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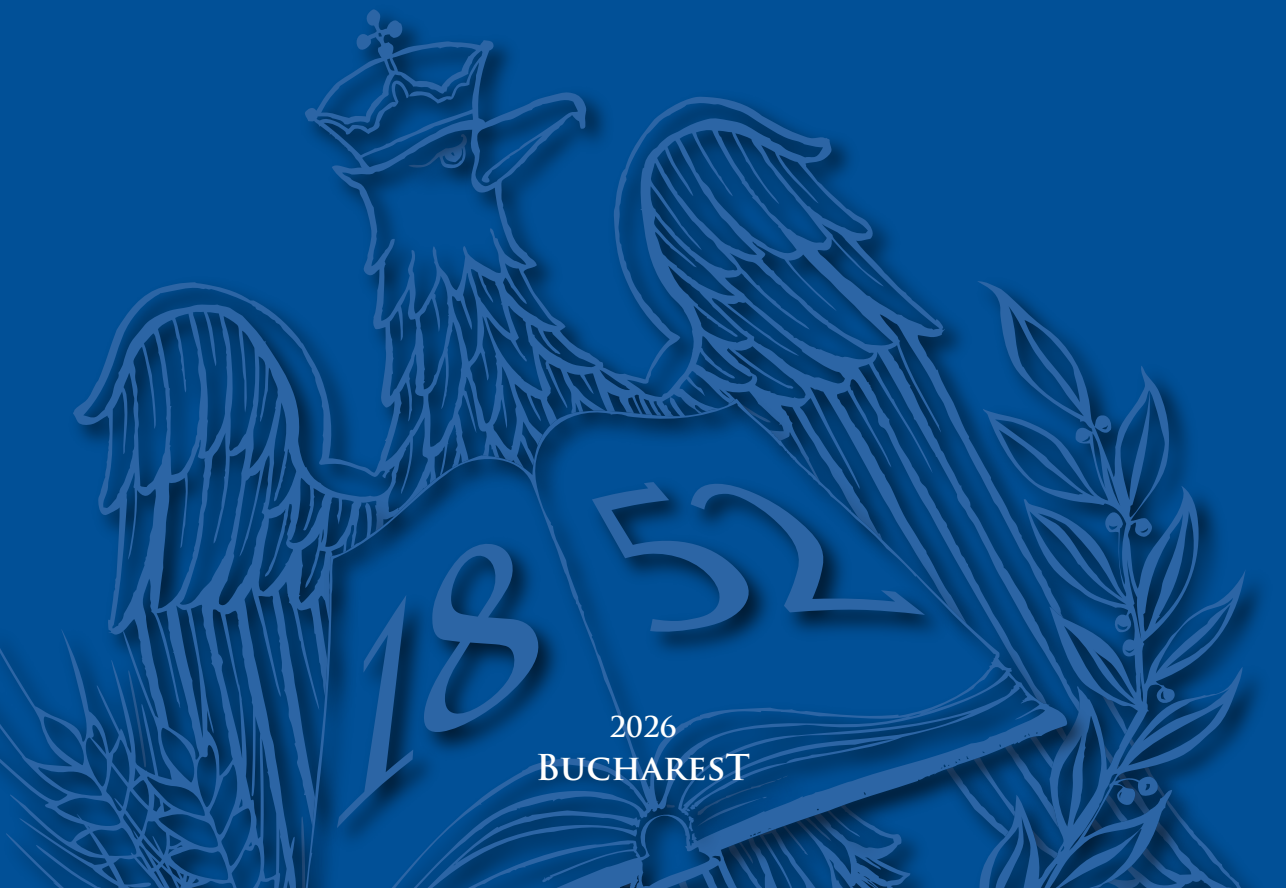

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MOLECULAR DETECTION OF CORONAVIRUSES (*CORONAVIRIDAE*) BY RT-qPCR IN BATS FROM A WILDLIFE REHABILITATION CENTRE IN ROMANIA

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Abstract

Bats are recognized as natural reservoirs for numerous viruses, including the family Coronaviridae. The aim of this study was to investigate the presence of coronaviruses related to the Sarbecovirus subgenus in bats admitted to the Wildlife Rescue and Rehabilitation Center. A total of 22 samples from 18 bats were analyzed, including 14 intestinal samples and 8 oral swabs. For two larger individuals, three intestinal segments were analyzed separately. RNA extraction was performed using the Auto-Pure32 system with the Nucleic Acid Extraction or Purification Reagent (MedicalSystem), followed by RT-qPCR detection (CFX96 Touch Real-Time PCR Detection System) using the STAT-NAT-SARS-CoV-2 kit (Sentinel-Diagnostics) targeting the E gene common to Sarbecovirus and the N and RdRp genes specific to SARS-CoV-2. Testing with the 2019-nCoV kit (PrimerDesign) confirmed the absence of SARS-CoV-2. Four bats (22.2%), identified as Pipistrellus kuhli, Nyctalus noctula, and Vespertilio murinus, were positive for the E gene (4/14 intestinal samples and 2/8 oral swabs). These findings indicate the presence of Sarbecovirus in bats from the Bucharest-Ilfov and Prahova region, highlighting the importance of continued molecular surveillance within a One Health framework.

Key words*: Chiroptera, coronaviruses, Sarbecovirus, RT-qPCR, wildlife surveillance.*

INTRODUCTION

Bats (order *Chiroptera*) comprise approximately 1,690 species with a broad geographic distribution and represent the second most diverse group of mammals after rodents (Burgin et al., 2018). Their social organization, particularly the formation of large multispecies colonies, facilitates the maintenance and transmission of viral pathogens within bat populations (Kerth, 2008). In addition to their essential ecological roles in insect control, pollination, and seed dispersal, bats have attracted increasing scientific interest due to their capacity to act as natural reservoirs for numerous viruses, including members of the family *Coronaviridae* (Woo et al., 2006; Wang & Anderson, 2019).

Interest in bat-associated coronaviruses (CoVs) increased considerably following major global outbreaks such as Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS) (Lelli et al., 2013), and, more recently, the COVID-19 pandemic (Pisoschi et al., 2022; Marchi et al., 2024). Phylogenetic analyses demonstrated that the viruses responsible for these outbreaks are closely related to coronaviruses detected in bats, suggesting that bats are important natural reservoirs for viruses in the subgenus *Sarbecovirus* (Lau et al., 2005; Zhou et al., 2020). Extensive studies have highlighted the remarkable diversity of bat coronaviruses and their important role in coronavirus evolution (Woo et al., 2006).

Coronaviruses are enveloped positive-sense single-stranded RNA [(+), ssRNA] viruses classified within the order *Nidovirales*, family *Coronaviridae*, and subfamily *Orthocoronavirinae* (Woo, De Groot et al., 2023). They are divided into four genera: *Alphacoronavirus* (α-CoV), *Betacoronavirus* (β-CoV), *Gammacoronavirus* (γ-CoV) and *Deltacoronavirus* (δ-CoV). All mammalian coronaviruses, including those detected in bats, belong to the α-and β-CoV genera (Balboni et al., 2011). Importantly, highly pathogenic

human coronaviruses such as SARS-CoV, MERS-CoV, and SARS-CoV-2 belong to the β-CoV genus. Previous studies identified bats from the genus *Rhinolophus* as natural hosts of SARS-CoV and SARS-like coronaviruses (Gouilh et al., 2018), while more recent investigations suggested that *Rhinolophus ferrumequinum* populations in Italy may act as reservoirs for SARS-CoV-2-related viruses (Briggs et al., 2023).

In Europe, surveillance studies have reported the presence of coronaviruses in several bat genera, including *Pipistrellus*, *Nyctalus*, *Vespertilio*, and *Rhinolophus* (Lelli et al., 2013), and have identified both α-CoVs and β-CoVs in bat populations from Italy, significantly expanding knowledge of bat-CoV ecology in Europe. Additional studies from Sardinia and other European regions further confirmed the circulation of β-CoVs in bat populations and emphasized the importance of bats as wildlife hosts for coronaviruses within the "One Health" framework (Lecis et al., 2019; Leopardi et al., 2023; Mira et al., 2025). However, data regarding the circulation of bat-associated coronaviruses in Eastern Europe, particularly in Romania, remains limited.

The present study aimed to investigate the presence of coronaviruses belonging to the family *Coronaviridae*, subgenus *Sarbecovirus*, in bats admitted to the Wildlife Rescue and Rehabilitation Centre of the "Visul Luanei" Foundation using RT-qPCR-based molecular detection methods. Intestinal samples were collected post-mortem from bats that died naturally during rehabilitation, while oral swabs were obtained from live individuals during routine examinations; therefore, no animals were euthanized or otherwise distressed for this study. Although the study is limited by the relatively small sample size and the absence of genomic sequencing, the results contribute to the current understanding of coronavirus circulation in Romanian bat populations and highlight the importance of continued wildlife surveillance for emerging zoonotic pathogens.

MATERIALS AND METHODS

Study Design and Sample Collection

This study investigated the presence of viral RNA belonging to the family *Coronaviridae*, genus *Betacoronavirus*, subgenus *Sarbecovirus*, in samples collected from bats admitted to the Wildlife Rescue and Rehabilitation Centre of the "Visul Luanei" Foundation, Romania. The bats originated from different regions of the country and were brought to the centre for rehabilitation and veterinary care. A total of 22 biological samples were collected from 18 bats, including 14 post-mortem intestinal samples from 10 bats that died naturally during rehabilitation (Table 1), while oral swabs were obtained from 8 live bats during routine clinical examination and care procedures (Table 2). For two larger individuals, three intestinal segments (jejunum, ileum, and colon) were sampled separately. All samples were collected under appropriate biosafety conditions. Oral swabs were immediately immersed in 350 µL RNA Stabilization Solution (ELK-Biotechnology), while intestinal tissues were preserved in 500 µL of the same solution and stored at −80°C until analysis. No animals were euthanized for the purposes of this study.

Table 1. Description of the bat group from which intestinal mass samples were collected (original photos)

ID & Species	Sex	Weight (g)	Evaluation	Location	Death
25-2585 *Pipistrellus kuhlii* 	M	3.9	Emaciated, moderate dehydration	Bucharest	25.07.25
25-3092 *Pipistrellus kuhlii* 	M	6.2	Suspected septicemia (cat attack), agony, physical trauma	Ilfov (county)	04.09.25

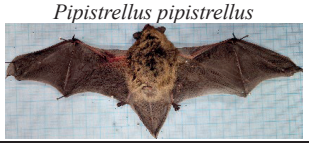
ID & Species	Sex	Weight (g)	Evaluation	Location	Death
25-2840 *Pipistrellus nathusii* 	F	3.7	Inflammation of the left radiocarpal region and the right 2nd phalanx	Bucharest	12.08.25
25-204 *Pipistrellus kuhlii* 	M	4.5	Underweight, severely dehydrated	Bucharest	16.02.25
25-547 *Vespertilio murinus* 	M	8.9	Mite infestation, severe dehydration and underweight, open fracture of the right femur	Brasov (county)	22.04.25
25-87 *Nyctalus noctula* 	M	22.1	Fracture at the level of phalanx II and II, necrotic tissue, patagium tears, right eye anophthalmia	Prahova (Sinaia)	20.01.25
24-3131 *Nyctalus noctula* 	F	27.3	Hematoma in the right-wing radio-ulnar region, inflamed right carpal joint	Brasov (county)	12.03.25
25-594 *Pipistrellus pipistrellus* 	M	5.3	Proximal open radio-ulnar fracture, right-wing	Bucharest	22.04.25
25-712 *Hypsugo savii* 	F	3.4	Missing phalanges on the right-wing	Timisoara (county)	05.05.25
25-3081 *Pipistrellus pipistrellus* 	F	4.0	Radiocarpal dislocation on the left-wing	Prahova (Ploiesti)	03.09.25

Table 2. Description of the bat group from which saliva samples were collected (*original photos*)

ID & Species	Sex	Weight (g)	Location	Diagnosis
25-3260 *Vespertilio murinus* 	F	17.0	Bucharest	Clinically healthy
25-3281 *Vespertilio murinus* 	F	12.0	Prahova (county)	Clinically healthy, slightly malnourished
25-3029 *Vespertilio murinus* 	M	14.0	Bucharest	Clinically healthy, slightly malnourished
25-852 *Nyctalus lasiopterus* 	F	58.1	Bucharest	Born in captivity, clinically healthy
25-3045 *Pipistrellus kuhlii* 	M	5.0	Bucharest	Clinically healthy
25-402 *Nyctalus lasiopterus* 	F	81.9	Bucharest	Clinically healthy

ID & Species	Sex	Weight (g)	Location	Diagnosis
25-3659 *Vespertilio murinus* 	F	14.0	Bucharest	Clinically healthy
25-3718 *Pipistrellus kuhlii* 	M	6.0	Bucharest	Old wound on the right patagium, clinically healthy

Sample Processing, RNA Extraction, and Purification

Intestinal tissues (25 mg) were mechanically homogenized in 500 µL lysis buffer (Nucleic Acid Extraction or Purification Reagent, MedicalSystem) using a Bioprep 24R homogenizer (Hangzhou Allsheng Instruments Co.) with ceramic beads. Tissue lysates were centrifuged at 10,000 × g for clarification, and the supernatants were used for RNA extraction. Following homogenization (4,000 rpm), the swab was removed from the collection tube, and 250 µL of the resulting suspension was used for RNA extraction.

Total RNA was extracted and purified using the Nucleic Acid Extraction or Purification Reagent (MedicalSystem) and the Auto-Pure32 automated extractor (Hangzhou Allsheng Instruments Co.). The method is based on magnetic bead technology and involves nucleic acid binding, washing steps, and final elution in a dedicated buffer.

To monitor extraction efficiency and detect potential PCR inhibition, an exogenous extraction control supplied with the 2019-nCoV Genesig® Advanced Kit (Primerdesign Ltd., UK) was added (10 µL/sample) during the extraction procedure. The control RNA was co-purified with the sample RNA and subsequently detected by RT-qPCR through the HEX channel. Successful amplification of the exogenous control (Ct 28 ± 3) confirmed both extraction performance and the absence of significant PCR inhibition. This approach was adopted because the internal control included in the STAT-NAT® SARS-CoV-2 MULTI Assay is based on the human *RNase P* gene and is therefore not suitable for bat-derived samples.

Molecular Detection of Coronaviruses by RT-qPCR Using the STAT-NAT® SARS-CoV-2 MULTI Assay

The STAT-NAT® SARS-CoV-2 MULTI Assay (Ref. 1N044, Sentinel Diagnostics) is a multiplex one-step RT-qPCR assay targeting the *N* and *RdRp* genes of SARS-CoV-2 (FAM and HEX channels, respectively) and the *E* gene conserved among *Sarbecovirus* members (Texas Red channel). The assay also includes a human *RNase P* internal control (Cy5 channel), which was not applicable to bat-derived samples. Reactions were prepared according to the manufacturer's instructions using 10 µL of extracted RNA in a final volume of 25 µL. Positive and no-template controls (NTC) supplied with the kit were included in each run. Amplification was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The thermal profile consisted of reverse transcription at 50°C for 10 min, followed by enzyme activation at 95°C for 2 min, 10 cycles at 95°C for 15 s and 58°C for 30 s without fluorescence acquisition, and 35 cycles under the same conditions with fluorescence detection in the FAM, HEX, Texas Red, and Cy5 channels.

Independent Confirmation Using the 2019-nCoV genesig® Advanced Kit

To independently verify the results, samples were analyzed using the 2019-nCoV genesig® Advanced Kit (Primerdesign Ltd., UK), a one-step RT-qPCR assay targeting the *RdRp* gene of SARS-CoV-2 (FAM channel), along with the exogenous extraction control (IEC, HEX channel). Reactions were prepared according to the manufacturer's instructions using the PrecisionPLUS™ OneStep 2X RT-qPCR Master Mix (Primerdesign Ltd., UK) in a final reaction volume of 20 µL, including 5 µL of extracted RNA. Positive, NTC and IEC controls were included in each run. RT-qPCR amplification was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with reverse transcription at 55°C for 10 min, enzyme activation at 95°C for 2 min, and 50 cycles of 95°C for 10 s and 60°C for 60 s, with fluorescence acquisition in the FAM and HEX channels.

Validation and Interpretation Criteria

RT-qPCR runs were considered valid when the positive control amplified within the expected Ct range, the NTC showed no amplification, and the IEC exogenous control included in the 2019-nCoV genesig® Advanced Kit amplified within the manufacturer's specified range (Ct 28 ± 5). Amplification results were expressed as cycle threshold (Ct) values generated by the instrument software. Samples with Ct values ≤ 40 were considered positive, whereas samples with Ct values > 40 or showing no amplification were considered negative.

