meat product was diluted 1:10 in Buffered peptone water, with successive decimal dilutions in MRD. After that, 1 mL was transferred from each decimal dilution into three 90 diameter Petri dishes with selective agar. Ottaviani–Agosti agar (Biolife Italiana S.r.l., Italy) was employed for *L. monocytogenes*, Xylose Lysine Deoxycholate (XLD) agar (Biolife Italiana S.r.l., Italy) for *S. Enteritidis*, and Tryptone-Bile-X-Glucuronide (TBX) agar (Biolife Italiana S.r.l., Italy) for *Escherichia coli*. *L. monocytogenes* and *S.* Enteritidis agars were incubated at 37 °C for 24 hours and then counted, while E. coli was incubated at 44 °C for 24 hours and then counted. The results were expressed as log cfu/g, and from the three tests of each combination the mean value was calculated.

Statistical analysis

The experiments were conducted in triplicate and data was analyzed using SPSS Statistics (IBM, USA). Following ANOVA, significant differences in the treatments were determined with Tukey's LSD test at $p \leq 0.05$.

RESULTS AND DISCUSSION

For the purposes of this study a total of 244 samples of RTE sausages were tested. 122 of them were CH, and same number were BH. No bacterial growth was observed in any of the negative controls.

Table 2. Chicken ham – *L. monocytogenes* (CH-LM)

LM (log cfu/g)											
Without liquid smoke			**1%**			**2.5%**			**5%**		
2 hours	7 days	14 days	2 hours	7 days	14 days	2 hours	7 days	14 days	2 hours	7 days	14 days
4.72 ± 0.3	5.11 ± 0.26	6.94 ± 0.31	4.65 ± 0.3	4.96 ± 0.25	4.88 ± 0.3	4.59 ± 0.16	4.72 ± 0.26	3.61 ± 0.37	4.04 ± 0.19	4.48 ± 0.16	2.32 ± 0.28

Listeria monocytogenes inoculated in the CH which were not infused with liquid smoke entered log phase and increased from 4.72 ± 0.3 to 5.11 ± 0.26 log cfu/g in 7 days, to 6.94 ± 0.31 log cfu/g in 14 days. In CH treatment with 1% liquid smoke resulted in modest suppression, with day 14 counts of 4.88 ± 0.3 log cfu/g, while 2.5% liquid smoke showed greater efficacy, reducing counts to 3.61 ± 0.37 log cfu/g by day 14. The 5% liquid smoke treatment produced the greatest reduction, lowering the Listeria count to 2.32 ± 0.28 log cfu/g (Table 2.).

Table 3. Beef ham – *L. monocytogenes* (BH-LM)

LM (log cfu/g)											
Without liquid smoke			**1%**			**2.5%**			**5%**		
2 hours	7 days	14 days	2 hours	7 days	14 days	2 hours	7 days	14 days	2 hours	7 days	14 days
4.62 ± 0.3	4.95 ± 0.31	6.86 ± 0.35	4.99 ± 0.26	5.35 ± 0.31	5.51 ± 0.3	4.93 ± 0.29	5.03 ± 0.29	4.99 ± 0.3	4.00 ± 0.26	4.48 ± 0.26	2.51 ± 0.3

Similarly to the CH, BH inoculated with *L. monocytogenes* entered log phase and the number increased from 4.62 ± 0.3 to 4.95 ± 0.31 log cfu/g in 7 days, to 6.86 ± 0.35 log cfu/g in 14 days, while the BH treated with 1% liquid smoke showed modest suppression of 5.51 ± 0.3 log cfu/g in 14 days. The 2.5% smoke treated BH had 4.99 ± 0.3 log cfu/g by day 14 showing greater efficacy, and the highest efficacy was observed in 5% smoke treated BH with 2.51 ± 0.3 log cfu/g (Table 3.).

The ability of *L. monocytogenes* to adapt and grow rapidly under psychrotrophic conditions is well documented (Chan and Wiedmann, 2009). Some authors (Buchanan and Klawitter, 1991) observed that at 5 °C L. monocytogenes can easily adapt and exhibit exponential growth rates. The growth rate of *L. monocytogenes* was suppressed ($p < 0.05\%$) with the addition of the liquid smoke over the period of 14 days. All infused concentrations had this effect. The 2.5 % liquid smoke infusion showed decrease in the cfu/g in CH, but for BH the results were similar as with the 1% infusion in 14 days. Both types of products that were infused with 5% liquid smoke exhibited bactericidal effect on *L. monocytogenes* over the 14 days.

The findings of this study are consistent with those reported by (Morey et al., 2012), who investigated the incorporation of liquid smoke into meat products. Liquid smoke has been widely applied in RTE products as a strategy to mitigate the risk of *L. monocytogenes*. Its antimicrobial activity is primarily attributed to compounds such as phenols, carbonyls, and organic acids, which are typically condensed in water or other carriers and subsequently applied to products, such as frankfurters, by spraying or dipping (Vitt et al., 2001; Holley and Patel, 2005). The efficacy of commercial liquid smoke formulations against *L. monocytogenes* has been well documented. For example, (Murphy et al., 2005) found that treatment of frankfurters with Select 23P reduced inoculated *L. monocytogenes* populations by 3.2 log cfu/cm² within 4 h, and to below 1 log cfu/cm² after 12 days of storage at 4 °C. This effect was largely attributed to the low pH (2.4) and the presence of phenolic constituents in the condensate. Similarly, (Gedela et al., 2007) reported that topical application of the commercial preparation Zesti-B reduced *L. monocytogenes* on frankfurters and fully cooked turkey chubs by approximately 2 log cfu/g ($p < 0.05$) within one week.

Table 4. Chicken ham – *Salmonella* Enteritidis (CH-SE)

SE (log cfu/g)											
Without liquid smoke			**1%**			**2.5%**			**5%**		
2 hours	7 days	14 days	2 hours	7 days	14 days	2 hours	7 days	14 days	2 hours	7 days	14 days
4.90 ± 0.14	5.40 ± 0.28	6.30 ± 0.14	4.81 ± 0.45	4.24 ± 0.46	4.88 ± 0.10	4.74 ± 0.38	4.15 ± 0.49	3.76 ± 0.31	3.90 ± 0.33	4.06 ± 0.11	3.49 ± 0.27

The CH samples inoculated with *S.* Enteritidis not treated with liquid smoke entered log phase and increased from 4.90 ± 0.14 log cfu/g on day 0 to 5.40 ± 0.28 log cfu/g on day 7 and 6.30 ± 0.14 log cfu/g on day 14. Significant reduction in bacterial count was observed in CH treated with 2.5% and 5% liquid smoke from 4.74 ± 0.38 log cfu/g and 3.9 ± 0.33 log cfu/g on day 0 to 3.76 ± 0.31 log cfu/g and 3.49 ± 0.27 log cfu/g on day 14, respectively (Table 4.).

Table 5. Beef ham – *Salmonella* Enteritidis (BH-SE)

SE (log cfu/g)											
Without liquid smoke			**1%**			**2.5%**			**5%**		
2 hours	7 days	14 days	2 hours	7 days	14 days	2 hours	7 days	14 days	2 hours	7 days	14 days
3.96 ± 0.35	4.76 ± 0.29	6.69 ± 0.07	5.09 ± 0.10	4.68 ± 0.61	4.26 ± 0.32	4.79 ± 0.41	4.24 ± 0.02	3.79 ± 0.39	3.91 ± 0.36	4.00 ± 0.92	2.78 ± 0.09

Similarly to CH, BH inoculated SE without smoke entered log phase and from 3.96 ± 0.35 log cfu/g on day 0 the bacterial count increased to 4.76 ± 0.29 log cfu/g on day 7 and 6.69 ± 0.07 log cfu/g on day 14. BH treated with 1% and 2.5% liquid smoke showed significant reduction in bacterial count from 4.09 ± 0.10 log cfu/g and 4.79 ± 0.41 log cfu/g on day 0 to 4.26 ± 0.32 log cfu/g and 3.79 ± 0.39 log cfu/g on day 14, respectively. Higher suppression was observed for BH treated with 5% liquid smoke with counts of 3.91 ± 0.36 log cfu/g on day 0 to 2.78 ± 0.09 log cfu/g on day 14 (Table 5.).