RESULTS AND DISCUSSIONS

Validation of RT-qPCR Assays

All RT-qPCR runs met the predefined validation criteria. For the STAT-NAT® SARS-CoV-2 MULTI Assay, the positive control showed successful amplification of the *N*, *RdRp*, and *E* target genes within the expected Ct range (23.47, 23.44 and 23.12 respectively), whereas the NTC remained negative. Similarly, for the 2019-nCoV genesig® Advanced Kit, the positive control amplified within the manufacturer's specified range (Ct=15.66), while no amplification was observed in the NTC. In addition, the IEC was successfully detected in all intestinal and oral swab samples, confirming efficient RNA extraction and the absence of significant PCR inhibition. Ct values for the IEC ranged from 23 to 28 cycles in intestinal samples and from 25 to 28 cycles in oral swabs. Overall, these results confirmed the analytical validity of all RT-qPCR assays performed in this study.

Detection of *Sarbecovirus*es-Associated RNA

Using the STAT-NAT® SARS-CoV-2 MULTI Assay, amplification of the *E* gene, a target conserved among *Sarbecovirus* members, was detected in samples from four of the 18 bats analyzed (22.2%) (Table 3). Positive results were obtained in both intestinal and oral samples, with Ct values ranging from 22.0 to 23.69. Among intestinal samples, *Sarbecoviruses* associated RNA was detected in one *Pipistrellus kuhlii* individual and in all three intestinal segments (jejunum, ileum, and colon) collected from one *Nyctalus noctula* bat. In addition, two oral swab samples, obtained from *Vespertilio murinus* and *Pipistrellus kuhlii* individuals, were positive for the *E* gene. No amplification of the *N* or *RdRp* genes specific to SARS-CoV-2 was observed in any sample.

Results Obtained with the 2019-nCoV genesig® Advanced Kit

All 22 samples collected from the 18 bats included in the study were additionally analyzed using the 2019-nCoV genesig® Advanced Kit (Primerdesign Ltd., UK), which specifically targets the *RdRp* gene of SARS-CoV-2. No amplification of the *RdRp* target was detected in any of the analyzed samples. The IEC control amplified successfully in all reactions. The assay was considered valid, as the positive control amplified with a Ct value of 15.66, whereas no amplification curve was observed for the negative control. These results did not confirm the presence of SARS-CoV-2 RNA in any of the samples tested.

Table 3. Characteristics of bat samples positive for the *E* gene detected using the STAT-NAT® SARS-CoV-2 MULTI Assay

Bat ID	Species	Sample Type	Location	Ct Value
25-3092	*Pipistrellus kuhlii*	Intestinal content	Ilfov County	22.50
25-87	*Nyctalus noctula*	Jejunum	Sinaia, Prahova County	23.69
25-87	*Nyctalus noctula*	Ileum	Sinaia, Prahova County	23.15
25-87	*Nyctalus noctula*	Colon	Sinaia, Prahova County	22.66
25-3260	*Vespertilio murinus*	Oral swab	Bucharest	23.15
25-3718	*Pipistrellus kuhlii*	Oral swab	Bucharest	22.00

Correlation of Results Obtained with the Two RT-qPCR Assays

The STAT-NAT® SARS-CoV-2 MULTI Assay detected amplification of the *E* gene, a marker common to members of the subgenus *Sarbecovirus*, in samples from four bats. In contrast, no amplification of the SARS-CoV-2-specific *N* or *RdRp* genes was observed with the STAT-NAT assay, and all samples tested negative for the *RdRp* gene when analyzed using the independent 2019-nCoV genesig® Advanced Kit. Taken together, these findings indicate the presence of RNA consistent with Sarbecovirus-related coronaviruses but provide no evidence of SARS-CoV-2 circulation in the analyzed bat samples.

Discussions

The exclusive detection of the *E* gene, a molecular target conserved among members of the subgenus *Sarbecovirus*, together with the absence of amplification of the SARS-CoV-2-specific *N* and *RdRp* genes in two independent RT-qPCR assays, suggests the presence of a bat-associated *Sarbecovirus* distinct from SARS-CoV-2. This interpretation is supported by the Ct values observed in positive samples (Ct range value from 22 to 24), indicating moderated viral RNA amount, and by the detection of positive amplification curve in both intestinal and oral samples.

The analytical validation of the results was further supported by successfully amplification of the IEC positive control in all samples, with Ct values ranging from 23 to 28, indicating efficient RNA extraction and the absence of significant PCR inhibition. In addition, contamination is unlikely, as no amplification was detected in the NTC of either RT-qPCR assay, and no amplification of the SARS-CoV-2-specific *N* or *RdRp* targets was observed.

An interesting finding was the detection of *Sarbecoviruses* associated RNA in all three intestinal segments (jejunum, ileum, and colon) analyzed from a single *Nyctalus noctula* individual, supporting a genuine enteric distribution of the virus. Furthermore, two *Pipistrellus kuhlii* individuals tested positive, one in an intestinal sample and the other in an oral swab. This species is among the most common urban bats in Romania and has frequently been reported in coronavirus surveillance studies conducted across Europe.

The detection of viral RNA in both intestinal and oral samples is consistent with previous reports showing that bat coronaviruses may be shed through the gastrointestinal and respiratory tracts without causing apparent clinical disease in their hosts. The positive individuals originated from Bucharest, Ilfov County, and Sinaia (Prahova County), suggesting that *Sarbecoviruses* strains might be present in bat populations from different regions of Romania.

The overall prevalence observed in the present study (22.2%) falls within the range reported in bat coronavirus surveillance studies conducted in Europe and other geographic regions (Lelli, Papetti et al., 2013; Lecis, Mucedda et al., 2019; Kandeil, Abi-Said et al., 2023; Leopardi, Desiato et al., 2023; Mira, Gucciardi et al., 2025). The detection of viral RNA in *Pipistrellus kuhlii* and *Nyctalus noctula* is consistent with previous findings from Italy, where both α- and β-coronaviruses were identified in these species, supporting their role as hosts of bat-associated coronaviruses in Europe (Lelli, Papetti et al., 2013).

Similarly, Lecis et al. (2019) reported the presence of a bat betacoronavirus in Sardinia and demonstrated phylogenetic relationships with previously described European strains, suggesting regional circulation of related

coronaviruses among bat populations (Lecis, Mucedda et al., 2019). Within a broader One Health framework, Leopardi et al. (2023) identified bats as the vertebrate group harboring the highest coronavirus diversity in Italian wildlife, highlighting the importance of species such as *Pipistrellus kuhlii* in maintaining coronavirus diversity in European ecosystems (Leopardi, Desiato et al., 2023). Comparable findings have also been reported outside Europe. For example, Kandeil et al., (2023) detected both α- and β-CoV in bat populations from Lebanon, further emphasizing the widespread distribution and diversity of coronaviruses in chiropteran hosts (Kandeil, Abi-Said et al., 2023). Earlier studies similarly demonstrated the remarkable genetic diversity of bat coronaviruses and their importance in coronavirus evolution (Woo, Lau et al., 2006; Platto, Zhou et al., 2021).

The absence of SARS-CoV-2-specific targets in all analyzed samples is consistent with recent investigations conducted during the COVID-19 pandemic. In Sicily, Mira et al. (2025) detected bat coronaviruses but found no evidence of SARS-CoV-2 circulation despite extensive molecular screening and phylogenetic analyses (Mira, Gucciardi et al., 2025). Likewise, our results indicate that none of the bats included in the present study harbored detectable SARS-CoV-2 RNA.

Several studies have reported higher prevalence values, particularly in species of the genus *Rhinolophus*, which are considered major reservoirs of SARS-related coronaviruses (Lau, Woo et al., 2005; Balboni, Gallina et al., 2012). Such differences may reflect variation in host species composition, geographic location, sample type, and methodological approaches. Unlike those investigations, the present study included multiple bat species across different genera and focused primarily on molecular screening rather than on viral characterization.

An important limitation of the present study is the lack of genomic sequencing. Although amplification of the *E* gene strongly suggests the presence of a *Sarbecovirus*-related coronavirus, definitive taxonomic identification and phylogenetic characterization were not possible. Many surveillance studies have combined molecular detection with sequencing approaches to confirm viral identity and investigate evolutionary relationships (Lecis, Mucedda et al., 2019; Cerri, Bolatti et al., 2023). Therefore, future studies should include amplification and sequencing of conserved genomic regions, such as *RdRp*, together with larger sample sizes and broader geographic coverage.

CONCLUSIONS

This study confirms the active circulation of coronaviruses from the *Sarbecovirus* subgenus among insectivorous bats in urban and peri-urban areas of Romania, specifically in the Bucharest-Ilfov and Prahova counties.

The detection of viral RNA loads in synanthropic species, specifically *Pipistrellus kuhlii*, *Nyctalus noctula*, and *Vespertilio murinus*, highlights their likely role as local reservoirs for SARS-like coronaviruses. Furthermore, our findings suggest a dual route of potential transmission, with evidence pointing toward both an enteric tropism and viral shedding via oral secretions. While molecular screening confirmed the absence of SARS-CoV-2, the presence of related *Sarbecovirus* strains aligns with international data establishing bats as critical hosts for diverse CoV populations.

Although this study is limited by a small sample size and the absence of genomic sequencing for precise phylogenetic resolution, it provides crucial baseline data for Romania. These findings strongly emphasize the necessity of sustained, large-scale wildlife surveillance integrated within a "One Health" framework to monitor viral diversity and assess zoonotic risks in human-dominated landscapes.

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SEVERE MULTISYSTEMIC FELINE CALICIVIRUS INFECTION WITH EXTENSIVE ORAL AND DERMATOLOGICAL INVOLVEMENT: A CASE REPORT

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Abstract

Feline calicivirus (FCV) commonly causes upper respiratory and oral disease, but rare cases may involve severe systemic manifestations. This report describes an atypical multisystemic FCV infection in a 5-year-old neutered male cat, marked by mucosal necrosis, peripheral oedema, hepatic dysfunction, and non-regenerative anaemia. Clinical signs included lethargy, anorexia, severe lingual ulceration, and limb swelling, with trauma ruled out radiographically. FCV infection was confirmed by PCR. The cat required intensive hospitalisation with fluid therapy, antibiotics, immunomodulation, vitamin supplementation, and assisted enteral nutrition. Complications included hypoalbuminemia, elevated liver enzymes, ulcerative skin lesions, and non-regenerative anaemia requiring erythropoietin therapy. After three months of multidisciplinary management, the cat made a full clinical recovery. This case highlights the potential severity of systemic FCV and the importance of early, comprehensive treatment.

Key words*: anaemia, calicivirus, oedema, multisystemic, ulceration.*

INTRODUCTION

Feline Calicivirus (FCV) is a highly prevalent, non-enveloped, single-stranded RNA virus of the family *Caliciviridae* and a major contributor to feline upper respiratory tract disease worldwide (Radford et al., 2007; Sykes et al., 2014). Marked genetic heterogeneity and prolonged environmental survival facilitate viral persistence in both household and multi-cat settings, despite widespread vaccination (Radford et al., 2009).

Typical FCV infection manifests as oral ulceration, upper respiratory signs, and transient lameness associated with synovitis; however, clinical outcomes are heterogeneous, ranging from subclinical infection to chronic inflammatory oral disease and persistent carrier states (Hurley and Sykes, 2003; Lommer and Verstraete, 2003; Radford et al., 2007).

Rarely, highly virulent systemic variants of FCV give rise to virulent systemic feline calicivirus (VS-FCV) disease, a fulminant syndrome characterised by acute fever, peripheral oedema, cutaneous ulceration, icterus, coagulopathies, and multi-organ failure, with reported mortality rates exceeding 30-50% in outbreak settings (Pedersen et al., 2000; Hurley et al., 2004; Coyne et al., 2006). The pathogenesis of this entity remains incompletely elucidated and is believed to reflect strain-specific virulence rather than predictable host factors (Radford et al., 2007).

Because no targeted antiviral therapy exists, survival depends on early recognition and aggressive, multidisciplinary supportive care (Hurley and Sykes, 2003; Sykes et al., 2014).

Here, we report an atypical multisystemic FCV infection in an adult cat with prolonged hospitalisation and full clinical recovery, underscoring the diagnostic complexity and therapeutic intensity required to successfully manage severe systemic disease.

MATERIALS AND METHODS

A 5-year-old neutered male domestic shorthair cat (body weight: 7.1 kg) was presented with a 3-day history of progressive lethargy, anorexia, hypersalivation, swelling, and pain in the humero-radio-ulnar joint of the front left limb. The cat had indoor and outdoor access and was reportedly vaccinated annually with a modified-live core vaccine, including feline calicivirus. No recent trauma, toxin exposure, or dietary changes were reported.

At presentation, rectal temperature was 39.5°C (reference interval [RI]: 38.0-39.2°C). The patient was estimated to be 5-7% dehydrated based on mucous membrane tackiness and skin turgor. Heart rate was 180-190 bpm (RI: 140-220 bpm), and respiratory rate was 40 breaths/min (RI: 20-30 breaths/min).

Severe ulcerative lesions were observed affecting the ventral and lateral aspects of the tongue, with surrounding mucosal necrosis (Figure 1). Mild peripheral lymphadenomegaly was detected. No cardiac abnormalities or pulmonary crackles were auscultated.

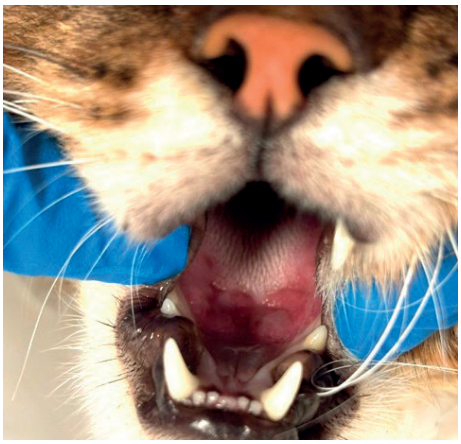

Figure 1. Severe lingual ulceration and mucosal necrosis at initial presentation (5-year-old DSH cat, VS-FCV)

Thoracic and appendicular radiographs were obtained using standard orthogonal views. No fractures, luxation, or osteolytic changes were identified (Figure 2). Soft tissue swelling was evident without underlying skeletal abnormalities.

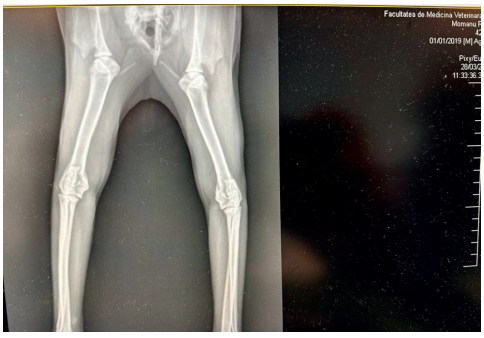

Figure 2. Orthogonal radiographic views of the thoracic limbs showing soft tissue swelling without skeletal abnormalities

Abdominal ultrasonography revealed mild hepatomegaly and diffusely hypoechoic hepatic parenchyma. No free abdominal fluid was detected. Oropharyngeal swabs were collected using sterile nylon swabs. Samples were submitted to a reference diagnostic laboratory for molecular confirmation of feline calicivirus infection. Real-time polymerase chain reaction (RT-PCR) was performed on oropharyngeal and conjunctival swabs using a commercial assay targeting conserved regions of the FCV capsid gene (VP1). FCV RNA was detected, with amplification curves consistent with a positive result, confirming active infection at the time of sampling.

Given the severity and multisystemic nature of the clinical presentation, the diagnostic approach was guided by the need to differentiate between the main viral agents associated with oral and systemic disease in cats.

Feline herpesvirus type 1 (FHV-1) was initially considered due to its common involvement in feline upper respiratory tract disease. However, the absence of characteristic ocular lesions, such as keratoconjunctivitis and corneal ulceration, and the lack of a predominantly oculo-nasal clinical pattern made this diagnosis less likely.

Feline immunodeficiency virus (FIV) was also a key differential diagnosis due to its well-recognised association with chronic oral disease, including gingivostomatitis and periodontitis, and its role in progressive immunosuppression with secondary infections. However, the temporal pattern of disease was not compatible with FIV infection, as FIV-associated oral pathology is typically chronic and progressive, whereas this case presented as an acute, rapidly developing multisystemic ulcerative syndrome with extensive dermatological involvement. In addition, FIV infection was excluded based on a negative enzyme-linked immunosorbent assay (ELISA) result.

Following systematic evaluation of these differentials, the detection of FCV RNA by RT-PCR, in combination with the acute multisystemic clinical phenotype and the exclusion of FHV-1 and FIV, supported feline calicivirus as the most probable primary aetiological agent in this case.

A complete blood count (CBC) at admission (day 0) was performed (Table 1).

Table 1. Complete blood count (CBC) parameters at the time of admission (Day 0) in a cat with suspected systemic feline calicivirus

Parameter	Reference interval	Results
Haematocrit	30.3-52.3%	36.8
Haemoglobin	9.8-16.2 g/dL	11.3
RBC	6.54-12.20 M/µL	8.41
Reticulocytes	3.0-50.0 K/µL	26.1
WBC	2.87-17.02 K/µL	21.24
Neutrophils	2.30-10.29 K/µL	15.43
Lymphocytes	0.92-6.88 K/µL	5.21
Platelets	151-600 K/µL	151

The patient was hospitalized in an isolation ward and managed with intensive supportive therapy. Intravenous isotonic crystalloid solution (Lactated Ringer's Solution) was administered at an initial rate of 4 mL/kg/h for correction of estimated dehydration (5-7%) and maintenance requirements. Fluid therapy was subsequently adjusted according to body weight, urine output, perfusion parameters, and serial electrolyte measurements.

Empirical broad-spectrum antimicrobial therapy was initiated due to the presence of extensive mucosal ulceration and risk of secondary bacterial infection. Amoxicillin–clavulanic acid was administered subcutaneously at 25 mg/kg every 24 hours. Treatment duration was 10 days and was reassessed based on clinical progression and laboratory findings (Table 1).

Analgesic management consisted of buprenorphine administered at 20 mcg/kg intravenously every 12 hours and meloxicam given at 0.1 mg/kg every 24 h subcutaneously. Pain was assessed using a validated feline pain scoring system (Glasgow Composite Measure Pain Scale - Feline), and analgesic dosing was adjusted accordingly. Due to persistent anorexia and severe lingual ulceration impairing voluntary food intake, a nasal feeding tube was placed. Enteral nutrition was initiated using a commercially available recovery diet at 25-33% of the calculated resting energy requirement (RER) on day 1 and progressively increased to full caloric requirements over 5 days. In addition to intensive supportive care, the patient received parenteral vitamin supplementation consisting of B-complex vitamins, including thiamine (vitamin B1, 10 mg/kg), pyridoxine (vitamin B6, 10 mg/kg), and cyanocobalamin (vitamin B12, 0.5 mg/kg), administered to support metabolic function, appetite recovery, and neurological and haematopoietic homeostasis during the acute phase of disease.

The diet was supplemented with an immunonutritional support formulation containing polysaccharides such as arabinogalactan, which exerts immunomodulatory effects by enhancing natural killer (NK) cell activity and also extracts from *Ganoderma lucidum* (Reishi mushroom), which have demonstrated potent immunomodulatory properties through the stimulation of lymphocyte activity. Local management of oral ulcerations included gentle cleansing of the oral cavity and topical application of a gel containing lactoferrin and chlorhexidine, intended to reduce bacterial load, limit secondary infection, and promote mucosal healing. Lactoferrin modulates the host immune response by enhancing the activity of neutrophils, macrophages, and natural killer (NK) cells, while regulating cytokine production to reduce excessive inflammation. It has been shown to support mucosal immunity and promote tissue repair and epithelial healing, particularly in inflamed or ulcerated tissues. Hypoalbuminemia was monitored through serial serum biochemistry analyses. Nutritional support was optimized, and fluid therapy was adjusted to avoid exacerbation of peripheral oedema. Hepatoprotective supplementation (e.g., S-adenosylmethionine) was introduced following detection of elevated hepatic enzyme activity. Progressive non-regenerative anaemia prompted the initiation of recombinant human erythropoietin at 100 IU/kg subcutaneously every 48 hours, in combination with iron supplementation. Hematologic parameters were monitored weekly to assess response and detect potential adverse effects.

Figure 3. Progression of vasculopathic lesions on the distal limbs, characterized by focal epidermal necrosis and marked peripheral oedema

Progressive ulcerative and necrotic cutaneous lesions developed during hospitalization,

initially affecting the distal thoracic limbs and subsequently involving all four limbs.
Lesions were characterized by focal epidermal necrosis, sero-cellular exudation, and peripheral oedema (Figures 3 and 4).
Wound management was conducted under aseptic conditions and followed principles of moist wound healing. Lesions were gently irrigated with sterile isotonic saline solution to remove surface debris and exudate. Mechanical debridement was limited to clearly demarcated non-viable tissue in order to preserve viable granulation tissue.

Figure 4. Extensive digital pad necrosis and ulcerative dermatitis affecting the thoracic limbs

Topical treatment consisted of medical-grade honey–based ointment (L-Mesitran® Soft). L-Mesitran contains standardized medical honey enriched with hypoallergenic lanolin, polyethylene glycol, propylene glycol, and vitamins C and E. The high osmolarity of honey promotes autolytic debridement and reduces local oedema through osmotic fluid shift, while its low pH and hydrogen peroxide–mediated activity contributes to broad-spectrum antimicrobial effects. Additionally, honey has been shown to stimulate angiogenesis, fibroblast proliferation, and epithelialization, thereby supporting granulation tissue formation and wound contraction (Vogt et al., 2021). A sterile non-adherent contact layer was applied over the ointment, followed by secondary absorbent padding and conforming bandage material. Bandages were changed every 48 hours, or earlier in cases of saturation, leakage, or contamination. At each bandage change, wound surface area, degree of exudation, presence of necrotic tissue, and granulation quality were assessed and documented. As lesions progressed to involve all four limbs, care was taken to avoid excessive compression in order to prevent vascular compromise in the context of peripheral oedema. Limb perfusion and distal temperature were monitored at each dressing change.
Systemic antimicrobial therapy and analgesia were continued concurrently, and nutritional support was maintained to optimize tissue repair. Progressive epithelialization and reduction of inflammatory oedema were observed over subsequent weeks, and no surgical wound closure was required (Figure 5).

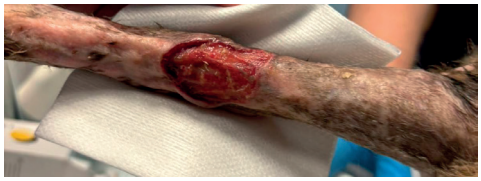

Figure 5. Clinical resolution of full-thickness cutaneous ulceration following protocolized topical medical-grade honey therapy (5-year-old DSH cat, recovery phase)

RESULTS AND DISCUSSIONS

Following admission, the patient demonstrated rapid clinical deterioration characterized by progression from lingual ulceration and pyrexia to peripheral oedema and multifocal ulcerative dermatitis. Within the first two weeks of hospitalization, erythematous distal limb lesions evolved into focal epidermal necrosis and full-thickness ulceration, ultimately affecting all four limbs (Figures 3-5). The anatomical distribution, bilateral symmetry, and absence of radiographic evidence of trauma supported a systemic inflammatory and vasculopathic process rather than mechanical injury.
Virulent systemic feline calicivirus (VS-FCV) has been described as a severe manifestation of FCV infection, characterized by cutaneous ulceration, facial and limb oedema, hepatic dysfunction, and high mortality rates in outbreak settings (Pedersen et al., 2000; Hurley et al., 2004; Coyne et al., 2006). The cutaneous lesions observed in the present case - particularly distal extremity necrosis and digital pad involvement - are consistent with previously reported vasculopathic and endothelial injury patterns associated with highly virulent FCV strains (Hurley et al., 2004; Radford et al., 2007) (Table 2).

Serial hematologic monitoring revealed a progressive decline in haematocrit from 36.8% to a minimum value of 22.6%, consistent with non-regenerative anaemia. Reticulocyte counts remained low during the acute phase, indicating insufficient bone marrow response. Non-regenerative anaemia in systemic viral infections may be multifactorial, potentially involving inflammatory cytokine-mediated suppression of erythropoiesis, transient bone marrow suppression, or anaemia of inflammatory disease (Weiss and Goodnough, 2005). Gradual hematologic recovery following erythropoietin administration suggests reversible marrow suppression rather than irreversible aplasia (Figure 6).

Table 2: Comparative analysis of clinical manifestations between the present case and reported literature for virulent systemic feline calicivirus (VS-FCV)

Parameter	Reported VS-FCV (Literature)	Present Case
Fever	Common	Yes
Peripheral oedema	Frequent	Yes
Cutaneous ulceration	Reported	Severe, 4 limbs
Hepatic involvement	Variable	Elevated enzymes
Anaemia	Occasionally reported	Non-regenerative
Mortality rate	30-50%	Survival
Outbreak setting	Common	Isolated case

The clinical presentation of the present case shows several similarities with virulent systemic feline calicivirus (VS-FCV) reported in the literature. Fever, peripheral oedema, and cutaneous ulceration are commonly described features of VS-FCV infection and were also present in this case, consistent with previously reported outbreak-associated cases (Pedersen et al., 2000; Hurley et al., 2004; Coyne et al., 2006). Hepatic involvement has been variably reported in severe systemic infections and was reflected in this case by increased liver enzyme activity. Anaemia has been occasionally described in VS-FCV cases, although it is not considered a consistent clinical feature; in the present case, a non-regenerative anaemia was identified. A central pathogenic mechanism is generalised vascular endothelial damage, which results in increased vascular permeability, capillary leakage, and tissue oedema. This explains the frequent clinical findings of peripheral oedema, facial swelling, and cutaneous ulceration. Histopathological studies have demonstrated systemic neutrophilic vasculitis and fibrinoid vascular necrosis, suggesting that endothelial injury is a key driver of disease severity (Pedersen et al., 2000; Hurley et al., 2004).

The resulting capillary leak syndrome leads to hypovolaemia, hypoperfusion, and secondary shock, which can progress to multi-organ failure. In parallel, epithelial necrosis in multiple tissues (oral cavity, skin, and occasionally internal organs) reflects both direct viral effects and immune-mediated damage (Hurley et al., 2004; Radford et al., 2007).

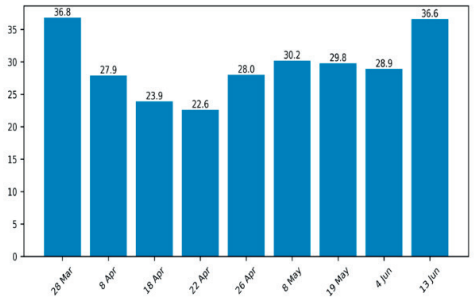

Figure 6. Temporal evolution of haematocrit (%) in a cat diagnosed with systemic feline calicivirus infection over a three-month monitoring period

Biochemical analysis demonstrated progressive hypoalbuminemia concurrent with the development of peripheral oedema. Albumin is the principal determinant of plasma colloid oncotic pressure; therefore, decreased serum albumin concentration likely contributed to interstitial fluid accumulation (DiBartola, 2011). In systemic inflammatory states, hypoalbuminemia may result from decreased hepatic synthesis secondary to acute-phase protein redistribution, increased vascular permeability, and enhanced extravascular protein loss (Radford et al., 2007). The temporal correlation between declining albumin levels and worsening oedema in this patient supports a pathophysiologic contribution of oncotic imbalance in addition to inflammatory endothelial dysfunction (Figure 7.).

Serial monitoring revealed a concurrent decline in haematocrit and serum albumin during the

acute phase of disease. Haematocrit decreased from 36.8% to 22.6% (38.6% relative reduction), while albumin reached a minimum value of 1.9 g/dL, below the reference interval (2.2-4.4 g/dL). The lowest albumin concentration temporally coincided with maximal peripheral oedema and progression of distal limb lesions.

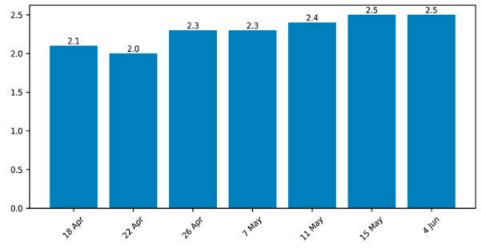

Figure 7. Fluctuations in serum albumin concentration (g/dL) in relation to the clinical development of peripheral oedema

This parallel deterioration supports a systemic inflammatory process. Pro-inflammatory cytokines are known to suppress hepatic albumin synthesis and inhibit erythropoiesis, contributing to hypoalbuminemia and anaemia of inflammation (Weiss & Goodnough, 2005). Increased vascular permeability likely facilitated transcapillary albumin loss, reducing plasma oncotic pressure and promoting interstitial fluid accumulation (Table 3).

Biochemical improvement preceded full hematologic recovery, consistent with restoration of hepatic synthetic function prior to bone marrow regenerative response. These findings indicate coordinated inflammatory, hepatic, and hematopoietic involvement in severe systemic FCV infection and support the reversibility of inflammation-mediated dysfunction under sustained supportive therapy.

Despite the severity of multisystemic involvement, the patient achieved complete clinical recovery following prolonged intensive supportive management. Mortality rates in reported VS-FCV outbreaks have ranged from 30% to over 50% (Hurley et al., 2004; Coyne et al., 2006), underscoring the clinical significance of this favourable outcome. The successful resolution of full-thickness limb ulcerations without surgical intervention further emphasizes the importance of optimized wound management, including the use of medical-grade honey-based topical therapy, which has demonstrated antimicrobial, anti-inflammatory, and pro-angiogenic properties in veterinary wound care (Molan, 2001; Dart et al., 2015).

Table 3. Chronological correlation between clinical progression, hematologic shifts (HCT), and biochemical markers (ALB, ALT)

Time Point	Clinical Findings	HCT (%)	ALB (2.2-4.0 mg/dl)	ALP (14-111 U/L)	Comments
Admission	Lingual ulcer	36.8	2.4	170	Stable
Week 2	Limb oedema	23.9	2.1	215	Anaemia progressing
Week 3	Full limb lesions	22.6	1.9	255	Minimum value
Week 8	Healing phase	30.2	2.0	218	Recovery

HCT – haematocrit; ALB – albumin; ALT – alanine aminotransferase.

This case illustrates the complex interplay between systemic inflammation, vascular permeability alterations, oncotic imbalance, and secondary tissue injury in severe FCV infection. Moreover, it highlights that early recognition, aggressive supportive therapy, and sustained multidisciplinary monitoring can significantly improve prognosis, even in cases exhibiting features consistent with virulent systemic disease.

CONCLUSIONS

This case highlights the potential for severe multisystemic involvement in feline calicivirus infection, extending beyond the classical presentation of upper respiratory and oral disease. The progression to peripheral oedema, full-thickness cutaneous ulceration, hepatic dysfunction, and non-regenerative anaemia

underscores the complex pathophysiology associated with virulent systemic FCV variants. Early recognition of systemic manifestations, close hematologic and biochemical monitoring, and aggressive multidisciplinary supportive management were critical to achieving a favourable outcome in this case. The temporal association between hypoalbuminemia and peripheral oedema further emphasizes the importance of understanding oncotic and inflammatory mechanisms in severe viral disease.

Despite features consistent with virulent systemic FCV, prolonged intensive therapy resulted in complete clinical recovery without surgical intervention. This case reinforces the necessity for prompt diagnosis and sustained therapeutic support in managing severe FCV-associated systemic disease.

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THE USE OF SERUM BIOMARKERS IN MONITORING CARDIAC ONCOPATHIES IN COMPANION ANIMALS

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Abstract

Cardiac oncopathies in companion animals, particularly dogs, are often underdiagnosed due to nonspecific clinical signs and limitations of standard diagnostic tools. In our study involving 42 dogs, we evaluated the diagnostic potential of several serum cardiac biomarkers for detecting myocardial damage or dysfunction of neoplastic origin. The biomarkers tested included cardiac troponin (cTnI), creatine kinase (CK), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and NT-proBNP. Among these, cTnI and NT-proBNP proved to be the most specific and valuable markers for identifying cardiac involvement, whether from primary cardiac tumors or metastatic infiltration. NT-proBNP was particularly useful in differentiating cardiac causes from non-cardiac causes of clinical signs, while elevated cTnI levels reflected direct cardiomyocyte injury. Supporting enzymes such as CK, AST, and LDH indicated tissue damage but lacked specificity for cardiac lesions. The combined profiling of these biomarkers in our cohort of 42 dogs enhanced diagnostic accuracy and may serve as a useful tool for monitoring disease progression and therapeutic response in veterinary cardiac oncology. Further research is needed to establish reference ranges and to tailor applications for specific species.