In the absence of liquid smoke infusion, meat products inoculated with S. Enteritidis showed a notable increase in bacterial growth over the 14-day storage period. These results indicate active bacterial proliferation and entry into the logarithmic growth phase in untreated products, highlighting the risk associated with unmitigated *Salmonella* contamination in RTE meat products during storage.

Conversely, the inclusion of liquid smoke significantly suppressed the growth of *S. Enteritidis* ($p < 0.05$). In CH infused with 1% liquid smoke, bacterial counts increased only slightly over the 14-day period, suggesting a strong bacteriostatic effect. In BH, the same concentration resulted in a moderate reduction. These findings show that liquid smoke, even at low levels, is capable of inhibiting Salmonella proliferation, particularly in beef.

A study by Soares et al. (2016) mentions that liquid smoke had an inhibitory effect on *S. Choleraesuis* with a minimal bactericidal concentration ranging from 7.5 to 15%. This is line with our study where we predominantly observed only bacteriostatic effect of the smoke on *Salmonella*. Another study screened MICs of eight commercial liquid smoke samples against *S.* Enteritidis and found ranges from 0.5 to 8.0% (Van Loo et al., 2012) The same study concluded that the effect of liquid smoke in *Salmo*nella and *E. coli* is similar.

However, it is important to note that while reductions were evident, complete elimination of pathogens was not achieved, reinforcing the notion that liquid smoke may function primarily as a bacteriostatic agent at lower concentrations, rather than exhibiting full bactericidal activity.

Table 6. Chicken ham – *E. coli* (CH-EC)

EC (log cfu/g)											
Without liquid smoke			**1%**			**2.5%**			**5%**		
2 hours	7 days	14 days	2 hours	7 days	14 days	2 hours	7 days	14 days	2 hours	7 days	14 days
4.60 ± 0.33	5.37 ± 0.35	6.70 ± 0.3	4.32 ± 0.3	4.40 ± 0.3	4.46 ± 0.35	4.26 ± 0.28	4.30 ± 0.3	4.28 ± 0.26	3.96 ± 0.25	4.18 ± 0.26	2.72 ± 0.3

E. coli inoculated CH samples which were not treated with liquid smoke entered log phase and increased from 4.60 ± 0.33 log cfu/g on day 0 to 5.37 ± 0.35 log cfu/g on day 7 and 6.70 ± 0.30 log cfu/g on day 14. In CH samples, treatment with 1% and 2.5% liquid smoke produced only modest suppression, yielding day-14 counts of 4.46 ± 0.35 log CFU/g and 4.28 ± 0.26 log CFU/g, respectively. Significant suppression was observed with 5% liquid smoke treated CH with count of 2.72 ± 0.30 log cfu/g (Table 6.).

Table 7. Beef ham – *E. coli* (BH-EC)

EC (log cfu/g)											
Without liquid smoke			**1%**			**2.5%**			**5%**		
2 hours	7 days	14 days	2 hours	7 days	14 days	2 hours	7 days	14 days	2 hours	7 days	14 days
4.30 ± 0.42	4.57 ± 0.04	6.58 ± 0.56	4.28 ± 0.36	4.81 ± 0.18	4.86 ± 0.18	4.20 ± 0.38	4.70 ± 0.72	4.71 ± 0.29	3.93 ± 0.26	4.68 ± 0.30	4.36 ± 0.26

Similarly to CH, modest suppression was observed on BH treated with 1% and 2.5 % liquid smoke with counts of 4.86 ± 0.34 log cfu/g and 4.71 ± 0.29 log cfu/g in 14 days, respectively. Greater suppression was observed in BH treated with 5% liquid smoke with counts of 4.36 ± 0.26 log cfu/g (Table 7).

The results of this study clearly demonstrate that the application of liquid smoke has a significant inhibitory effect on the growth of *E. coli* in both CH and BH. In the control samples (without liquid smoke infusion), there was a rapid and consistent increase in *E. coli* counts over the 14-day period. These results confirm that under favourable conditions, *E. coli* can multiply rapidly in meat products, posing a serious food safety concern. In contrast, the addition of liquid smoke, even at low concentrations (1%), significantly suppressed the growth of *E. coli*. One percent infusion reduced the growth rate in CH to a near-stagnant level for 14 days, while the BH saw a slower but more pronounced increase. Higher concentrations of liquid smoke (2.5% and 5%) had an even stronger inhibitory effect, especially in chicken meat products. Notably, the 5% concentration not only suppressed growth but reduced the *E. coli* count from 3.96 to 2.72 log CFU/g, suggesting potential bactericidal activity of the smoke components.

These findings are consistent with previous studies that have shown smoke extracts to be effective in controlling microbial growth in food products. Estrada-Muñoz et al., (1998) demonstrated similar suppression in beef products, while Dien et al. (2022) found comparable results in tuna and sashimi, suggesting that the antimicrobial effects of smoke are not limited to red meat but may extend to seafood as well.

These results not only confirm the antimicrobial efficacy of liquid smoke but also suggest a dose-dependent response, particularly against *E. coli*. The reduction in *E. coli* was more pronounced in beef products compared to chicken products, which could be due to matrix-specific interactions between the meat composition and the antimicrobial components of liquid smoke.

Interestingly, the data also supports the observation by Asita and Campbell (1990) that Gram-negative bacteria such as *E. coli* tend to be less sensitive to smoke extracts compared to Gram-positive organisms. Despite this general resistance, the fact that high concentrations of liquid smoke were able to reduce bacterial counts shows its potential as a natural preservative, especially in RTE and processed meat products.

CONCLUSION

This study demonstrated that liquid smoke has effects on *L. monocytogenes*, *E. coli* and *Salmonella* Enteritidis. Additionally, although the number of pathogenic bacteria was reduced, they were not eliminated. These findings emphasize that liquid smoke can help improve food safety, but proper hygiene practices are still crucial.

The present study demonstrates the potential of liquid smoke as a natural antimicrobial additive for improving the microbiological safety of meat products during refrigerated storage. Its use may act as an additional hurdle within a multi-intervention food safety strategy for RTE meats.

However, this study is not without limitations. The observed antimicrobial effects were primarily bacteriostatic at the concentrations used, and further research is required in order to determine the sensory impact, optimal dosing, and potential synergies with other preservation techniques. Moreover, evaluating its effects under different packaging conditions (e.g., vacuum vs. modified atmosphere) and storage temperatures could further clarify the practical utility of liquid smoke in industrial applications.

Author's Contribution

KP and MP made contributions to conception and design of the article; KP, MP and MRM conducted the experiments; KP, MP and MRM wrote the manuscript; MP and DJ coordinated the work and revised the manuscript. KP and MRM prepared the final draft; MP and MRM contributed to the analysis and presentation of the results.

Competing interest

The authors declare that they have no competing interests.

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TEFLUTHRIN-INDUCED MASS MORTALITY OF FARMED RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) IN SERBIA: A CASE STUDY

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Abstract

We present the first documented case of mass mortality in farmed rainbow trout (*Oncorhynchus mykiss*) linked to tefluthrin contamination in a freshwater stream. In June 2013, a twelve-day pollution event occurred in the Rogačica River, Republic of Serbia, resulting in the death of approximately 160,000 trout fingerlings and yearlings, with a total biomass of 4,190 kg. Water samples were analyzed for pesticide residues using liquid chromatography–tandem mass spectrometry (LC-MS/MS) with an Agilent 1260 Infinity LC system (Agilent Technologies, USA) coupled to a 6460 Triple Quad LC/MS mass spectrometer. Water analysis revealed tefluthrin concentrations of 2.04 and 0.52 μg/L, representing 9- to 34-fold exceedances of the reported 96 h LC50 for rainbow trout (0.06 μg/L). The contamination originated from washing pesticide-coated corn seed in a local distillery, representing a previously undocumented source of aquatic pesticide pollution. This case highlights the vulnerability of aquaculture to agricultural pesticides, the particular sensitivity of early life stages of salmonids to pyrethroids, and the need for improved regulatory and biosecurity measures to prevent similar events.