Key words*: cardiac biomarkers, NT-proBNP, cTnI, creatine kinase, cardiac oncopathies.*

INTRODUCTION

Cardiac oncopathies in companion animals represent an insufficiently explored field in veterinary medicine, despite their significant impact on patient health status and unfavorable prognosis. In dogs, cardiac tumors, whether primary or metastatic, are frequently diagnosed in advanced stages, mainly due to the nonspecific nature of clinical signs and the limitations of current diagnostic methods. This highlights the need for more sensitive, accessible, and minimally invasive diagnostic tools capable of early detection of cardiac involvement and timely referral of the patient for cardiologic evaluation (Oyama et al., 2008; Boswood et al., 2008; Langhorn & Willesen, 2016a; Baisan et al., 2016).

In recent years, the use of serum cardiac biomarkers has gained increasing interest as a promising method for detecting cardiac injury and dysfunction. Biomarkers such as cardiac troponins and natriuretic peptides have already demonstrated their utility in human cardiology, and their applicability in veterinary medicine is becoming increasingly recognized (Langhorn & Willesen, 2016b; Langhorn & Willesen, 2016a; de Lima et al., 2017; Pelander et al., 2017; Baisan et al., 2016; Lobetti et al., 2012; Ljungvall, 2018; Peyron, 2020; Illinois College of Veterinary Medicine, 2019; Rebez et al., 2024). However, their specific role in the context of cardiac oncopathies in companion animals remains insufficiently clarified.

The aim of the present study was to evaluate the diagnostic relevance of a panel of serum cardiac biomarkers, including cardiac troponin I (cTnI), N-terminal pro-B-type natriuretic peptide (NT-proBNP), creatine kinase (CK), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH), in identifying myocardial lesions or dysfunctions of neoplastic origin in dogs. By analyzing their specificity and combined utility, this research aims to contribute to the improvement of early diagnosis, differential diagnosis, and disease progression monitoring in veterinary cardiac oncology (Oyama et al., 2008; Langhorn & Willesen, 2016a; Langhorn & Willesen, 2016b; Smith, 2023; Rebez et al., 2024; Lee et al., 2025; Chompoosan et al., 2025).

MATERIALS AND METHODS

The study was conducted within the "Prof. Univ. Dr. Alin Bîrțoiu" University Emergency Hospital, with the aim of evaluating the diagnostic utility of serum cardiac biomarkers in identifying myocardial damage associated with cardiac oncopathies in dogs. The research included 42 dogs diagnosed or suspected with neoplasms involving primary or secondary cardiac involvement, which were investigated through clinical, imaging, and paraclinical examinations.

The study protocol included the evaluation of the following serum biomarkers: cardiac troponin I (cTnI), N-terminal pro-B-type natriuretic peptide (NT-proBNP), creatine kinase (CK), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH), selected due to their relevance in detecting myocardial injury and cardiac dysfunction.

Figure 1. Automated biochemical analyzer Seamaty SMT-120V used in the study for the determination of serum biochemical parameters and enzymes associated with tissue and myocardial damage (CK, AST, LDH) in the dogs included in the research

Biological samples were collected through peripheral venipuncture using whole blood and serum samples obtained according to standardized laboratory protocols. Biochemical analyses were performed using the automated Seamaty SMT-120V analyzer, employed for the determination of serum enzymatic and metabolic parameters, while the assessment of specific cardiac biomarkers was carried out using immunological methods with the Vcheck V200 (Bionote) system.

Figure 2. Immunological system Vcheck V200 (Bionote) used for the quantification of specific cardiac biomarkers, namely cardiac troponin I (cTnI) and NT-proBNP

The obtained results were centralized and subjected to comparative statistical analysis in order to determine the sensitivity and specificity of each biomarker in detecting cardiac involvement of neoplastic origin. Furthermore, the diagnostic value of the association between cTnI and NT-proBNP was analyzed, together with the relationship between serum biomarker variations and the severity of cardiac changes identified clinically and through imaging investigations. The integrated interpretation of clinical, imaging, and biochemical data allowed the evaluation of the potential of cardiac biomarkers as minimally invasive tools for early diagnosis, differential diagnosis, and monitoring of disease progression in veterinary cardiac oncology.

RESULTS AND DISCUSSIONS

In the present study, 42 dogs diagnosed or suspected with cardiac oncopathies were evaluated, investigating the serum alterations of cardiac biomarkers and their correlation with the degree of myocardial involvement. The obtained results highlighted significant variations in biomarker values depending on the type and severity of cardiac lesions identified clinically and through imaging examinations.

Increased values of cardiac troponin I (cTnI) were consistently associated with the presence of active myocardial lesions, suggesting direct cardiomyocyte injury caused by tumor infiltration, local ischemia, or tissue

compression. Elevated cTnI concentrations were predominantly observed in patients with primary cardiac tumors and in cases with extensive cardiac metastases, confirming the sensitivity of this biomarker for detecting myocardial injury. The obtained results are consistent with the scientific literature, where cTnI is considered one of the most relevant markers of myocardial damage in veterinary medicine.

NT-proBNP showed significantly increased values in patients presenting with cardiac chamber dilation, pericardial effusion, and clinical signs suggestive of heart failure secondary to neoplastic involvement. This biomarker proved useful in differentiating cardiac from non-cardiac respiratory and circulatory manifestations, an important aspect in oncologic patients presenting nonspecific clinical signs. The results support previously reported data regarding the usefulness of NT-proBNP in identifying occult cardiomyopathies and evaluating patients with respiratory signs of cardiac origin. An important finding of the present study was that NT-proBNP did not show markedly elevated values (>200 pmol/L above the maximum reference value) in cases of non-infiltrative chemodectomas.

Serum enzymes CK, AST, and LDH showed moderate to severe increases in most investigated cases, reflecting processes of cellular necrosis, tissue hypoxia, and muscular destruction. However, the reduced specificity of these parameters limits their individual diagnostic value in cardiac oncopathies, requiring interpretation in correlation with specific cardiac biomarkers and complementary imaging investigations. Another important aspect is the limited applicability of these serum enzymes in patients with concomitant hepatic or muscular dysfunctions.

Comparative analysis of the results demonstrated that the combined use of cTnI and NT-proBNP provides the highest diagnostic accuracy in identifying cardiac involvement of neoplastic origin. The combined biomarker profile allowed a more precise assessment of myocardial injury severity and may represent a useful tool for both early diagnosis and monitoring disease progression and therapeutic response in veterinary cardiac oncology.

The use of these parameters should be complemented by echocardiographic examination in order to establish the diagnosis of cardiac oncopathy.

Table 1. Correlation of cardiac biomarkers with the type and severity of neoplastic cardiac involvement in dogs, according to the analyzer reference values

Parameter	Analyzer reference values	Active cardiac tumors	Inactive / stable cardiac tumors	Cardiac tamponade	Arrhythmias associated with neoplastic involvement	Pericardial effusion
cTnI	<0.1 ng/ml = normal 0.1-0.2 ng/ml = suspect >0.2 ng/ml = abnormal	Marked increase (>0.2 ng/ml), indicating active myocardial injury	Normal or slightly increased values	Severe increase due to myocardial compression and ischemia	Moderate increase in conduction system lesions	Moderate increase due to compression and local hypoxia
NT-proBNP	<900 pmol/L = normal 900-1800 pmol/L = suspect >1800 pmol/L = abnormal	Significant increases due to ventricular stress and cardiac remodeling	Values close to normal or slightly increased	Marked increase caused by acute hemodynamic overload	Variable increase depending on the degree of cardiac dysfunction	Moderate to severe increase due to atrial and ventricular distension
CK	0-559 U/L	Moderate increase associated with tissue necrosis	Generally within physiological limits	Increase due to hypoperfusion and secondary muscular injury	May increase during severe arrhythmic episodes	Mild to moderate increase
AST	0-48 U/L	Moderate increase in active tumoral infiltration	Stable values	Increase due to hypoxia and systemic congestion	Variable	Mild to moderate increase
LDH	0-798 U/L	Significant increase in extensive neoplastic processes and necrosis	Slightly increased or normal	Severe increase in tissue hypoxia and circulatory collapse	Moderately increased	Moderate increase due to secondary tissue damage

The use of these parameters does not establish or confirm the diagnosis of cardiac oncopathies; however, they allow the assessment of disease severity, therapeutic evolution under treatment, and the degree of myocardial involvement.

CONCLUSIONS

The results obtained in the present study highlight the usefulness of serum cardiac biomarkers in the evaluation of myocardial damage associated with cardiac oncopathies in dogs. Among the investigated parameters, cardiac troponin I (cTnI) and NT-proBNP demonstrated the highest diagnostic relevance, showing significant correlations with the severity of cardiac lesions identified clinically and through imaging examinations.

Increased cTnI values were mainly associated with active myocardial injury, tumor infiltration, and cardiac ischemia, while NT-proBNP reflected the degree of hemodynamic stress and cardiac remodeling secondary to neoplastic involvement.

The study demonstrated that cardiac biomarker values vary depending on the type and severity of cardiovascular complications associated with the neoplastic process. The most significant increases in cTnI and NT-proBNP were observed in cases complicated by cardiac tamponade, pericardial effusion, and heart failure, suggesting severe myocardial involvement and a guarded prognosis. In contrast, inactive or stable tumor lesions showed values close to physiological limits, indicating reduced pathological activity. An important finding observed during the study was the absence of significant NT-proBNP increases in chemodectomas, suggesting a possible variability in biomarker response depending on tumor type.

Serum enzymes CK, AST, and LDH showed alterations compatible with cellular necrosis and tissue hypoxia; however, their reduced specificity limits their individual diagnostic value in cardiac oncopathies. Furthermore, interpretation of these parameters should be performed cautiously in patients presenting concomitant hepatic or muscular disorders, as these conditions may significantly influence the obtained serum values.

The combination of specific cardiac biomarkers with echocardiographic examination represents the most effective approach for evaluating patients suspected of cardiac oncopathies. The use of these parameters does not establish or directly confirm the diagnosis of cardiac neoplasia; however, they provide important information regarding the severity of myocardial involvement, disease progression, and therapeutic response monitoring. In conclusion, serum cardiac biomarkers may represent valuable complementary tools in the diagnosis and management of veterinary cardiac oncology, although further studies are necessary to standardize their use and interpretation in clinical practice.

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PERIANESTHETIC MANAGEMENT OF DOGS WITH INTRACRANIAL PATHOLOGY UNDERGOING MAGNETIC RESONANCE IMAGING

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Abstract

Magnetic resonance imaging (MRI) is an essential diagnostic modality in veterinary neurology and requires close collaboration among neurologists, anesthesiologists, and radiologists. This study aimed to evaluate the perianesthetic management of 20 dogs with intracranial disorders undergoing MRI, with particular emphasis on protocols and the incidence of anesthesia-related complications. The dogs were assigned to three age-based groups. All patients were referred for MRI following a specialized neurological examination and underwent a comprehensive preanesthetic assessment. After premedication, anesthesia was induced and maintained for 25-38 minutes. The recorded complications included hypothermia (n = 5), delayed recovery (n = 3), hypotension (n = 2), and hypertension (n = 2). These findings highlight that a tailored anesthetic approach, guided by thorough preanesthetic evaluation and proactive management, is essential for optimizing patient safety and clinical outcomes in dogs with neurological disorders.

Key words*: anesthesia, MRI, dogs, neurolog*

INTRODUCTION

Perianesthetic management of patients with intracranial pathology undergoing MRI is of critical importance. In contrast to human medicine, where anesthesiology support may not be routinely required, veterinary MRI examinations necessitate complete patient immobilization to achieve optimal image resolution and diagnostic accuracy. This requirement is reliably fulfilled through the administration of general anesthesia (Pavel et al., 2024).

MRI has significantly contributed to the evolution of veterinary medical research through its application in the study of central nervous system (CNS) and spinal cord disorders (Labruyère & Schwarz, 2013).

This paper aims to demonstrate the importance of tailored anesthetic protocols for patients with intracranial disorders, with particular attention to optimizing welfare and ensuring safety throughout anesthesia.

MATERIALS AND METHODS

This prospective clinical observational study was performed between November 2024 and April 2025 at the Faculty of Veterinary Medicine in Bucharest. Informed consent for inclusion in the study was obtained from all owners of the patients involved. This study included 20 dogs, aged between 3 and 14 years old, both males and females, of the following breeds: French Bulldog (n = 5), mixed breeds (n = 5), Maltese dog (n = 3), Beagle (n = 2), Chihuahua (n = 2), Cane Corso (n = 1), Amstaff (n = 1) and Labrador Retriever (n = 1).

Dogs meeting the following inclusion criteria were enrolled in the study: requirement for a head magnetic resonance imaging (MRI) examination along with a complete preanesthetic evaluation consisting of a neurological assessment, completion of a cardiological examination, biochemical and hematological analyses. Following the preanesthetic examination, all patients were classified according to the American Society of Anesthesiologists (ASA) into ASA II and ASA III status to assess anesthetic-related risks. Before premedication, patients were stratified into three age groups. The classification was performed in order to evaluate age-related differences in anesthetic approach and perioperative management. The anesthetic protocol was adapted according to patient age,

physiological characteristics, and underlying intracranial pathology. Standard monitoring was applied in all cases, and anesthetic techniques were selected based on current clinical practice and individual patient needs. The three groups are:

- Group 1 (G1)- between 3 and 5 years, ASA II status
- Group 2 (G2)- between 6 and 9 years, ASA II & III status
- Group 3 (G3)- between 11 and 14 years, ASA II & III status.

All dogs assigned to this study received intramuscular (IM) premedication. This choice of IM premedication reduced stress and calmed the patients, which is crucial in neurological patients (Costea, 2016). The anesthetic agents selected for this stage were dexmedetomidine (Dexdomitor®, 0.5 mg/mL), butorphanol (Butomidor®, 10 mg/mL) and midazolam (Midazolam®, 5 mg/mL) at the doses presented in Table 1.

Table 1. Premedication protocols and dosages

	G1	G2	G3
Dexmedetomidine	2 µg/kg	-	-
Butorphanol	0.3 mg/kg	0.3 mg/kg	0.2 mg/kg
Midazolam	-	-	0.2 mg/kg

After a 10-minute waiting period, an intravenous (IV) catheter was inserted to facilitate the administration of anesthetic agents as well as fluid therapy. Before induction, all patients were preoxygenated for 2-3 minutes and after that, induction was performed intravenously with 2-3 mg/kg of propofol (Propomitor®, 1%) (IV), followed by endotracheal intubation. Anesthesia was maintained with 2-4% Isoflurane (Isothesia®, 1000 mg/g) and 100% oxygen with a total flow of (500 mL/min + 10 mL/kg/min), through an anesthesia machine (AEON 7200A) with the use of a rebreathing system.

End-tidal carbon dioxide ($EtCO_2$) was continuously monitored using a side stream capnograph. To maintain $EtCO_2$ values within the target range of 35-45 mmHg, patients were managed either with spontaneous ventilation or intermittent positive pressure ventilation (IPPV) at a respiratory rate of 12-15 breaths per minute. Blood pressure was monitored non-invasively using the oscillometric method systematically at baseline, at 2-minute intervals through anesthesia, and upon emergence. The threshold for hypotension was a Mean Arterial Pressure (MAP) of 60 mmHg and for hypertension a Systolic Arterial Pressure (SAP) of 182 mmHg. Corneal protection during the MRI examination was ensured by the application of an ocular lubricant in all patients. All dogs received intravenous fluid therapy with Ringer's solution at a rate of 3 mL/kg/h. Intrarectal temperature was measured at two time points: before the induction of anesthesia and during the recovery phase with a hypothermia threshold set at 37°C. A silicone hot water bottle was placed beneath the abdomen of each patient to prevent hypothermia, as the ambient temperature in the MRI suite was maintained at approximately 20°C and scan durations ranged from 25 to 38 minutes. According to the MRI protocol that included image acquisition both before and after contrast agent administration, all patients in this study received gadolinium-based contrast medium (Clariscan®, *acidum gadotericum* 0.5 mmol/mL) at a dose of 0.2 mL/kg, approximately 15-20 minutes after the induction of anesthesia.

RESULTS AND DISCUSSIONS

The 20 cases included in the study were diagnosed with a range of cerebral pathologies following MRI examination. In G1, MRI diagnoses included: encephalitis (n = 1), ventriculomegaly (n = 1), meningioma (n = 1) and frontal lobe glioma (n = 1). In this group delayed recovery was observed beyond 15 minutes (n = 2) and hypothermia (n = 1).

For G2 the MRI diagnosis included: intraventricular mass (n = 2), hydrocephalus with secondary cerebellar herniation (n = 2), intracranial mass in the left cerebral hemisphere (n = 2), encephalitis (n = 1), thalamic glioma (n = 1), frontal lobe glioma (n = 1) and thalamic cystic lesion (n = 1). In this group we identified hypotension after contrast administration (n = 2), hypothermia (n = 1), delayed recovery beyond 15 minutes (n = 1), and hypertension during recovery (n = 1).

For G3 the MRI diagnoses included: subarachnoid diverticulum (n = 2), temporal lobe glioma (n = 2), encephalitis (n = 1) and

left tympanic bulla mass (n = 1). In this group we identified hypothermia (n = 3) and hypertension (n = 1). All cases of delayed recovery (n = 3), were considered likely to be associated with the underlying cerebral pathology.

The study population consisted primarily of dogs classified as ASA II and ASA III, reflecting the increased anesthetic risk associated with intracranial neurological disorders. Magnetic resonance imaging revealed a wide spectrum of neurological conditions, including inflammatory diseases, congenital abnormalities, and intracranial neoplasms with various anatomical localizations. Hypothermia was the most frequently observed peri-anesthetic complication (n = 5). All cases of hypothermia were associated with prolonged MRI scan durations exceeding 32-38 minutes, regardless of ASA classification. Delayed anesthetic recovery, defined as recovery exceeding 15 minutes, was recorded in three cases. In all cases, delayed recovery was considered likely to be related to the underlying cerebral pathology, suggesting an influence of central nervous system disease on anesthetic drug metabolism and recovery dynamics. Hypotension (n = 2) occurred following intravenous administration of the gadolinium-based contrast agent, with recorded mean arterial blood pressure values ranging between 58 and 60 mm Hg, but cannot be totally related to its administration, since it can also be considered a general anesthesia complication and it was not accompanied by other specific side effects.

Hypertension was an infrequent finding, observed in two cases, occurring either during the recovery phase or intra-procedurally. These events were transient and did not require interruption of the MRI examination.

A higher incidence of hemodynamic disturbances was observed in dogs classified as ASA III compared to ASA II patients, indicating reduced physiological reserve in animals with more severe systemic compromise.

The findings of this study indicate that general anesthesia in dogs with intracranial neurological disorders is associated with an increased risk of peri- and post-anesthetic complications, even in patients classified as ASA II.

Hypothermia and delayed recovery were the most commonly encountered adverse events, possibly influenced by prolonged procedure duration and the presence of underlying cerebral disease. Additionally, intravenous contrast administration represents a period during which the patient must be closely monitored, as it was associated with transient hypotensive episodes in a subset of patients in this study. Correlation of the present study's results with those reported in the literature shows that no severe reactions have been reported after administration, such as changes in pulse or respiratory rate (Pavel et al., 2018). Anaphylactic or anaphylactoid reactions following IV gadolinium injections may include vomiting, facial, periorbital and sublingual oedema, soft feces but also severe cardiovascular and respiratory dysfunctions (Girard & Leece, 2010).

Based on the findings reported in this study, the results suggest that perioperative management is particularly important in patients with cerebral disorders, as the risks are higher both during anesthesia and in the post-anesthetic period. According to previous studies, anesthetic complications for neurological patients occur more frequently in dogs with intracranial lesions than in those without. Through detailed assessment of perioperative risk factors and the procedures performed, outcomes in these patients can be improved (Hicks et al., 2013).

Assessing perianesthetic risk in veterinary patients with suspected intracranial lesions undergoing diagnostic procedures is of critical importance, as it enables both clinicians and owners to make well-informed anesthetic decisions. Identifying patient-specific risk factors allows for the consideration and application of targeted protocols or therapeutic interventions before or during anesthesia, intending to improve anesthetic recovery (Jiménez et al., 2012).

CONCLUSIONS

Although this research was limited by the small number of cases, we can state that a personalized anesthetic approach, guided by comprehensive preanesthetic evaluation and

proactive management, is essential for optimizing patient safety and outcomes in this high-risk population. The careful selection of a premedication protocol for patients with cerebral disorders is crucial, employing agents with minimal impact on key physiological functions, including the nervous, cardiovascular, and respiratory systems. Collaboration between the anesthesiologist and the neurologist is vital in the management of patients with cerebral pathologies, as it can enhance patient safety and minimize perioperative risks. This study emphasizes the importance of individualized perioperative anesthetic management in dogs with neurological pathology, with particular attention to thermoregulation, hemodynamic stability, and close monitoring during both anesthesia and recovery.

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OPTIMIZATION OF THE DIAGNOSIS AND THERAPY OF PAIN IN DIFFERENT SPECIES

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Abstract

Pain represents an essential component of modern veterinary practice, significantly influencing diagnosis, clinical progression, and patients' quality of life. Recognized as the fourth vital sign, pain requires accurate and systematic assessment to establish a precise diagnosis and implement appropriate treatment. This article analyses the neurophysiological mechanisms of pain, methods for its recognition and evaluation across different species, as well as current therapeutic strategies used in the management of acute and chronic pain. The behavioural characteristics of pain in dogs, cats, and cattle are presented, along with the advantages of multimodal analgesia, which combines pharmacological and non-pharmacological approaches. Understanding the complexity of pain and applying therapeutic protocols tailored to each patient contribute to improving animal welfare and increasing the effectiveness of veterinary medical practice.

Key words*: pain, nociception, multimodal analgesia, animal welfare.*

INTRODUCTION

Pain, according to the International Association for the Study of Pain (I.A.S.P.), is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (Gaynor & Muir, 2014). The correct identification of pain has a considerable impact on diagnostic accuracy, therapy planning, as well as the patient's healing and recovery. In modern veterinary medicine, pain is recognized as the "fourth vital sign", alongside temperature, pulse, and respiration, emphasizing the need for its systematic evaluation in clinical practice. Untreated pain leads to stress, suffering, and physiological changes that may worsen the progression of the disease. The correct detection of pain contributes to establishing an accurate differential diagnosis and to identifying the primary condition. A detailed clinical examination, combined with modern paraclinical methods, allows for the localization of the source of pain and helps avoid diagnostic errors (Mathews et al., 2014).

MATERIALS AND METHODS

In this research, a structured review of scientific studies was conducted with the aim of highlighting the importance of optimizing pain assessment and therapy across different animal species. A total of 33 bibliographic sources were analyzed, selected according to well defined criteria to ensure the relevance and up to date nature of the information used. Studies published between 1987 and 2026 were analyzed in order to cover both foundational research and the most recent scientific advances in the field. The databases used to identify relevant literature included PubMed, ScienceDirect, Google Scholar, and Wiley Online Library, as well as textbooks and guidelines published by recognized veterinary organizations (Allweiler, 2023). The keywords used in the search process also included pain transmission pathways, pain in animals, multimodal analgesia, and non-pharmacological approaches. The selection criteria included scientific articles published in peer reviewed journals, specialized textbooks, and guidelines issued by recognized veterinary organizations, written in English. The selected works focused on pain transmission pathways, recognition of pain in different species, and the optimization of pain management protocols.

RESULTS AND DISCUSSIONS

Pain acts as a crucial built-in protective mechanism capable of having a significant impact on the quality of life. Understanding the

physiological impact of pain is crucial for developing new therapies. Nociceptor neurons are central to the processes of pain and inflammation. Interactions between nociceptors and the immune system take place both at the injury site and within the central nervous system. Targeting chemical mediators and modulating nociceptor activity present promising strategies for pain management.

Essentially, the sensory nervous system is crucial for adapting the body's protective response, making the understanding of these interactions essential for identifying new pain treatment strategies (Karcz et al., 2024).

Pain is a multifaceted experience, encompassing both physical and physiological aspects that affect the whole organism.

To facilitate the understanding of the neurophysiological process of pain, it is divided into five consecutive processes, beginning when the painful or nociceptive stimulus comes into contact with the organism (Muir & Woolf, 2001)

Transduction: the transmission of pain begins with the stimulation of the physiological receptors known as nociceptors, which are extensively located in the skin, mucosa, membranes, deep fascias, connective tissues of visceral organs, ligaments, and articular capsules, as well as in the periosteum, muscles, tendons, and arterial vessels. These receptors are associated with free nerve endings and constitute the more distal segment of a first-order afferent neuron made up of small-diameter fibers. The nociceptive stimulus is converted into an electrical signal within nociceptors (Lamont et al., 2000).

Transmission: it refers to the transmission of the electrical signal produced by nociceptors along the axons of first-order neurons, which form synapses with second-order neurons in the dorsal horn of the spinal cord (Muir, 2009). The message of "pain" is encoded within the pattern and frequency of impulses transmitted through the axons of primary afferent nociceptors. A direct correlation exists between the intensity of the stimulus and the discharge frequency of nociceptors (Osterweis et al., 1987).

Modulation: the process by which excitatory and inhibitory mechanisms alter the transmission of the nerve impulse (Lamont et al., 2000).

Projection: nociceptive information is conveyed to the brain via nerve tracts originating in the dorsal horn, particularly through spinothalamic and supraspinal structures. The dorsal columns transmit somatic sensory information to the brain. The intermediate column comprises sensory motor and autonomic nerve fibers that descend from the brain, as well as sensory pathways that ascend to the brain (Gainor & Muir, 2014).

Perception: the processing and integration of information occurring in multiple specific brain regions, such as the thalamus and cerebral cortex, where sensory characteristics including onset, location, and type of the nociceptive stimulus are defined (Hernandez-Avalos et al., 2019).

Acute pain is common as a result of surgery, illness or injury and it is undeniably unpleasant, it is typically linked to actual or potential tissue damage and plays a crucial role in quickly altering an animal's behavior to prevent or reduce injury, while creating optimal condition for healing.

The treatment of acute pain aims to address the underlying cause and to interrupt nociceptive signaling at multiple levels throughout the nervous system, whereas therapeutic approaches to chronic pain must be based on a multidisciplinary strategy and a holistic management of the patient's quality of life (Mathews et al., 2014).

Chronic pain is defined as pain that persists beyond the normal period of tissue healing, usually longer than 3-6 months. Unlike acute pain, it is maladaptive in nature and it is associated with neuroplastic changes in the central nervous system, including central sensitization, reorganization of neuronal circuits, and altered processing of nociceptive signals.

Chronic pain may have inflammatory, neuropathic, or mixed components and it is commonly encountered in conditions such as osteoarthritis, degenerative diseases, neoplasms, or nerve injuries (Costea, 2016).

Recognition of pain in animals is not intuitive and involves the assessment of behavioral and physiological signs, including the use of pain scales, however physiological parameters do not always distinguish pain from other physiological stimuli such as stress or anxiety.

Physiological parameters (e.g., changes in heart rate, respiratory rate, blood pressure, or pupil

dilation) can be used to assess responses to an acute noxious (painful) stimulus, especially during anesthesia, and to evaluate pain in specific clinical scenarios (Allweiler, 2023).

Pain evaluation in veterinary medicine must rely on a multimodal framework that extends beyond physiological and endocrine biomarkers, reflecting the subjective and multidimensional character of pain (Walsh et al., 2024; Mota-Rojas et al., 2025). Efforts to standardize pain assessment criteria have led to the development of various guidelines used to recognize pain based on physical and biological changes, such as behavioral alterations (Holton et al., 2001), facial expression, chemical mediators, and physiological parameters.

Behavioral changes to be considered in animals include social interaction aggression, vocalization, self-mutilation, sleep disturbances, restlessness and lethargy (Wiseman-Orr et al., 2006).

Body language changes involve variations in posture, limb positioning, gestures, and facial expressions (Ponzio, 2006). Importantly, these behavioral and postural modifications are species-specific and have been consistently documented in animals exposed to noxious or painful stimuli (De Grauw et al., 2016).

In cats, reports describe reduced activity levels, reduced appetite, a tendency to hide or avoid interaction, and excessive licking of the affected area, which interferes with normal grooming behavior (Reid et al, 2018). Cats experiencing pain may manifest through subtle behavioral changes, including increased aggression, hiding, reduced appetite, weight loss, inappropriate urination and decreased activity.

In dogs, the behavioral manifestation of pain varies by species and it is influenced by factors such as age, breed, individual temperament, and the presence of additional stressors like anxiety or fear (Mathews et al., 2014). Behavioral signs of pain in dogs include changes in posture or body position, altered behavior, vocalization, altered response to touch, modified interaction with humans (e.g., reduced interaction, aggression), impaired mobility (e.g., lameness, reluctance to move), and decreased appetite (Mathews et al., 2014).

Pain management in farm animals is commonly evaluated through alterations in clinical parameters accompanied by behavioral disturbances (Abrahamsen, 2008).

Affected animals may exhibit signs such as expiratory grunting, teeth grinding, reduced appetite and rumination, increased vocalization, prolonged periods of lying down, or diminished grooming behavior.

In sheep, elevations in respiratory and heart rates represent sensitive, non-invasive indicators for the assessment of mild to moderate pain (Cohen et al., 2024). Animals experiencing pain may also withdraw from the group and appear apathetic, depressed, or lethargic.

Inadequate analgesic management can lead to decreased feed and water intake, resulting in reduced daily weight gain and milk yield, diminished locomotor activity, endocrine disturbances, a catabolic metabolic state, fever, leukocytosis, and the development of behavioral abnormalities (Costea et al., 2024).

In cattle, pain related behaviors are often associated with conditions considered extremely painful, such as acute toxic mastitis, fractures, septic arthritis, and peritonitis (Huxley & Whay, 2006). These pain behaviors include changes in posture (crouching, arched back, lowered head position), severe lameness, attention directed toward the painful area, vocalization, teeth grinding (bruxism), and changes in social behavior (Gleerup et al., 2015).

It should be noted that these behavioral changes are often more noticeable when animals are alone, whereas during interactions with others, their behavior may be barely detectable or they may show no obvious signs at all (Brondani et al., 2013).

Establishing an accurate pain diagnosis requires a detailed evaluation that includes physical examinations, reflex testing, and review of the medical history to identify possible causes or contributing factors to pain.

Studies suggest that multimodal analgesia, which involves combining different classes of analgesics, provides improved pain control while minimizing the adverse effects associated with high doses of single agents.

Pain management protocols often include both pharmacological and non-pharmacological approaches, thus emphasizing the importance of multimodal strategies.

Pharmacological approaches consist of pain analgesia and are divided into three main categories: opioids, non-opioids, and adjuvants. *Opioids* are utilized in the management of moderate to severe pain with high efficacy. However, their use carries considerable risks, including the potential for dependence and a range of adverse effects, thus necessitating careful monitoring (Alorfi, 2023). Opioids attach to opioid receptors in both the central and peripheral nervous systems, reducing the release of excitatory neurotransmitters from afferent fibers in the spinal cord and consequently inhibiting the synaptic transmission of pain signals. Postsynaptically, the increased K^+ efflux causes the neuronal hyperpolarization of spinal projection neurons and inhibits ascending nociceptive pathways (Mathews et al., 2014). *Nonsteroidal anti-inflammatory* drugs (NSAIDs) reduce the production of pain-inducing prostaglandins and are effective in managing inflammatory pain.

Combining nonsteroidal anti-inflammatory drugs (NSAIDs) with opioids can improve overall pain control and allow for reduced opioid dosages, thereby lowering the risk of opioid-related adverse effects (Schwenk & Mariano, 2018). These therapeutic regimens must be tailored to each individual patient, taking into account the performed procedure, the side effects of individual medications, and the patient's pre-existing medical conditions (Buvanendran & Kroin, 2009). *Local anesthetics* utilized in clinical practice impede nerve conduction by disrupting the influx of sodium ions across the nerve cell membrane, provide localized pain relief by inhibiting nerve signals transmission at the administration site and are commonly used for nerve blocks and regional anesthesia.

The Alpha-2 adrenergic agonists activation of these receptors inhibits the release of norepinephrine in the form of a negative feedback loop in both the central and peripheral nervous systems and modulates pain signals in the spinal cord and brain, inducing sedation and analgesia, and they are particularly useful for procedural pain. Alpha-2 adrenergic agonists are indicated for the purposes of anxiolysis, sedation, analgesia, treatment of delirium and shivering, as well as numerous other applications within perioperative medical practice. Alpha-2 adrenergic agonist medications have been shown to reduce the need for anesthetics and analgesics during the perioperative phase.