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Key words: Oncorhynchus mykiss, aquaculture, pesticide residues, pollution, pyrethroid insecticides, tefluthrin

TEFLUTRINOM IZAZVANA MASOVNA UGINUĆA KALIFORNIJSKE PASTRMKE (*ONCORHYNCHUS MYKISS*) U SRBIJI: STUDIJA SLUČAJA

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Kratak sadržaj

U radu je opisan prvi dokumentovani slučaj masovnog uginuća kalifornijske pastrmke (*Oncorhynchus mykiss*) u akvakulturi, povezan sa kontaminacijom površinskih voda teflutrinom. U junu 2013. godine došlo je do dvanaestodnevnog zagađenja u reci Rogačici (Srbija), što je rezultiralo uginućem oko 160.000 jedinki mlađi i jednogodišnjaka pastrmke, ukupne biomase od 4.190 kg. Uzorci vode su analizirani na prisustvo ostataka pesticida primenom tečne hromatografije u kombinaciji sa tandemskom masenom spektrometrijom (LC-MS/MS), korišćenjem sistema Agilent 1260 Infinity LC (Agilent Technologies, SAD) povezanog sa masenim spektrometrom 6460 Triple Quad LC/MS. Analiza vode potvrdila je koncentracije teflutrina od 2,04 i 0,52 µg/L, što je od 9 do 34 puta premašilo LC50 vrednost za kalifornijsku pastrmku (0,06 µg/L). Izvor kontaminacije bilo je pranje semena kukuruza obloženog pesticidom u lokalnoj destileriji, što predstavlja do sada nedokumentovan izvor unosa pesticida u vodene ekosisteme. Ovaj slučaj ukazuje na rizik kojem je akvakultura izložena usled neadekvatne upotrebe poljoprivrednih pesticida, izuzetnu osetljivost ranih razvojnih stadijuma salmonidnih vrsta na piretroide, kao i na potrebu za unapređenjem regulatornih i biosigurnosnih mera radi prevencije sličnih događaja.

Ključne reči: Oncorhynchus mykiss, akvakultura, ostaci pesticida, zagađenje, piretroidni insekticidi, teflutrin

INTRODUCTION

Aquatic ecosystems and aquaculture operations are increasingly exposed to pesticides originating from agricultural practices. Among them, pyrethroids are widely applied because of their effectiveness against insect pests, yet they pose significant risks to non-target aquatic organisms, especially fish (Haya, 1989; Ullah et al., 2019). Rainbow trout (Oncorhynchus mykiss), one of the most widely farmed salmonids in Europe, is particularly sensitive to water quality deterioration and pesticide contamination (Velisek et al., 2006; Besser et al., 2020).

Previous studies have demonstrated that pyrethroids disrupt fish physiology, behavior, and survival, with early life stages being particularly susceptible (Werner and Moran, 2008; Richterova and Svobodova, 2012). Although pyrethroid toxicity is well documented, field-documented cases of large-scale fish mortality directly attributable to tefluthrin remain absent. Tefluthrin, a contact and vapor-phase pyrethroid insecticide, is considered one of the most acute toxic pyrethroids for vertebrates (EFSA, 2010; Soderlund et al., 2002). Recent experimental studies have provided further insights into its mode of action and toxic potential. For example, enantioselective effects of tefluthrin in zebrafish revealed differential developmental and physiological toxicities (Wang et al., 2023), while exposure during early life stages was shown to impair cardiac and skeletal development, increase oxidative stress, and induce apoptosis (Wu et al., 2025). Furthermore, pyrethroid-associated nephrotoxicity has been reported in catfish species under controlled conditions, highlighting the broader risks of this pesticide class for aquaculture species (Stilwell et al., 2024). Despite these findings, no aquaculture poisoning incidents involving tefluthrin have been officially reported to date.

We report the first confirmed case in Serbia of widespread rainbow trout mortality associated with tefluthrin contamination. The study details the extent of the fish losses, examines water residues, identifies the source of pollution, and highlights the implications of this previously unrecognized contamination pathway for aquaculture biosecurity and environmental management.

MATERIAL AND METHODS

The Rogačica River, a tributary of the Drina River in western Serbia, supports several small- to medium-scale rainbow trout farms (Figure 1.).

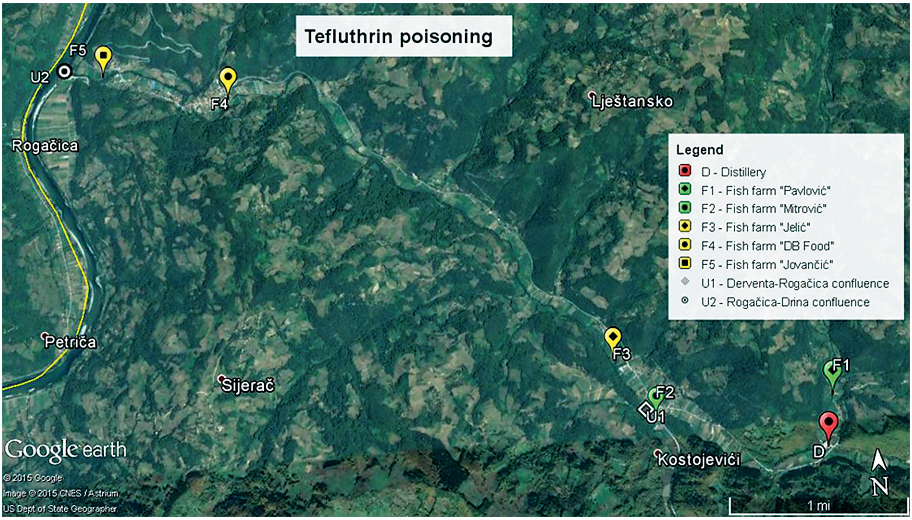

Figure 1. Location of trout farms (F1–F5) and the distillery (D) along the Rogačica River.

In June 2013, three downstream farms reported sudden fish mortality events, prompting an on-site investigation.

Mortality data were obtained through interviews with farm owners and verified by direct on-site observations. Dead fish were collected daily, weighed in bulk, and the number of individuals was estimated by dividing the total biomass by the mean body weight of representative size classes. To ensure accuracy, size-class distributions were determined from random subsamples, and estimates were cross-checked against farm records where available (Figure 2.).

Figure 2. Preparing dead fish for weighting at F4 fish farm on 21.6.2013.

Water samples were collected on 28 June 2013 from the pre-treatment precipitation facility and the initial rearing pond at the most severely affected farm (Figure 3.).

Figure 3. Water sampling location (R, S) at the F4 fish farm.

Samples were analyzed for pesticide residues using liquid chromatography–tandem mass spectrometry (LC-MS/MS) with an Agilent 1260 Infinity LC system (Agilent Technologies, USA) coupled to a 6460 Triple Quad LC/MS mass spectrometer. Prior to analysis, samples were filtered through 0.45 μm membranes and subjected to solid-phase extraction (SPE) for pre-concentration and purification. Chromatographic separation was performed on a reverse-phase column, and compounds were detected using multiple reaction monitoring (MRM) in both positive and negative electrospray ionization modes. Quantification was achieved using external calibration with matrix-matched standards, while method validation included assessments of recovery (70 – 120%), repeatability (RSD < 20%), and limits of detection and quantification consistent with EU guidelines for pesticide residue analysis (European Commission, 2017; EFSA, 2010).

RESULTS AND DISCUSSION

The twelve-day pollution event began on 20 June 2013. At farm F4, white foam appeared on the water surface, followed by abnormal trout behavior: disorientation, surface swimming, reduced respiration, and lethargy, consistent with pyrethroid poisoning (Farag et al. 2021). Mortality occurred within 30–60 minutes, primarily affecting fingerlings (<100 g). Although some recovery was observed the following morning, repeated evening pollution episodes caused cumulative losses. Total mortality at farm F4 was 3,350 kg of rainbow trout (average 30 -100 g). Additional losses occurred at farm F3 (658 kg, mixed size classes) and farm F5 (182 kg, fry and fingerlings of 5–15 g). In total, approximately 160,000 fish (4,190 kg) died across the three farms, predominantly fry and fingerlings (Figure 4.).

Figure 4. Mortality of rainbow trout yearlings at the F4 fish farm on 21 June 2013

Necropsy of moribund and freshly dead rainbow trout revealed no significant pathomorphological changes, and no lesions indicative of infectious disease were observed, supporting chemical intoxication as the primary cause of mortality.

Water samples collected on 28 June 2013 confirmed tefluthrin residues at 2.04 μg/L (pre-treatment facility) and 0.52 μg/L (fish pool B14). These values exceeded the reported 96 h LC50 for rainbow trout (0.06 μg/L) by 34- and 9-fold, respectively (EFSA, 2010). Other pesticides (metalaxyl, metiram, imidacloprid, thiamethoxam) were detected but at concentrations well below their LC50 values, making tefluthrin the most likely cause of mortality (Table 1.).

Table 1. Results of water analysis from the Rogačica River in Serbia

Common name	Class of Pesticide	Purpose	LC50 value for rainbow trout	Water Analysis
Tefluthrin	Pyrethroid	Insecticide	0.06 μg/L (96 h; static)	pre-treatment: 2.04 μg/L B14: 0.52 μg/L
Carbofuran	Carbamate	Insecticide	0.3625 mg/L (96 h; semi-static)	Not detected
Thiram	Dithiocarbamate	Fungicide	0.046 mg/L (96 h)	Not detected
Metiram	Dithiocarbamate	Fungicide	0.33 mg/L (formulation; 96 h)	pre-treatment: 0.01 μg/L B14: 0
Metalaxyl	Phenylamide	Fungicide	> 100 mg/L (96 h)	pre-treatment: 0.02 μg/L B14: 0.01 μg/L
Imidacloprid	Neonicotinoid	Insecticide	211 mg/L (96 h)	pre-treatment: 0.04 μg/L B14: 0.09 μg/L
Clothianidin	Neonicotinoid	Insecticide	> 104.2 mg/L (96 h)	Not detected
Thiamethoxam	Neonicotinoid	Insecticide	> 125 mg/L (96 h)	pre-treatment: not detected B14: 0.02 μg/L

The official investigation identified the pollution source as a distillery upstream of the affected farms. Seed corn coated with tefluthrin had been washed prior to ethanol production, releasing contaminated wastewater directly into the river. This contamination pathway is unprecedented; although pesticide runoff from fields is well known, this study provides the first evidence that industrial seed washing can directly cause mass fish mortality (Weston and Lydy, 2010; Méjanelle et al. 2020).

The observed mortality pattern, characterized by rapid onset, behavioral abnormalities, and a disproportionate impact on young trout, aligns with the established toxicological profile of pyrethroids, which affect the nervous system and are especially detrimental to early life stages (Ullah et al., 2019; Werner and Moran, 2008; Richterova and Svobodova, 2012). The disproportionate impact on fry and fingerlings supports previous laboratory findings showing that early life stages of rainbow trout are especially sensitive to pyrethroids (Haya, 1989). The loss of over 160,000 fish in less than two weeks highlights the economic and ecological risks of pesticide mismanagement. For farmers, such events result in severe financial damage, while for aquatic ecosystems, they emphasize the vulnerability of aquaculture operations located on small streams affected by upstream industrial or agricultural activities (Varela et al., 2023).

Pyrethroids are recognized for their persistence and potential to bioaccumulate. Even at sublethal levels, they can disrupt fish physiology and compromise ecosystem health (Hartz et al., 2019; Ullah et al., 2019). The exceptionally high concentrations observed in this case underscore the heightened vulnerability of aquaculture operations in small riverine systems to sudden contamination events. From a regulatory standpoint, this case highlights the need for stricter oversight of pesticide-treated seed handling. Industrial seed washing should be recognized as a high-risk activity. Recommended preventive measures include mandatory wastewater treatment at seed-processing facilities, regular monitoring of aquaculture water supplies in regions with intensive pesticide use, and incorporation of pesticide risk assessments into aquaculture biosecurity frameworks (Burch et al., 2025; Carvalho, 2017).

From the perspective of aquaculture operations, such events are situated within the broader framework of risks and uncertainties. A clear conceptual distinction between these terms is essential. Risk denotes situations in which the distribution of potential outcomes is either known a priori or can be statistically inferred from prior experience. Uncertainty, by contrast, arises when the probabilities of outcomes cannot be quantified (Knight, 1921). Importantly, risk assessments are often subjective, as probability estimates depend on the evaluator knowledge and perception (Hardaker, 2016). The incident under consideration could, in principle, be classified as a production risk. However, the poisoning was triggered by the presence of a substance that, under normal production conditions, would not be expected in corn used for alcohol manufacturing. This atypical causative factor indicates that the event could not have been reasonably foreseen. Consequently, it is more appropriately characterized as an uncertainty in trout production. Recognizing such distinctions is critical, as the management of uncertainty requires adaptive strategies that differ fundamentally from conventional risk-mitigation approaches in aquaculture biosecurity and farm management.

CONCLUSION

This study provides the first documented evidence of mass mortality of farmed rainbow trout caused by tefluthrin contamination. The incident resulted from washing pesticide-coated corn seed in a distillery, introducing toxic residues into a stream supplying multiple fish farms. Tefluthrin concentrations exceeded the LC_{50} for rainbow trout by more than an order of magnitude, causing the loss of approximately 160,000 fish, predominantly fingerlings. The findings highlight three critical points: (i) rainbow trout early life stages are highly susceptible to pyrethroids, (ii) unconventional agricultural practices such as seed washing can serve as acute source of water pollution, and (iii) aquaculture remains highly vulnerable to external environmental hazards despite internal biosecurity measures. To prevent similar incidents, strict regulations on pesticide-treated seed disposal, routine monitoring of aquaculture water supplies, and improved integration of environmental toxicology into aquaculture management are needed.

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Author's Contribution

SČ, PS, DB and VR made contributions to conception and design of the study, involved in data collection and drafting the manuscript. VR revised the manuscript critically and together with SČ prepared the final draft of the manuscript. All authors read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

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SMOKE INHALATION INJURIES IN A DOG - CASE REPORT

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Abstract

Smoke inhalation injuries are relatively uncommon in veterinary pathology and the current literature on spontaneous carbon monoxide intoxication in companion animals is limited. The majority of available data are derived from isolated case reports or extrapolated from human forensic studies. Consequently, there is a need for systematic documentation and analysis of such cases to improve diagnostic accuracy and support legal investigations involving animal fatalities in fire-related incidents. In this case, five mixed-breed dogs (two males and three females), ranging in age from one to five years, were found deceased as a result of a residential structure fire. All animals were in good nutritional condition and did not have microchip identification. One dog, at the request of the public prosecutor, was submitted for necropsy to the Department of Forensic Veterinary Medicine and Legislation, Faculty of Veterinary Medicine, University of Belgrade, Serbia, in order to determine the cause of death. Gross examination revealed discoloration of the coat due to soot deposition, cherry-red oral mucosa and the presence of soot particles on the oral mucosa, including the teeth and tongue, as well as in the external ear canal. No external thermal

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injuries, such as burns or signs of carbonization were found. Histopathological findings demonstrated pulmonary edema, soot particles in airways and generalized hyperemia. Toxicological examination of postmortem blood samples collected from the heart and major vessels demonstrated markedly elevated carboxyhemoglobin concentrations (68.67%), consistent with fatal carbon monoxide poisoning.