Adjuvant analgesics (e.g., gabapentin, tramadol, and ketamine) are frequently utilized in small animal practice. Most of these drugs are usually prescribed for outpatient patients when pain does not respond to standard analgesics medications (e.g., local anesthetics, opioids, and NSAIDs) or when the use of other pain-relieving medications, such as NSAIDs, is contraindicated. These drugs should be considered as alternatives intended to reduce or replace opioid use (Ruel & Steagall, 2019). Gabapentin modifies neurotransmitter release and is effective in managing neuropathic pain resulting from nerve injury. NMDA antagonists: NMDA receptors play an essential role in pain sensitization. NMDA antagonists, such as ketamine and amantadine, block these receptors, preventing pain amplification and providing profound analgesia (Ruel & Steagall, 2019).

Non-pharmacological approaches include thermotherapy, cryotherapy massage, therapeutic exercises, aquatic therapy, acupuncture and electrical stimulation (Grubb, 2022).

Thermotherapy consists in applying both heat and cold to manage pain, injuries, and surgical wounds. Superficial heat helps increase blood circulation in the muscles, encourages muscle relaxation, and improves the flexibility of fibrous tissues. It can be delivered through warm compresses or infrared heat lamps (Bockstahler et al, 2004). *Cryotherapy* induces vasoconstriction, counteracts inflammation, provides analgesia, and reduces muscle spasms (Bockstahler et al., 2004).

Massage is increasingly recognized as a beneficial treatment modality (Corti, 2014). Massage, defined as the therapeutic manipulation of soft tissues, produces multiple effects on the muscles, circulatory system, autonomic nervous system, and psychological wellbeing.

Various techniques are applied to obtain specific therapeutic results in the management of numerous conditions, including swelling and edema, critical illness and extended periods of immobility, osteoarthritis and chronic pain, as well as in palliative and hospice care (Corti, 2014).

Therapeutic exercises, as part of *physiotherapy*, are designed to enhance pain-free range of motion and active flexibility, encourage the use of the limb while decreasing lameness, increase muscle mass and strength, improve everyday functional ability, and reduce the risk of future injuries (Bockstahler et al., 2004). Therapeutic exercise encompasses passive movements and proprioceptive training, as well as active exercises aimed at enhancing limb function, increasing speed, and building strength (Grubb, 2022).

Aquatic therapy refers to any exercise or manual therapy carried out in a water based environment. The benefits of aquatic therapy include the ability to provide prescriptive, functional exercises without causing pain. Balance exercises can be safely performed in water long before they can be attempted on land. The increased metabolic demand resulting from water resistance helps accelerate weight loss while simultaneously increasing muscle strength (Chiquoine et al., 2018).

Acupuncture is a popular complementary treatment option in human medicine, and increasingly, pet owners are also turning to acupuncture for their animals (Habacher et al., 2006). It is a widely used therapeutic technique that involves the insertion of fine needles into specific points on the body to stimulate the flow of vital energy ("Qi") and support the restoration of both physical and mental balance in the organism (Lindley & Cummings, 2006).

Acupuncture therapy is individualized according to the patient's specific needs, with the goal of targeting the underlying cause of the condition and preventing recurrence by restoring energetic balance (Matern, 2022). In animals, acupuncture aims to reduce pain, promote anesthesia, and treat musculoskeletal conditions (such as arthritis, hip dysplasia, and traumatic nerve injuries), gastrointestinal disorders (including abnormalities of gastric motility and irritable bowel syndrome), inflammation, as well as neurological, respiratory, and reproductive disorders (Rose et al., 2017).

Electrical stimulation has proven beneficial in the management of a wide range of painful conditions and in cases of muscle atrophy (Rushton, 2002). Indications include pain management especially in conditions such as arthritis, spondylosis, and spondylarthrosis as well as during recovery after an orthopedic surgery and in nerve regeneration. Additional uses involve promoting fracture healing, reducing muscle tension, preventing muscle atrophy caused by inactivity, and improving muscle strength (Bockstahler et al., 2004). Therapeutic approaches including neuromuscular electrical stimulation and laser therapy, have been shown to alleviate pain and inflammation, promote tissue repair, and shorten recovery following orthopedic surgery. Treatment modalities include uromuscular electrical stimulation (NMES), transcutaneous electrical nerve stimulation (TENS), and electrical muscle stimulation (EMS). Although these modalities provide a range of frequencies and currents, low-frequency pulsed alternating currents are most frequently applied for therapeutic purposes (Bockstahler et al., 2004).

CONCLUSIONS

Understanding the sequential processes of pain provides a comprehensive framework for interpreting how painful stimuli are generated, processed, and ultimately experienced by animals.

Differentiating between acute and chronic pain is fundamental for effective clinical management. While acute pain serves as an adaptive and protective function by promoting healing and preventing further injury, chronic pain is maladaptive and associated with long term neuroplastic changes that significantly compromise animal welfare and the quality of life.

These findings emphasize the necessity of early recognition and appropriate intervention to avoid the progression of acute pain into chronic pain.

Accurate pain assessment in animals remains a major challenge, as pain expression is often subtle, species specific, and influenced by behavioral, physiological, and environmental factors.

The concealment of pain as a survival mechanism further complicates diagnosis, underscoring the importance of combining behavioral observations with physiological indicators and validated pain assessment tools.

Species specific behavioral changes observed in companion animals and farm animals reinforce the need for tailored assessment protocols.

Effective pain management requires a multimodal approach that integrates medication and non-medication strategies. The combined use of opioids, non-opioid analgesics, adjuvant drugs, and regional anesthesia allows for improved analgesic efficacy while minimizing adverse effects.

Therapies without medication, such as physiotherapy, thermotherapy, aquatic therapy, acupuncture, massage, and electrical stimulation play a crucial complementary role, particularly in chronic pain management and rehabilitation.

Overall, this research underscores that comprehensive pain recognition and multimodal management are essential not only for improving clinical outcomes but also for safeguarding animal welfare.

Continued advancements in understanding pain mechanisms and refining assessment and treatment strategies are critical for the development of more effective, individualized, and humane pain management protocols in veterinary medicine.

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SURGICAL STABILIZATION OF FLAIL CHEST IN CATS USING FIRST-GENERATION U-GRIP ANATOMICAL PLATES: THREE CLINICAL CASES

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Abstract

Thoracic trauma in cats frequently results in rib fractures which may progress to flail chest and severe respiratory compromise. Three feline patients diagnosed with multiple rib fractures and flail chest were presented to the University Veterinary Emergency Hospital "Prof. univ. dr. Alin Bîrțoiu", Faculty of Veterinary Medicine, Bucharest. Diagnostic evaluation included thoracic radiography and computed tomography (CT) to assess the extent of rib fractures and associated thoracic injuries. Initial stabilization included oxygen therapy, analgesia and thoracocentesis in cases presenting pneumothorax. Due to severe thoracic wall instability, surgical stabilization of the fractured ribs was performed using U-GRIP™ first-generation plates developed in collaboration with Cabiomede, representing a novel fixation option in veterinary thoracic surgery. In one case, a fractured rib perforated the lung parenchyma requiring lung lobectomy, while another patient presented pulmonary bullae that also required lobectomy. The third cat underwent surgical stabilization of the rib fractures. Appropriate stabilization of the thoracic wall together with management of associated pulmonary injuries may improve respiratory function and clinical outcome in cats with severe thoracic trauma.

Key words*: rib fractures, flail chest, thoracic trauma, cats, U-GRIP plate.*

INTRODUCTION

Flail chest is a severe thoracic injury characterized by the fracture of multiple consecutive ribs, resulting in a segment of the thoracic wall that moves paradoxically during respiration. This condition leads to impaired ventilation, decreased pulmonary compliance, and significant respiratory compromise. In both human and veterinary patients, flail chest is associated with increased morbidity due to pain, pulmonary contusions, and compromised gas exchange (Olsen et al., 2002; Cabon et al., 2015).

In human medicine, surgical stabilization of rib fractures (SSRF) has become an increasingly accepted treatment modality for flail chest and severe thoracic trauma. Multiple studies have demonstrated that surgical fixation improves respiratory mechanics, reduces the duration of mechanical ventilation, decreases intensive care unit stay, and lowers complication rates compared to conservative management (Tanaka et al., 2002; Granetzny et al., 2005; Slobogean et al., 2013; Pieracci et al., 2017; Liu et al., 2019; Fokin et al., 2020). As a result, SSRF is now considered a standard or strongly recommended approach in selected human patients.

In contrast, veterinary management of flail chest remains predominantly conservative, relying on analgesia, oxygen therapy, and external stabilization techniques. Although these approaches may be effective in some cases, they do not directly restore the structural integrity of the thoracic wall and may be insufficient in patients with severe instability or associated pulmonary injury (Olsen et al., 2002; Cabon et al., 2015). Surgical stabilization techniques have been described in dogs, including interfragmentary wiring and external splinting; however, these methods present limitations and are not widely adopted in clinical practice (Ahn et al., 2016; Min et al., 2016).

The application of surgical stabilization in cats presents additional challenges. Feline ribs are

small in diameter, typically measuring approximately 3-4 mm, and exhibit pronounced curvature, making conventional fixation techniques such as wiring technically demanding and often unreliable. Furthermore, external splinting methods may lead to patient discomfort and inadequate stabilization, particularly in active or stressed patients (Ahn et al., 2016; Min et al., 2016). These anatomical and practical limitations highlight the need for alternative fixation methods specifically adapted to feline thoracic anatomy.

In response to these challenges, a first-generation U-GRIP™ anatomical plate system was developed to allow effective stabilization of fractured ribs in small patients. The design enables secure engagement of the rib while minimizing soft tissue trauma and facilitating a reproducible surgical technique (Uzun et al., 2023).

The aim of this study is to describe the clinical application of this novel fixation system in three feline cases presenting with flail chest, and to evaluate surgical feasibility and short-term outcomes.

MATERIALS AND METHODS

Study Design and Case Selection

This study describes three feline patients diagnosed with flail chest and treated surgically at the University Veterinary Emergency Hospital. Cases were selected based on the presence of thoracic wall instability characterized by multiple rib fractures resulting in paradoxical motion, accompanied by respiratory compromise requiring clinical intervention (Table 1).

Table 1: Clinical Case Summary

Case	Trauma Type	Ribs Fractured	Ribs Stabilized	Pulmonary Injury	Surgical Procedure
#1	Dog bite trauma	Bilateral rib fractures (multiple)	8th (R)	Right middle lung lobe perforation	Lung lobectomy + thoracic wall reconstruction
#2	Unknown trauma	Left ribs 9, 10, 11, 12	9th and 10th (L)	Pulmonary contusions	Thoracic wall reconstruction
#3	Hit-by-car (polytrauma – CMF + thoracic trauma)	Right ribs 4, 5, 6	6th (R)	Pulmonary bullae and blebs (right caudal, R-Cr and accessory Acc., lobes)	Partial lung lobectomy + thoracic wall reconstruction

Clinical Evaluation and Stabilization

All patients underwent emergency stabilization focused on restoration of respiratory function compromised by blunt thoracic trauma and associated pulmonary injury, as well as adequate pain control.

Supplemental oxygen was delivered using flow-by and mask techniques with high flow rates. Opioid analgesia was administered in all patients. Thoracentesis was performed in cases presenting pneumothorax to relieve respiratory distress.

Intercostal nerve blocks were applied both during emergency stabilization and intraoperatively in order to improve analgesia and reduce thoracic pain associated with rib fractures.

Clinical examination was followed by thoracic imaging. Thoracic radiography was performed after initial stabilization to identify rib fractures and assess thoracic wall integrity. Computed tomography (CT) was subsequently performed to accurately determine the number and location of fractured ribs and to identify associated pulmonary injuries, including lung parenchymal damage or bullae.

Indications for Surgical Intervention

Surgical stabilization was indicated in patients presenting with marked thoracic wall instability consistent with flail chest, as well as in cases where rib fractures resulted in penetration or risk of penetration into the thoracic cavity, potentially compromising pulmonary structures. Only ribs contributing to thoracic instability or posing a direct threat to intrathoracic structures were selected for surgical stabilization. Fractures that remained aligned and did not significantly affect respiratory mechanics were managed conservatively.

Surgical Technique

All procedures were performed under general anesthesia via intercostal thoracotomy. Surgical stabilization of rib fractures was performed using U-GRIP™ first-generation titanium plate (prototype system currently under intellectual property protection) developed in collaboration with Cabiomede. This titanium implant is specifically designed for small-diameter ribs and features a hook-based mechanism that allows the plate to engage the rib from the cranial aspect, thereby avoiding the intercostal neurovascular bundle located on the caudal margin. The system is applied without circumferential encirclement of the rib, reducing the risk of vascular or nerve injury.

Fixation was achieved using 1.5 mm locking screws adapted to the limited cortical thickness of feline ribs, providing stable anchorage while minimizing additional trauma to surrounding tissues. The implant design allows rapid application and facilitates reproducible stabilization in anatomically challenging conditions.
In cases where pulmonary injury was identified, additional procedures, including lung lobectomy, were performed as indicated.

Postoperative Management
Postoperative care included oxygen supplementation, analgesia, and continuous clinical monitoring. Thoracic drainage was performed when necessary, using chest tubes connected to a Heimlich valve system to manage residual pneumothorax.
Patients were closely monitored for respiratory function, pain control, and potential complications during the postoperative period.

RESULTS AND DISCUSSIONS

Three feline patients diagnosed with flail chest underwent surgical stabilization. Clinical outcomes, respiratory recovery, and postoperative evolution were evaluated.

Case 1 – Dog Bite Trauma with Bilateral Flail Chest
The first patient was presented following a dog bite injury affecting the thoracic region. Although no external penetrating wounds were identified, the compression force of the bite resulted in bilateral flail chest with multiple rib fractures. Clinical examination revealed severe respiratory distress and marked paradoxical thoracic wall movement.
Thoracic imaging confirmed multiple rib fractures affecting both hemithoraces. On the right side, a displaced rib fragment had penetrated the thoracic cavity and perforated the right middle lung lobe (R-M), while fractures on the left side did not result in pulmonary penetration. Pneumothorax was identified and managed with thoracentesis during emergency stabilization.
Surgical intervention was performed due to thoracic instability and lung injury. Lung lobectomy of the right middle lung lobe (R-M) was carried out because of traumatic perforation. Thoracic wall stabilization on the right side was achieved using a U-GRIP™ first-generation plate applied to the rib responsible for instability. On the left side, thoracic wall integrity was restored by reconstruction of the disrupted intercostal musculature.
Postoperatively, the patient demonstrated rapid improvement in respiratory function, with complete resolution of paradoxical thoracic wall movement. No recurrence of pneumothorax was observed. Thoracic wall stability was maintained, and no implant-related or infectious complications developed.

Case 2 – Unknown Thoracic Trauma with Unilateral Flail Chest
The second patient was presented following unknown thoracic trauma and exhibited respiratory distress and thoracic pain consistent with flail chest. Thoracic imaging revealed multiple rib fractures affecting the left thoracic wall, involving ribs 9, 10, 11 and 12 (Figure 1). Pulmonary contusions were also identified.

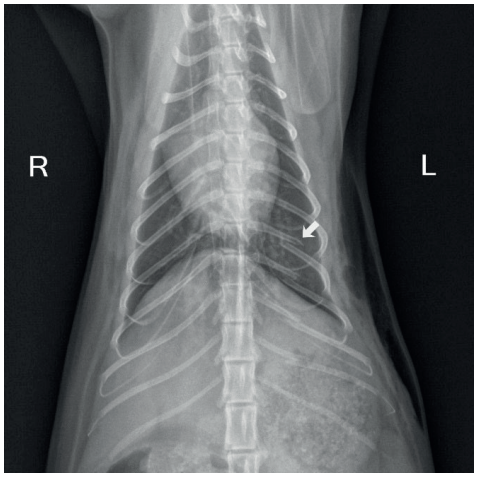

Figure 1. Thoracic radiograph showing multiple fractures of the left ribs (9-12)

Fractures of ribs 9 and 10 were displaced toward the thoracic cavity and considered at risk for penetration of intrathoracic structures. Due to thoracic wall instability and the risk of further displacement, surgical stabilization was indicated.
Intraoperative exploration confirmed the absence of lung parenchymal injury. Stabilization of ribs 9 and 10 was performed

using U-GRIP™ first-generation plates. Fractures of ribs 11 and 12, which remained aligned and did not contribute to thoracic instability, were managed conservatively.
Postoperatively, the patient showed progressive improvement in respiratory function, with resolution of thoracic pain and restoration of stable chest wall mechanics. No pneumothorax or respiratory deterioration was observed.