This case emphasizes the importance of a comprehensive forensic methodology integrating anamnestic data, post-mortem macroscopic and microscopic findings, and toxicological analysis, as essential and reliable tools for investigating fire-related fatalities in companion animals.

Key words: carbon monoxid, poisoning, pet animals, fire-related fatalities, necropsy, toxicology

POVREDE USLED UDISANJA DIMA KOD PSA – PRIKAZ SLUČAJA

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Kratak sadržaj

Povrede nastale usled udisanja dima su relativno retke u veterinarskoj patologiji, a literatura o spontanoj intoksikaciji kućnih ljubimaca ugljenmonoksidom je i dalje oskudna. Većina dostupnih podataka potiče iz pojedinačnih izveštaja ili je zasnovana na rezultatima studija sprovedenih na ljudima. Shodno tome, postoji potreba za sistematskom dokumentacijom i analizom takvih slučajeva kako bi se unapredila pouzdanost dijagnoze i pružila relevantna podrška pravnim postupcima u vezi sa smrtnim slučajevima životinja u incidentima povezanim sa požarima. U ovom slučaju, pet pasa mešanaca (dva mužjaka i tri ženke), starosti između jedne i pet godina, pronađeno je u stambenoj kući u kojoj je izbio požar. Svi su bili u dobroj kondiciji i bez mikročipa. Jedan pas je, na zahtev javnog tužioca, dostavljen na nekropsiju na Katedru za sudsku veterinarsku medicinu i zakonske propise, Fakulteta veterinarske medicine, Univerziteta u Beogradu, Srbija, radi utvrđivanja uzroka uginuća. Makroskopskim pregledom konstatovana je promena boje dlake usled taloženja čađi, crvenkasta sluzokoža usne duplje i prisustvo čestica čađi na sluzokoži usne duplje, uključujući zube i jezik, kao i u spoljašnjem ušnom kanalu. Spoljašnjim pregledom nisu ustanovljene termičke povrede u vidu opekotina ili karbonizacije. Histološki, ustanovljen je edem pluća, čestice čađi u disajnim putevima i generalizovana hiperemija. Toksikološka analiza uzoraka krvi iz srca i glavnih krvnih sudova nakon uginuća pokazala je značajno povišene koncentracije karboksihemoglobina (68,67%), što je u skladu sa fatalnim trovanjem ugljen-monoksidom.

Ovaj slučaj ističe značaj forenzičke metodologije koja obuhvata anamnestičke podatke, makroskopske i histopatološke nalaze nakon uginuća i toksikološku analizu, kao bitne i pouzdane alate za istraživanje slučajeva uginuća kućih ljubimaca u požarima.

Ključne reči: trovanje ugljen dioksidom, kućni ljubimci, fatalne povrede izazvane požarom, obdukcija, toksikologija

CASE PRESENTATION

Anamnestic Data

In this case, a mixed-breed female dog, approximately two years old, weighing 13.2 kg, with gray coat, was examined. The coat was heavily contaminated with soot, particularly in the head region and on the distal parts of the forelimbs and hindlimbs, suggesting that the dog was alive at the time of the fire and most likely attempting to escape from the smoke-filled environment (Figure 1a). This external finding indicates active contact with smoke and combustion products, while respiratory and circulatory functions were still preserved. Notably, the area around the neck, corresponding to where a collar would have been, remained clean and retained its original white color.

Macroscopic examination

Examination of the oral mucosa revealed no macroscopic lesions. However, it exhibited a cherry-red discoloration and contained soot particles, which were also present on the teeth and tongue (Figure 1b). A dark red discharge, composed of blood and soot particles, was observed flowing spontaneously and under pressure from the nostrils. The skin of the left ear displayed prominent soot deposition and erythema, indicative of thermal and smoke exposure. In contrast, the right ear remained unaffected, suggesting the animal had been positioned on its right side at the time of exposure. The skin on the ventral surface of the abdomen exhibited a cherry-red discoloration and was covered with soot particles.

The tongue is blackened and covered with a substantial amount of soot (Figure 1c). The esophageal mucosa was intact but hyperemic, with a small amount of soft, mushy content containing soot particles. The trachea was completely filled along its entire length with a dark, black-gray, foamy substance (Figure 1d). The lungs were dark red with mottled areas of lighter red, exhibited a rubbery consistency, and had rounded, blunt edges. Upon sectioning, the lung parenchyma was moist, with moderate amounts of frothy, blood-tinged fluid present in the airways.

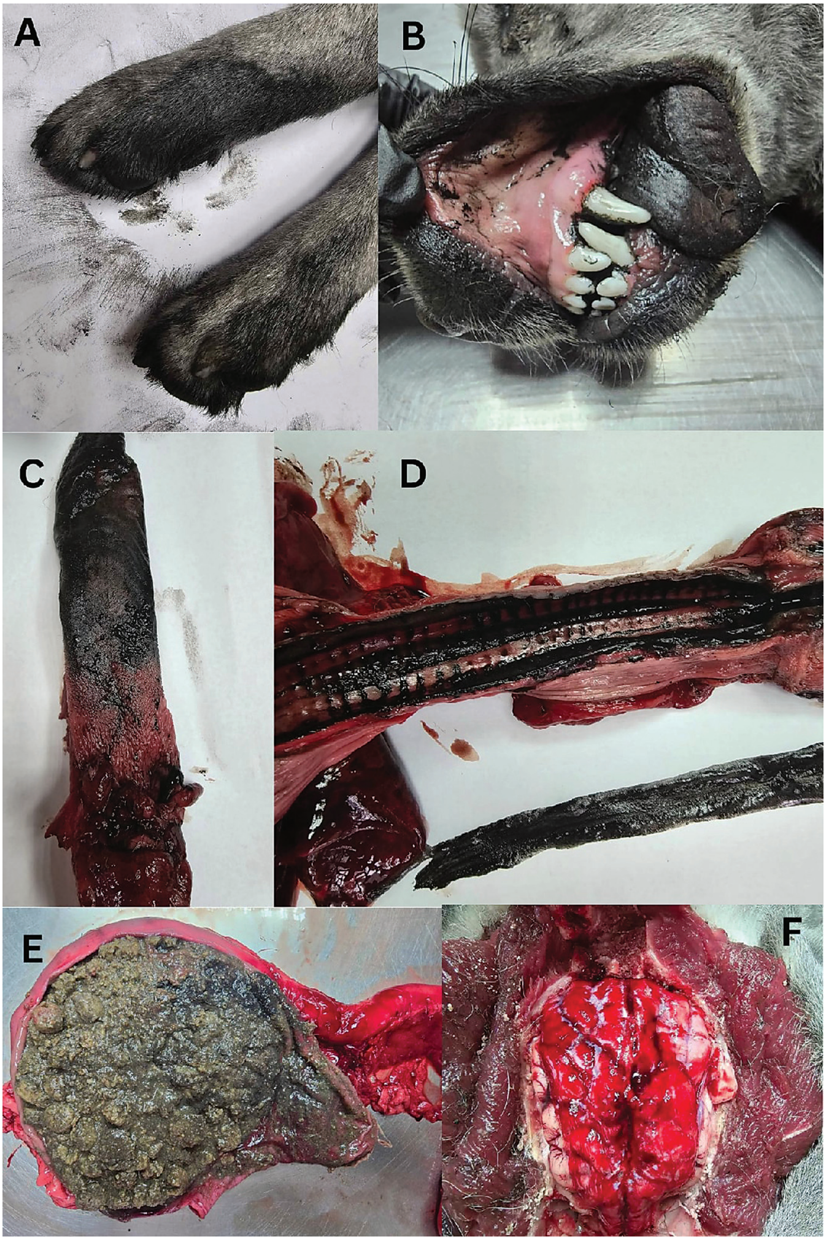

Figure 1. 1a) heavily contaminated coat with soot on the distal parts of the forelimbs; 1b) cherry-red discoloration and contained soot particles in oral mucosa; 1c) blackened and soot on the tongue; 1d) The trachea completely filled with a dark black-gray, foamy material; 1f) meningeal hyperemia, characterized by diffuse vascular congestion.

Gross examination of the stomach revealed black pigmentation of the gastric contents (Figure 1e), indicative of ingested soot, likely resulting from the animal's inhalation and subsequent swallowing of combustion by-products during the fire. Examination of the brain revealed marked meningeal hyperemia, characterized by diffuse vascular congestion, indicative of an acute hypoxic event, most likely resulting from smoke inhalation (Figure 1f).

Following necropsy, tissue samples (lungs, heart, liver, kidney, gaster, trachea) were collected for histopathological analysis and fixed in 10% neutral buffered formalin. The samples were embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E). For the toxicological evaluation of potential carbon monoxide poisoning, postmortem blood samples were collected from the heart and major blood vessels in order to determine carboxyhemoglobin (COHb) concentrations via spectrophotometric analysis.

Microscopic examination

Microscopic examination reveaed that the respiratory system is predominantly affected. In the lungs, pulmonary edema (Figure 2a) is evident, characterized by the accumulation of homogeneous eosinophilic fluid within the alveolar spaces. Anthracotic pigment (coal dust particles) is observed in the alveoli (Figure 2b), along with desquamated alveolar epithelial cells and intra-alveolar hemorrhages, indicated by the presence of extravasated erythrocytes. The vasculature of the alveolar septa and the larger interstitial vessels exhibited marked hyperemia with luminal spaces densely packed with erythrocytes. In the areas unaffected by edema, alveolar emphysema was observed, characterized by rupture of alveolar septa and coalescence of adjacent alveoli.

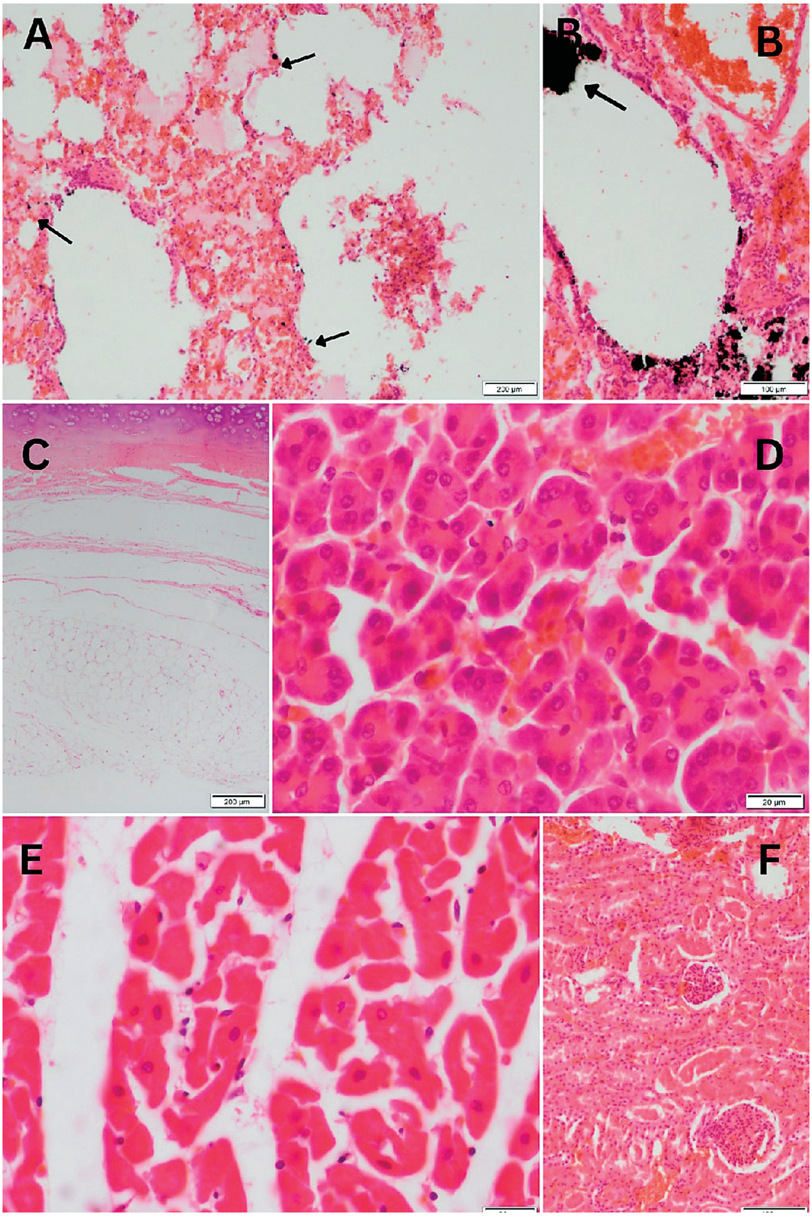

Figure 2. 2a) pulmonary edema characterized by the accumulation of homogeneous eosinophilic fluid within the alveolar spaces; 2b) desquamated alveolar epithelial cells and intra-alveolar hemorrhages; 2c) pronounced hyperemia and epithelial architectural distortion in trachea; 2d) pyknosis in the hepatocytes which are notably swollen; 2e) myocardial tissue revealed multifocal to coalescing areas of coagulative necrosis, accompanied by interstitial edema and neutrophilic infiltration.

The tracheal mucosa displayed significant structural damage, including pronounced hyperemia and epithelial architectural distortion. Surface accumulation of desquamated epithelial cells and particulate carbonaceous material is noted (Figure 2c). Additionally, a homogeneous, partially coagulated eosinophilic exudate was present on the epithelial surface.

In the hepatic tissue, hepatocytes are notably swollen, with cytoplasmic vacuolation and mildly cloudy appearance. Cell nuclei were prominent, with some displaying features consistent with pyknosis (Figure 2d). The sinusoidal capillaries are engorged with erythrocytes, indicating sinusoidal congestion.

Microscopic examination of the myocardial tissue revealed multifocal to coalescing areas of coagulative necrosis, accompanied by interstitial edema and neutrophilic infiltration (Figure 2e). Myocardial fibers exhibited wavy degeneration and occasional contraction band necrosis, suggestive of catecholamine-induced injury and hypoxia. Vascular congestion and perivascular hemorrhage were also noted.

Microscopic examination of renal tissues revealed mild interstitial edema and mononuclear inflammatory infiltrates along with occasional glomerular congestion (Figure 2f). The kidneys exhibited features consistent with acute tubular necrosis, including epithelial cell desquamation, cytoplasmic vacuolation, and loss of brush borders.

Histopathological examination of cerebral tissues revealed changes consistent with acute hypoxic-ischemic encephalopathy. Neurons, particularly within the hippocampus and cerebral cortex, showed eosinophilic cytoplasm and pyknotic nuclei, indicating irreversible injury (Figure 3a). Perivascular and perineuronal edema (Figure 3b) were common, along with congestion of meningeal and parenchymal vessels. Microglial nodules and reactive astrocytosis (Figure 3c) were observed, indicating an early glial response to injury.