Case 3 – Polytrauma with Persistent Pneumothorax and Flail Chest
The third patient was presented following hit-by-car (HBC) trauma and was diagnosed with polytrauma, including craniomaxillofacial injuries and thoracic trauma. Thoracic CT revealed fractures of ribs 4, 5 and 6 on the right thoracic wall, accompanied by severe pneumothorax resulting in respiratory compromise (Figure 2).
Initial stabilization included thoracentesis, which was performed twice due to rapid reaccumulation of air. Because pneumothorax persisted despite repeated thoracentesis, a thoracic drain (MILA® chest tube) connected to a Heimlich valve was placed to allow continuous evacuation of air from the pleural cavity.

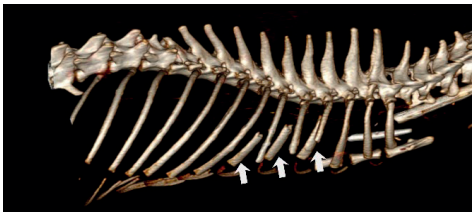

Figure 2. Three-dimensional CT reconstruction showing fractures of the right 5th–7th ribs (arrows)

Surgical intervention was subsequently performed via intercostal thoracotomy. Intraoperative examination revealed pulmonary bullae and blebs affecting the right caudal and accessory lung lobes, which required partial lung lobectomy. Thoracic wall stabilization was achieved by placement of a U-GRIP™ first-generation plate on rib 5.
Postoperatively, the patient showed marked improvement in respiratory function, with resolution of pneumothorax. The thoracic drain was removed following cessation of air leakage and stabilization of respiratory parameters.

Surgical Intervention
Surgical management was indicated in all three patients due to thoracic wall instability associated with flail chest and the presence of injuries threatening intrathoracic structures. All procedures were performed under general anesthesia using an intercostal thoracotomy approach, selected according to the location of the fractured ribs.

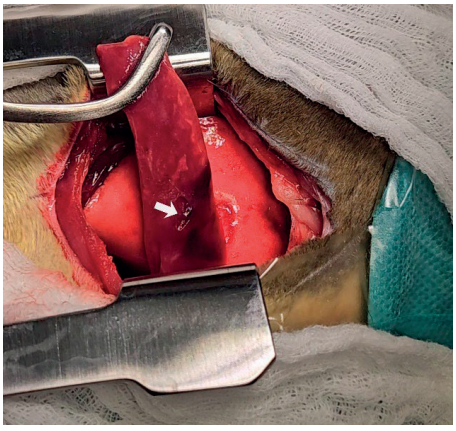

Figure 3. Intraoperative view of an atelectatic right middle lung lobe (R-M) exposed through intercostal thoracotomy. Darker areas of the lung parenchyma indicate severe pulmonary contusions, and the site of traumatic rupture caused by a displaced rib fragment is indicated by the white arrow

Thoracic exploration allowed direct assessment of rib displacement and associated pulmonary injuries.
In one patient, a displaced rib fragment had penetrated the thoracic cavity and perforated the R-M lung lobe, requiring lung lobectomy (Case #1, Figure 3).
In another patient (Case #3), persistent pneumothorax was associated with pulmonary bullae and blebs affecting the right caudal and accessory lung lobes, which required partial lung lobectomy during surgical exploration (Figure 4).

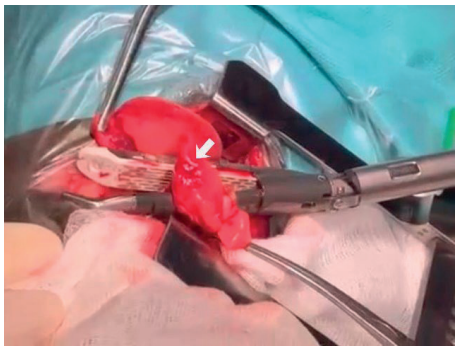

Figure 4. Intraoperative view of the right caudal lung lobe (R-Ca) demonstrating pulmonary blebs (white arrow). Partial lobectomy was performed using surgical staplers

Following surgical management of pulmonary injuries, attention was directed toward restoration of thoracic wall stability. Stabilization of rib fractures was performed using U-GRIP™ first-generation plates (prototype system currently under intellectual property protection). These titanium plates (2 mm thickness) use 1.5 mm locking screws and are designed
to grasp the rib from the cranial aspect, thereby avoiding compression of the intercostal neurovascular bundle located along the caudal rib margin. In this patient (Case #2), stabilization of a single rib restored thoracic wall alignment and improved the position of adjacent fractures.

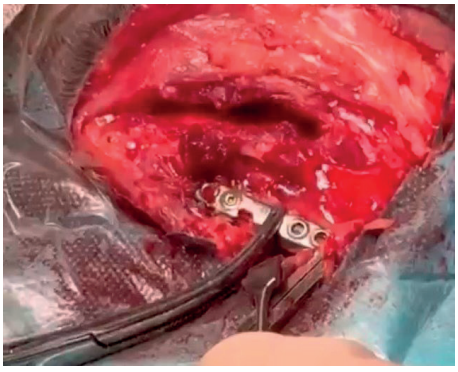

Figure 5. Intraoperative view demonstrating thoracic wall stabilization of a fractured rib using a single U-GRIP™ first-generation plate

Plates were applied only to ribs responsible for thoracic instability or posing a risk of intrathoracic penetration.

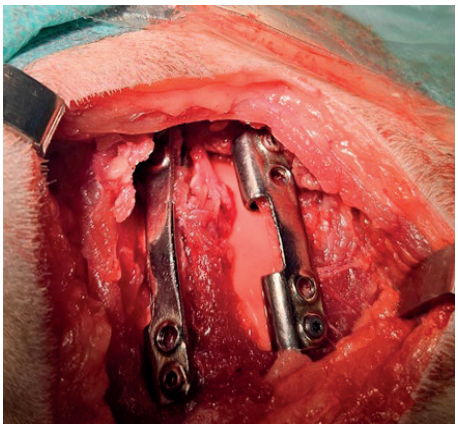

Figure 6. Intraoperative view demonstrating stabilization of multiple rib fractures using two U-GRIP™ first-generation plates, shown prior to reconstruction of the thoracic wall

Thoracic drainage was established in all patients using MILA® chest tubes, which were placed before complete thoracic closure. Heimlich valves were applied in one patient. The duration of surgical procedures ranged between 60 and 110 minutes, depending on the complexity of the pulmonary injuries and the number of ribs requiring surgical stabilization.

Outcomes

Postoperative recovery was monitored in all patients with particular attention to respiratory pattern, thoracic pain and chest tube output. Thoracic drainage allowed continuous evacuation of residual intrathoracic air during the early postoperative period and chest tubes were removed once respiratory stability and absence of further air leakage were confirmed (between 36 to 72 hours).

All patients showed improvement in respiratory function following surgical stabilization of the thoracic wall and management of associated pulmonary injuries.

Follow-up evaluations were performed at 15, 30 and 60 days after surgery, with further monitoring performed through communication with the owners.

Two patients recovered uneventfully and returned to normal respiratory function. One patient developed feline panleukopenia approximately 10 days after surgery and died due to complications unrelated to the thoracic surgical procedure.

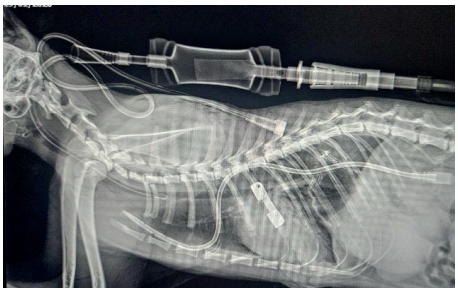

Figure 7. Postoperative lateral thoracic radiograph demonstrating thoracic wall stabilization using a U-GRIP™ first-generation plate. A MILA® chest tube connected to a Heimlich valve system is visible in situ for continued evacuation of intrathoracic air

Flail chest represents one of the most severe manifestations of thoracic trauma and is characterized by paradoxical movement of a segment of the thoracic wall caused by multiple consecutive rib fractures. This condition disrupts normal respiratory mechanics and can lead to significant respiratory compromise.

While surgical stabilization is well established in human medicine (Tanaka et al., 2002; Granetzny et al., 2005; Liu et al., 2019), veterinary literature remains limited (Olsen et al., 2002; Ahn et al., 2016; Min et al., 2016).

In human medicine, surgical stabilization of rib fractures (SSRF) has become an established therapeutic approach for severe thoracic wall instability. Multiple clinical studies have demonstrated that rib stabilization improves respiratory function, reduces ventilator dependence, and shortens intensive care unit stays (Tanaka et al., 2002; Slobogean et al., 2013; Liu et al., 2019). The development of specialized rib plating systems has facilitated reliable stabilization of fractured ribs in human trauma patients (Fokin et al., 2020; Pieracci et al., 2017).

In contrast, surgical stabilization of rib fractures in veterinary patients presents unique technical challenges. The ribs of cats are extremely small, highly curved, and fragile, which significantly limits the applicability of conventional fixation systems. Additionally, thoracic anatomy varies widely depending on patient size, breed, and body condition, further complicating implant positioning and stability.

Several techniques have been described for stabilization of rib fractures in veterinary medicine, including circumcostal sutures, interfragmentary wiring, and external thoracic bandaging (Olsen et al., 2002; Ahn et al., 2016; Min et al., 2016). However, these techniques often fail to provide sufficient rigidity in cases of true flail chest, particularly when multiple adjacent ribs are fractured (Olsen et al., 2002).

Interfragmentary wiring may be technically limited in feline ribs due to reduced bone stock and risk of iatrogenic damage, while external splinting may result in discomfort and inconsistent stabilization (Ahn et al., 2016; Min et al., 2016).

In the present case series, thoracic wall stabilization was achieved using a U-GRIP™ first-generation plate, a prototype implant specifically designed for the stabilization of small and curved ribs. The implant provides secure mechanical engagement without relying solely on screw purchase in minimal cortical bone, making it particularly suitable for feline thoracic anatomy.

This design allows faster application, improved stability, reduced surgical trauma, and greater reproducibility compared to traditional techniques.

In addition to thoracic wall stabilization, associated pulmonary injuries were addressed surgically when required. Lung lobectomy was performed in cases of traumatic pulmonary injury, allowing removal of non-viable lung tissue and contributing to overall respiratory stabilization.

Surgical stabilization resulted in restoration of thoracic wall integrity and allowed adequate lung expansion during respiration. These findings suggest that surgical management of severe flail chest in cats is feasible and may offer advantages over conservative treatment in selected cases, particularly in the presence of significant respiratory compromise.

The U-GRIP™ first-generation plate used in these cases represents an early prototype currently undergoing further development. Further biomechanical studies and larger clinical series are required to evaluate its performance and define its role in veterinary thoracic surgery.

Severe thoracic trauma in cats remains a significant clinical challenge. While surgical stabilization is widely adopted in human trauma care, its application in veterinary medicine has

been limited by anatomical and technical constraints. The present cases demonstrate that anatomically adapted stabilization systems may overcome these limitations and provide effective thoracic wall stabilization in feline patients.

This approach may represent an important step toward establishing surgical rib stabilization as a viable and reproducible treatment option in veterinary trauma surgery.

CONCLUSIONS

Flail chest represents a severe form of thoracic trauma that can result in significant respiratory compromise in feline patients. The present cases demonstrate that surgical stabilization of the thoracic wall can be successfully performed in cats with multiple rib fractures and flail chest, allowing restoration of thoracic stability and improvement of respiratory mechanics.

These findings support surgical intervention as a viable treatment option in selected feline trauma patients, particularly in cases of marked thoracic wall instability and persistent respiratory compromise.

The U-GRIP™ first-generation plate, used as a prototype stabilization system in these cases, proved adaptable to the anatomical characteristics of feline ribs and allowed effective surgical stabilization.

This implant design addresses key limitations of conventional techniques in small animal patients and may facilitate more consistent and reproducible thoracic wall stabilization.

Further development and evaluation of dedicated rib stabilization implants for veterinary patients are currently underway and may contribute to improved management of severe thoracic trauma in small animals.

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CONFLICTS OF INTEREST

The author declares no conflicts of interest related to the content of this article.

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CLINICAL MANAGEMENT OF PYOTHORAX IN CATS: DIAGNOSTIC AND THERAPEUTIC CONSIDERATIONS

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Abstract

Pyothorax, also termed empyema, is defined as the accumulation of purulent septic exudate within the pleural cavity and represents one of the most severe pleural diseases in cats. The condition may arise from thoracic bite wounds, penetrating trauma, extension of pulmonary infection, or other sources of pleural contamination. Affected patients commonly present with dyspnea, lethargy, fever, and systemic illness. Thoracentesis is both a diagnostic and therapeutic procedure, allowing confirmation of septic pleural effusion and immediate relief of respiratory distress. Cytologic evaluation typically reveals degenerate neutrophils with intracellular bacteria. Successful management requires aggressive pleural drainage through thoracostomy tubes combined with repeated pleural lavage and systemic broad-spectrum antimicrobial therapy. When clinical improvement is not achieved within 48-72 hours, surgical exploration may be required to eliminate persistent infectious foci or non-functional pulmonary tissue. Early recognition and decisive management are essential, as delayed treatment may result in pleural fibrosis and irreversible fibrothorax.

Key words*: pyothorax, pleural effusion, fibrosis, fibrothorax, cats.*

INTRODUCTION

Pyothorax, also referred to as empyema, represents the accumulation of purulent septic exudate within the pleural cavity and is considered one of the most severe pleural diseases encountered in feline patients. The condition results from bacterial contamination of the pleural space and may develop secondary to thoracic bite wounds, penetrating trauma, pulmonary infections, or other sources of pleural contamination. In cats, pyothorax frequently presents with acute respiratory distress, lethargy, fever, anorexia, and systemic illness, reflecting both respiratory compromise and systemic inflammatory response.

Feline pyothorax typically presents as a life-threatening pleural infection characterized by accumulation of septic exudate within the pleural cavity, leading to respiratory compromise and systemic inflammatory response. This infected pleural fluid interferes with normal lung expansion and leads to restrictive respiratory dysfunction. Accumulation of septic exudate within the pleural cavity may rapidly result in dyspnea and hypoxemia, requiring immediate diagnostic and therapeutic intervention. Thoracentesis remains a fundamental procedure for both diagnosis and initial stabilization, allowing removal of pleural fluid while providing samples for cytological and microbiological evaluation. Cytological examination typically reveals degenerate neutrophils and intracellular bacteria, confirming the septic nature of the effusion (Tobias & Johnston, 2017; Fossum, 2018).

Successful treatment of feline pyothorax relies on aggressive evacuation of infected pleural fluid through chest tube placement combined with repeated pleural lavage and systemic broad-spectrum antimicrobial therapy (Monnet, 2018).

Adequate pleural drainage plays a critical role in controlling infection, improving pulmonary expansion, and preventing further pleural inflammation.

However, in some cases medical management may fail due to persistent infection, fibrin deposition, or the development of pleural loculations that limit effective drainage. Progressive organization of pleural exudate may lead to pleural thickening and lung entrapment, eventually resulting in fibrothorax. In this advanced stage, restrictive pleural fibrosis may significantly impair lung expansion and worsen prognosis (Fossum, 2018). Early recognition of

treatment failure and timely clinical decision-making are therefore essential components in the management of this condition.
The aim of this paper is to present the diagnostic and therapeutic considerations involved in the clinical management of feline pyothorax, emphasizing early pleural drainage, structured reassessment of clinical response, timely surgical intervention when required, and the prevention of fibrothorax.