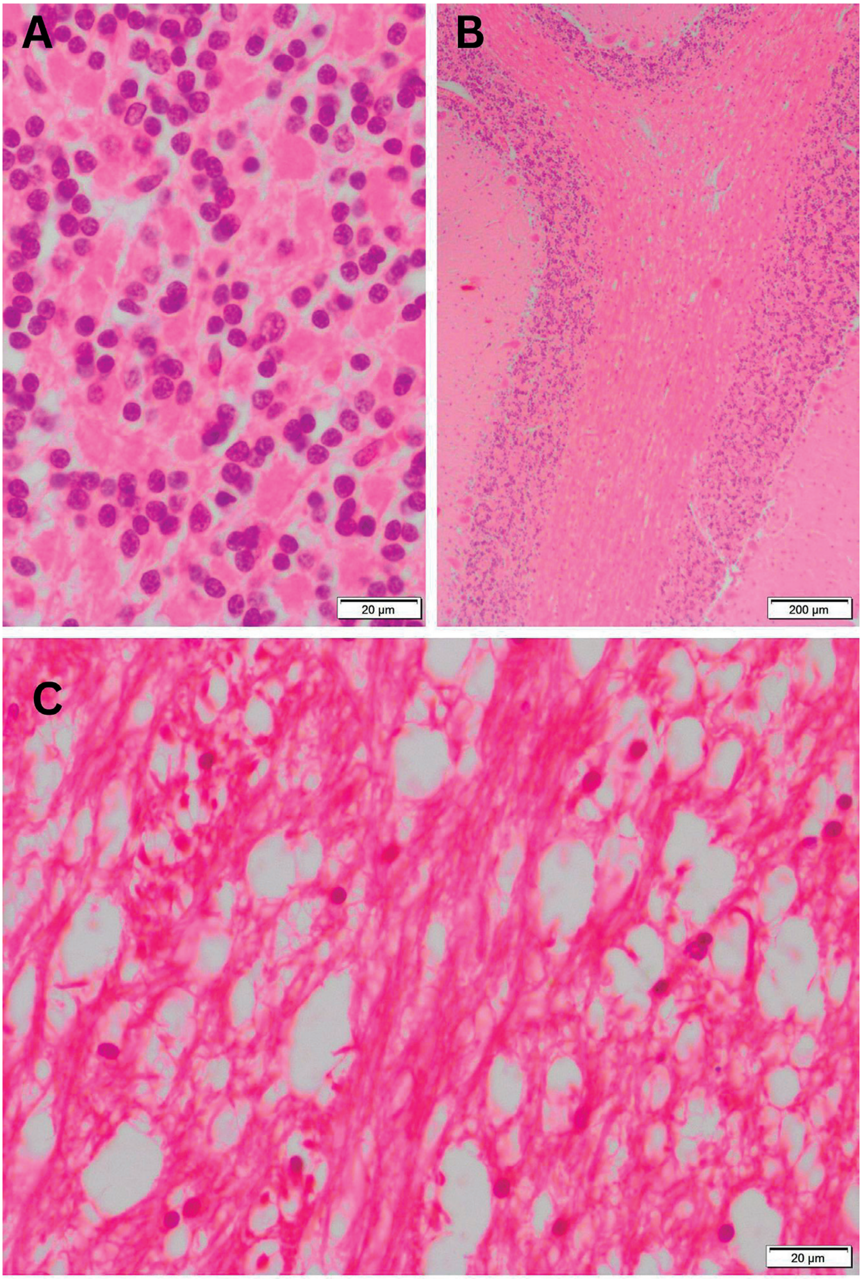

Figure 3. Histopathological examination of the brain

Toxicological analysis

Carboxyhemoglobin (COHb) levels were determined using a spectrophotometric method. To 0.5 mL of blood, 2 mL of distilled water was added. 1mL of obtained hemolysate was taken and mixed with 4 mL of acetate buffer pH = 5 (5 mol/dm^3 acetic acid and 3 mol/dm^3 sodium-acetate (1:3)) and sample was heated at 55 °C for 5 minutes. The sample was then cooled under a stream of water and subsequently centrifuged at 3000 rpm. Liquid sample was filtered and absorbance was measured at 533 nm. The concentration of COHb was calculated using the following formula: %COHb = Abs/0,0405.

DISCUSSION

Thermal injuries, although relatively uncommon in veterinary pathology (Munro and Thrusfield, 2001), pose a significant challenge in the field of veterinary forensics. In animals, this type of injuries can be caused by open fire, boiling water, chemicals, ionizing radiation, as well as by improper use of electric heaters or insufficiently grounded plates of electric scooters (Zhenikhova et al., 2024). In fire-related fatalities (FRFs), death may result directly from extensive thermal trauma, including cutaneous burns or indirectly from the inhalation of smoke and the consequent exposure to toxic combustion products (Madea et al., 2004, Jordaan et al., 2020).

Inhalation of hot and toxic gases is one of the primary contributors to mortality in fire victims, underscoring the importance of comprehensive toxicological investigations. During fires, smoke and combustion gases contain a complex mixture of toxic substances, including carbon monoxide (CO), cyanide (CN), acrolein and other pulmonary irritants (Alarie, 2002; Madea et al., 2004). These substances are produced during the combustion or smoldering of various materials (furnishings, clothing and plastics) leading to chemical injury of the tracheobronchial airways and pulmonary parenchyma (Farrow, 1983; Geor and Ames, 1991). Exposure to these toxic compounds can cause rapid unconsciousness or death, often occurring before any visible external injuries become apparent. Smoke inhalation injury (SII) refers to the inhalation of superheated gases, steam, chemical vapors, and particulate matter, leading to primary damage to the respiratory tract. Smoke inhalation induces three distinct physiological types of injury: (a) thermal injury, predominantly affecting the upper airway; (b) chemical injury, impacting both the upper and lower respiratory tract; and (c) systemic effects of toxic gases, such as CO and CN (Gupta et al., 2018) or any combination of these. The prevalence of smoke inhalation injury (SII) in small animals remains unclear, primarily due to high prehospital mortality rates and the lack of a centralized reporting database (Rosati et al., 2020).

Forensic indicators such as the presence of soot in the airways, esophagus or stomach can provide valuable information regarding the animal's physiological status at the time of the fire. Additionally, toxicological findings, especially the elevated levels of COHb or CN, are pivotal in establishing the cause of death (Madea et al., 2004).

The veterinary forensic expert plays a vital role in determining whether an animal was alive at the time of fire exposure - an essential factor in establishing the cause and manner of death during legal investigations. Evidence of vital reactions to fire, such as the inhalation of hot gases or steam, often coexists with varying degrees of postmortem thermal damage (Madea et al., 2004). One of the most significant indicators is the presence of soot on the mucous membranes of the oral and nasal cavities, as well as throughout the respiratory and upper digestive tracts, including the stomach, which suggests the animal was breathing and swallowing during the fire (Stern et al., 2014). However, the interpretation of soot deposition requires careful consideration. While the presence of soot in the deep airways and digestive tract serves as a strong indicator of life during the fire, its absence does not necessarily rule out antemortem exposure (Madea et al., 2004). Soot particles can passively enter the oral cavity of a deceased animal, potentially depositing on the tongue, pharynx or even the glottis. In contrast, the presence of soot deeper within the trachea or in the stomach provides more definitive evidence of vital exposure (Knight, 1996; Madea et al., 2004). Additional respiratory findings, including epithelial damage, loss of cilia, increased mucus production, and edema (particularly in the proximal trachea) further support the diagnosis of smoke inhalation and are most effectively evaluated through histological sampling at multiple levels (Galeiras, 2021).

Soot was identified within both airways and alveolar spaces in this case report. These findings are similar to those reported by Stern et al. (2014). The presence of pulmonary edema, characterized by homogeneous eosinophilic fluid accumulation within alveoli, aligns with observations in both canine and bear models. For instance, a study by Tunç and Kutsal (2023) on bears revealed similar pulmonary lesions, including edema and emphysema, following smoke exposure. These findings suggest that the severity of pulmonary damage is dose-dependent and can vary based on species and exposure duration.

Significant damage to the tracheal mucosa, including hyperemia, epithelial irregularities, and soot particle deposition, has been documented in this case report. A case report by Rosati et al. (2020) describing a dog with obstructive tracheal necrosis secondary to smoke inhalation documented similar mucosal injuries. Such lesions are indicative of the direct toxic effects of inhaled smoke on airway epithelium. The findings of surface accumulation of desquamated epithelial cells and particulate carbonaceous material in trachea in this case report are similar to those reported by Wohlsein et al. (2016).

The observed hepatic congestion, characterized by swollen hepatocytes and sinusoidal erythrocyte accumulation, is in line with the findings commonly reported in cases of smoke exposure. A study on toxic smoke inhalation in fire dog victims by Stern et al. (2014) reported hepatic congestion and cellular alterations consistent with the effects of systemic hypoxia and circulatory disturbances.

The skeletal muscle changes observed in this case report, including coagulative necrosis and interstitial edema, are notable findings this case report. These alterations are often attributed to ischemic injury and inflammatory responses following smoke inhalation.

Microscopic examination of renal tissues from dogs exposed to smoke inhalation in this case report revealed acute tubular necrosis, characterized by tubular epithelial cell necrosis, loss of brush borders, cytoplasmic vacuolation, and occasional glomerular congestion. Interstitial edema and mild infiltration of inflammatory cells were also noted. These alterations are likely secondary to systemic hypoxia, carbon monoxide toxicity, and oxidative stress resulting from exposure to smoke constituents.

The extent of renal damage appeared to correlate with the duration and severity of smoke exposure.

Myocardial lesions may reflect a combination of hypoxia, oxidative stress and sympathetic overactivity. Cerebral damage appears to be primarily hypoxic in nature; however, the potential for direct neurotoxic effects from combustion byproducts cannot be excluded. These findings are in line with changes reported in human and experimental models of smoke exposure (Gorgun et al., 2017).

CO is the primary cause of fatalities in residential and room fires (Rodkey et al., 1974). CO poisoning, a common and potentially fatal consequence of smoke inhalation during fires, occurs when CO binds to hemoglobin with an affinity approximately 250 times greater than that of oxygen, forming COHb and thereby severely impairing oxygen delivery to tissues. Clinical signs of CO toxicity typically emerge when COHb levels exceed 10% (Tintinalli et al., 2016), with progression to lethargy, disorientation and eventually cardiac arrest at concentrations above 50%. There is no direct correlation between

COHb levels and the presence or intensity of clinical, gross, or histologic signs, though levels above 10% are considered to be indicative of toxicity (Ashbaugh et al., 2012). The central nervous system and heart are particularly vulnerable to hypoxic injury (Merck and Miller, 2013). In animals which have higher metabolic rates than humans, lethal COHb levels can be reached more rapidly. Postmortem detection of elevated COHb levels serves as a reliable indicator that the animal was alive during the fire and had inhaled toxic gases (Rose et al., 2017; Ashbaugh et al., 2012; Piantadosi, 1999). CO has a high affinity for myoglobin, and binding to cardiac myoglobin can lead to myocardial depression, hypotension and arrhythmias. Cardiac decompensation further exacerbates tissue hypoxia, which ultimately leads to death (Blumenthal, 2001).

In forensic fire investigations involving animals, laboratory testing plays a critical role, providing crucial evidence for establishing the cause of death. The detection of COHb in postmortem blood samples serves as a key marker of antemortem inhalation, thereby reinforcing conclusions derived from gross pathology and histopathological findings.

The integration of gross pathology, microscopic examination and toxicology is indispensable for accurately distinguishing between various forms of fire-related trauma and for providing objective, scientifically grounded evidence essential for legal investigations into animal fatalities (Wohlsein et al., 2016). In order to establish a definitive diagnosis in cases of fire-related fatalities in animals, a thorough evaluation of the anamnestic data, in conjunction with both macroscopic and microscopic findings, as well as comprehensive toxicological analysis, is crucial.

This case highlights the importance of a thorough forensic veterinary approach in the investigation of animal deaths related to fire incidents and underscores the critical importance of a comprehensive forensic veterinary approach in determining the cause of death in animals exposed to fire-related incidents. In this case, the presence of soot on the mucosal surfaces of the trachea, esophagus and stomach indicates exposure to open fire (Wohlsein et al. 2016).

The presence of soot on the mucosal surfaces of the trachea, esophagus and stomach suggests vital reactions of the organism and confirms exposure to smoke and combustion products during the agonal or pre-mortem phase. This finding serves as a key indicator of vitality and respiration during the fire (Stern et al., 2014). The presence of inhaled smoke particles in the stomach further corroborates the likelihood of life during the smoky phase of the fire (Knight, 1996; Madea et al., 2004). Additionally, the detection of soot in the airways or upper gastrointestinal tract is nearly always associated with toxic or lethal concentrations of COHb and CN (Stoll et al., 2016), as confirmed by spectrophotometric analysis. This report also underscores the value of postmortem blood as a reliable sample for diagnostic confirmation.

In this case, the absence of thermal injuries, combined with the presence of soot particles in the respiratory and digestive tract and elevated COHb levels strongly supports the conclusion that the dog deceased due to indirect effects of fire - smoke inhalation and the associated exposure to toxic combustion products.

CONCLUSION

This case highlights the critical importance of comprehensive forensic veterinary examination integrating gross pathology, histopathology and toxicology for determining the cause of death in fire-related animal fatalities. It also underscores the evidentiary value of soot distribution and COHb quantification in differentiating antemortem from postmortem fire exposure.

Author's Contribution

JAR - performed the necropsies and wrote the manuscript, EN - English proof reading, ID - preparation of histological slides using the standard hematoxylin and eosin (H&E) staining method and the analysis and interpretation of histopathological findings, VN performed necropsy, DV participated in writing of the manuscript, SĐ performed toxicological analysis, DD designed the study and participated in writing of the manuscript.

Competing interest

The authors declare no conflict of interest.

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Chen J. and McClane B.A. 2015. Characterization of *Clostridium perfringens* TpeL toxin gene carriage, production, cytotoxic contributions, and trypsin sensitivity. Infection and Immunity, 83: 2369–2381. https://doi.org/10.1128/iai.03136-14

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

Books:

Ficken, M. D. and Wages, D. P. 1997. Necrotic enteritis in Diseases of Poultry, Eds. B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif, Iowa State University Press, Ames, Iowa, USA, 10th edition.

Chapters in books:

Plumb J.A. and Hanson L.A. 2011. Sturgeon viruses. In *Health maintenance and principal microbial diseases of cultured fishes*. Eds. J.A. Plumb, L.A. Hanson, 3rd edition, Blackwell Publishing, 219-225.

Articles in proceedings:

Giangaspero A., Marangi M., Pati S., Cafiero M.A., Camarda C., Sparagano O.A.E. 2011. Investigating the presence of acaricide residues in laying hens naturally infected by the red mite *Dermanyssus gallinae*. In *Book of Abstracts*, The 12th Asian food conference 2011, BITEC Bangna, Bangkok, Thailand, 27.

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Lazić G., Lazić S., Bugarski D., Grubač S., Lupulović D., Samojlović M., Petrović T. 2018. Human enteroviruses in river water and sewage in Vojvodina. In *Book of Abstracts*, International Scientific Conference "Green economy and environment protection", Belgrade, 23-25. April 2018, edited by Larisa Jovanović, Belgrade, Naučno stručno društvo za zaštitu životne sredine "ECOLOGICA", 95-96.

Lows and Regulations:

European Union. 2003. Commission Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition, Official Journal of the European Union, L 268:29. https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2003R1831:20100901:EN:PDF

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European Food Safety Authority. 2016. Peer review of the pesticide risk assessment of the active substance benzoic acid. EFSA Journal, 14(12):4657-n/a. http://dx.doi.org/10.2903/j.efsa.2016.4657.

Software:

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

Web Links:

OIE: Animal Diseases. Available at: http://www.oie.int/en/animal-health-in-the-world/information-on-aquatic-and-terrestrial-animal-diseases/. Accessed 07.08.2019.

European Centre for Disease Prevention and Control (ECDC). Historical data by year - West Nile fever seasonal surveillance. Available at: https://ecdc.europa.eu/en/west-nile-fever/surveillance-and-disease-data/historical Accessed 31.07.2019.

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