MATERIALS AND METHODS

This study presents the clinical management of feline patients diagnosed with pyothorax and treated at the "Prof. univ. dr. Alin Bîrţoiu" University Veterinary Emergency Hospital, Faculty of Veterinary Medicine Bucharest.
Diagnosis of pyothorax was established through a combination of clinical examination, thoracic imaging and analysis of pleural fluid obtained by thoracocentesis. Initial diagnostic evaluation included thoracic radiography and ultrasonography to confirm the presence of pleural effusion and assess lung expansion. In selected cases, computed tomography (CT) was performed to further evaluate the extent of pleural disease and identify possible underlying causes such as pulmonary abscesses, foreign bodies or localized pulmonary lesions.
Initial stabilization focused on restoration of respiratory function and hemodynamic stability. Oxygen supplementation was provided, and analgesia was administered to improve patient comfort and reduce respiratory compromise associated with thoracic pain. Diagnostic thoracocentesis was performed in all patients to confirm septic pleural effusion and to achieve immediate decompression of the thoracic cavity. Following confirmation of pyothorax by macroscopic examination of pleural fluid and rapid cytological assessment, thoracic drainage was established using chest tubes to allow continuous evacuation of purulent pleural fluid. Bilateral drainage was performed when pleural effusion was present in both hemithoraces. Thoracic lavage with sterile warmed isotonic fluids, such as Ringer's solution, was initiated to facilitate removal of inflammatory debris and bacterial contamination from the pleural space. Systemic antibiotics were administered intravenously and initially selected based on cytological findings, later adjusted according to bacterial culture and antimicrobial susceptibility testing when available.
Clinical progression was monitored through repeated physical examinations, assessment of respiratory function, and evaluation of drainage characteristics. Particular attention was given to the response during the first 48-72 hours of treatment, as failure to demonstrate clinical improvement within this period raised concern for persistent infection, pleural loculation or development of restrictive pleural disease.
Surgical intervention was considered in patients that failed to respond to conservative management, in cases with persistent pleural infection despite adequate drainage, or when imaging findings suggested the presence of pulmonary lesions requiring surgical exploration. Surgical procedures were performed through thoracotomy in order to allow debridement of infected tissue, exploration of the pleural cavity, and lung lobectomy when necessary. Follow-up evaluation included clinical reassessment and thoracic imaging to monitor lung re-expansion and detect possible complications such as pleural fibrosis or fibrothorax.

RESULTS AND DISCUSSIONS

Pleural Fluid Characteristics

Pleural fluid obtained from affected cats is typically turbid to purulent in appearance and consistent with septic exudate. In severe cases, the fluid contains a high burden of inflammatory cells, fibrinous material and necrotic debris, reflecting the intensity of pleural inflammation and bacterial contamination (Figure 1).

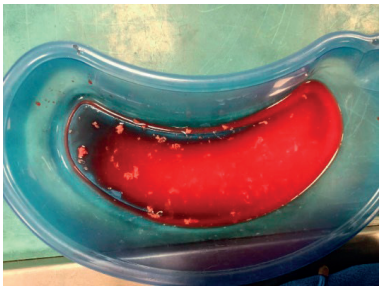

Figure 1. Gross appearance of pleural fluid obtained by thoracocentesis showing turbid reddish exudate with fibrinous debris consistent with septic pleural effusion (original Dr. Uzun, FMVB)

Cytological evaluation of the pleural fluid demonstrates septic exudate characterized predominantly by degenerated neutrophils and abundant bacterial organisms. Numerous bacteria are observed both free within the background and intracellularly within neutrophils, consistent with active bacterial phagocytosis. The smear also contains cellular debris and inflammatory material typical of severe pleural infection (Figure 2).

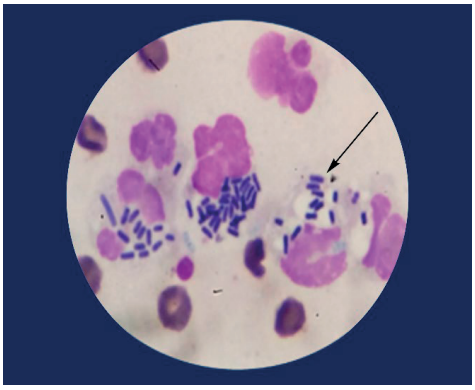

Figure 2. Thoracocentesis fluid cytology (MGG Quick stain, 1000×) demonstrating septic exudate characterized by degenerated neutrophils with intracellular bacteria consistent with bacterial phagocytosis
(Modified from Uzun S., Veteriner Tıp Acil Prosedürleri).

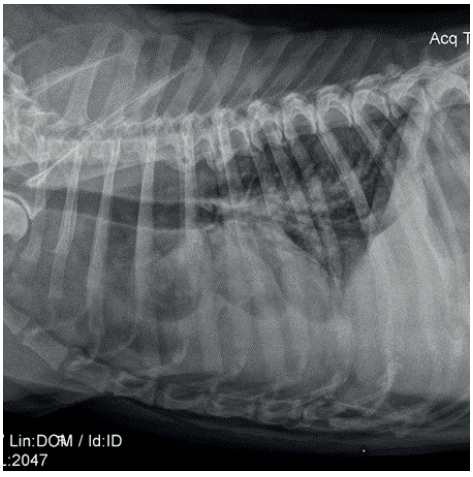

Figure 3. Right lateral thoracic radiograph of a cat with pyothorax. There is marked pleural effusion causing retraction of the lung lobes away from the thoracic wall, widened interlobar fissure lines, and obscuration of the cardiac silhouette and diaphragm. The overall "soft tissue opacity" pattern is typical of large-volume pleural fluid accumulation
(original radiology department FMVB)

Thoracic Radiography

Thoracic radiography reveals pleural effusion, fissure lines, lung lobe retraction, and loss of cardiac silhouette. However, in severe effusion, radiographs may only show a "white thorax" with limited detail (Figure 3).

Pleural Drainage and Thoracic Lavage

Following confirmation of pyothorax, chest tubes are placed in order to establish continuous drainage of the infected pleural fluid. Bilateral drainage is required in patients with fluid accumulation affecting both hemithoraces. Continuous evacuation of purulent pleural effusion improves lung expansion and facilitates subsequent pleural lavage.

Drainage characteristics are monitored closely during hospitalization, with particular attention to the volume, consistency and persistence of pleural exudate. In patients responding favorably to treatment, progressive reduction in drainage volume and improvement in respiratory pattern are observed (Figure 4).

Figure 4. Cat with pyothorax on the second day after chest tube placement. The patient shows improved comfort and respiratory stability following the third thoracic lavage (original Dr. Uzun, FMVB)

Thoracic lavage with sterile warmed isotonic fluids, such as sterile warmed Ringer's solution, was initiated to facilitate removal of inflammatory debris and bacterial contamination from the pleural space, in order to facilitate removal of purulent exudate, fibrinous material and cellular detritus from the pleural cavity. Repeated lavage improves clearance of the infected pleural space and enhances the effectiveness of chest tube drainage.

In cases with early therapeutic response, lavage contributes to progressive reduction of pleural contamination and improves lung re-expansion.

However, in more advanced disease, persistent fibrin deposition and pleural loculation limit the efficacy of lavage alone.

Surgical Management

Surgical intervention becomes necessary in patients that fail to respond adequately to pleural drainage and thoracic lavage or in cases where advanced pulmonary pathology and restrictive pleural changes are present. Additionally, patients that initially respond to medical management may subsequently develop recurrence of pyothorax over time. Operative treatment allows direct access to the pleural cavity for evacuation of purulent material, removal of fibrinous debris, disruption of pleural loculations and extensive lavage of the infected thoracic space. In addition, surgical exploration permits direct evaluation of pulmonary structures and identification of underlying lesions that may perpetuate infection.

In selected cases, pulmonary lesions require lung lobectomy in order to eliminate the primary source of infection and restore thoracic function. Severely affected lung lobes may demonstrate necrosis, abscessation or irreversible parenchymal destruction, making preservation impossible. Surgical resection of the diseased lung tissue, combined with thorough debridement of the pleural cavity and lavage, contributes to resolution of infection and facilitates re-expansion of the remaining pulmonary parenchyma (Figure 5).

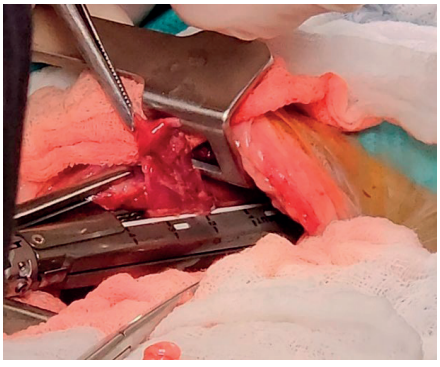

Figure 5. Intraoperative view during thoracic surgery showing lung lobectomy performed to remove severely diseased pulmonary tissue associated with pyothorax (original Dr. Uzun, FMVB)

In chronic or delayed cases, progressive pleural fibrosis may lead to fibrothorax, characterized by thick fibrous pleural membranes that severely restrict lung expansion. Intraoperatively, these patients demonstrate marked pleural thickening and lung entrapment, often preventing normal pulmonary re-expansion even after evacuation of infected material (Figure 6).

The development of fibrothorax represents one of the most severe complications of feline pyothorax and significantly limits the potential for complete pulmonary re-expansion. Once dense fibrous pleural membranes have formed, even aggressive surgical intervention may fail to restore normal lung function. These findings emphasize the importance of early diagnosis, rapid pleural drainage, and close monitoring of treatment response in order to prevent progression toward irreversible pleural organization.

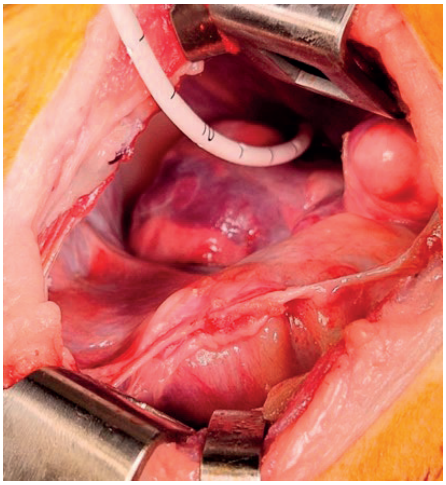

Figure 6. Intraoperative appearance of severely fibrotic lung lobes showing marked restriction of lung expansion consistent with fibrothorax and lung entrapment (original Dr. Uzun, FMVB)

Based on the clinical observations and therapeutic responses observed in these cases, a clinical decision-making algorithm was developed to assist in the diagnostic evaluation and management of pleural effusions and feline pyothorax (Figure 7).

Pyothorax represents a severe infectious process within the pleural cavity characterized by accumulation of purulent exudate, bacterial contamination, and progressive inflammatory changes affecting the pleural membranes. In cats, the condition may develop rapidly and commonly presents with respiratory distress, lethargy, and systemic inflammatory response.

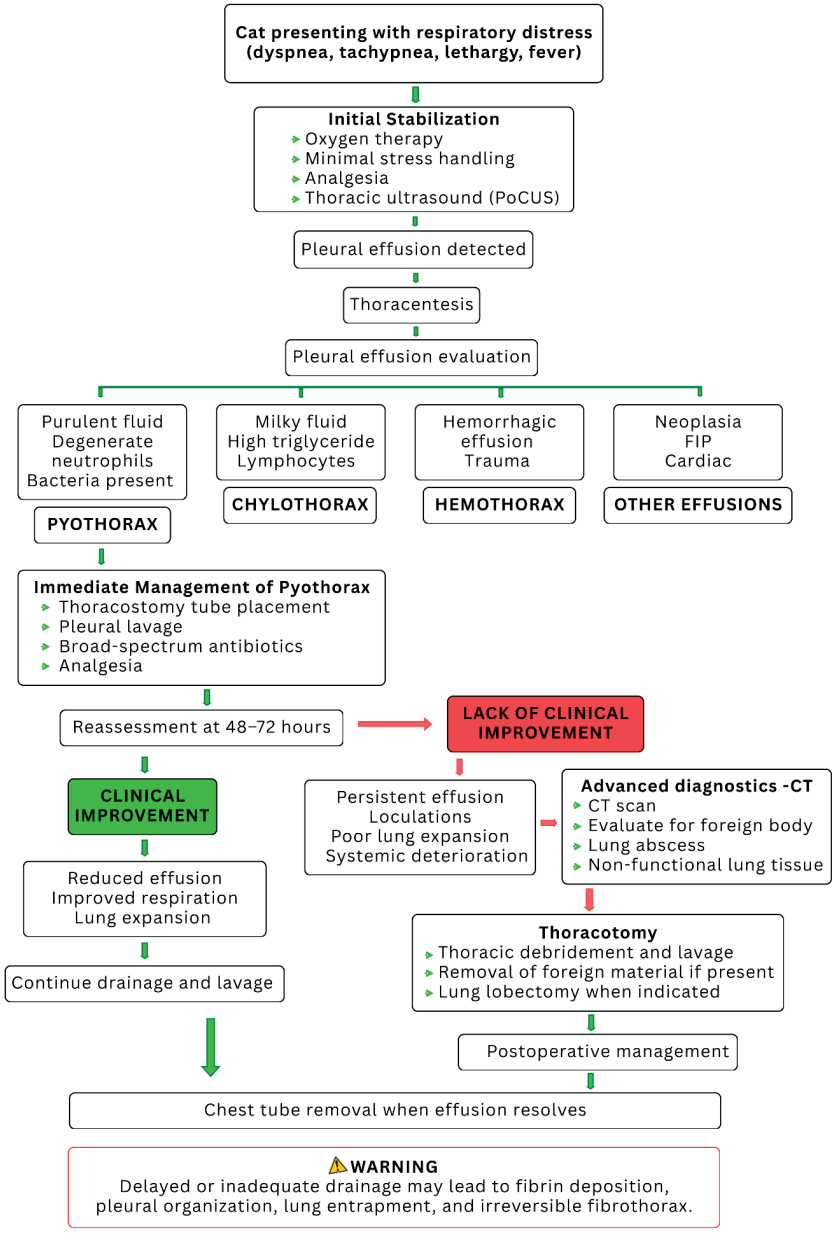

Figure 7. Clinical decision-making algorithm for the diagnostic evaluation and management of pleural effusions and feline pyothorax. The flowchart summarizes the stepwise clinical approach from initial stabilization and pleural fluid classification to medical management and indications for surgical intervention in cats with suspected pyothorax

Early recognition and prompt evacuation of infected pleural fluid are critical components of successful management. Delayed or inadequate drainage allows ongoing pleural inflammation, leading to fibrin deposition, pleural organization, and formation of loculations that interfere with effective drainage and lung re-expansion. Progressive pleural fibrosis may ultimately result in lung entrapment and irreversible fibrothorax, substantially

complicating clinical management and reducing the likelihood of successful conservative therapy (Fossum, 2018).

Effective treatment of pyothorax relies on rapid evacuation of infected pleural fluid, adequate drainage, and appropriate antimicrobial therapy. Thoracostomy tube placement enables continuous drainage of purulent material and facilitates repeated pleural lavage, which helps reduce bacterial load and remove inflammatory debris from the pleural cavity (Fossum, 2018). Early intervention is therefore essential to prevent progressive fibrin deposition and organization within the pleural space, allowing restoration of normal pulmonary expansion. Previous reports have shown that many feline pyothorax cases can be successfully managed with medical therapy when effective drainage and lavage are instituted promptly and combined with appropriate systemic antibiotic treatment (Monnet, 2018).

Surgical exploration should be considered in cases demonstrating persistent pleural effusion, formation of pleural loculations, poor pulmonary re-expansion, or systemic deterioration despite appropriate medical management. Progressive fibrin deposition and compartmentalization of purulent material may prevent effective drainage and lavage, limiting the success of conservative treatment approaches (Fossum, 2018; Johnston & Tobias, 2017). Advanced imaging, particularly computed tomography, may assist in identifying underlying causes such as foreign bodies, pulmonary abscesses, or non-functional pulmonary tissue. Thoracotomy allows direct removal of fibrinous debris, breakdown of loculations, and elimination of infectious sources, including pulmonary lobectomy when severely diseased lung tissue is present (Monnet, 2018; Fossum, 2018).

Pyothorax should be considered a time-sensitive thoracic emergency rather than a condition that allows prolonged therapeutic hesitation. Delayed or insufficient drainage permits rapid progression of pleural inflammation, fibrin deposition, and formation of pleural loculations, ultimately leading to lung entrapment and fibrothorax. Once this stage develops, conservative therapy becomes increasingly ineffective and surgical intervention may represent the only remaining option for survival (Fossum, 2018; Johnston & Tobias, 2017). Clinicians managing feline pyothorax should therefore closely monitor response to treatment and avoid prolonged medical management when clinical improvement is not evident. Early recognition of treatment failure and timely surgical consultation may be decisive in preventing irreversible pleural disease and improving patient outcome.

CONCLUSIONS

Feline pyothorax represents a severe pleural infection requiring rapid recognition and decisive clinical management.

Successful treatment depends on prompt evacuation of infected pleural fluid, effective drainage, and appropriate antimicrobial therapy. Delayed or inadequate treatment may allow progression of pleural inflammation, fibrin deposition, and loculation formation, ultimately resulting in lung entrapment and fibrothorax. Recognizing when medical therapy has failed and surgical management is required may be the most critical decision in the treatment of feline pyothorax.

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UROLITHIASIS IN WILD FELIDS

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Abstract

Urolithiasis in wild felids is uncommon under natural conditions but becomes clinically relevant in captivity due to dietary, environmental, and behavioral changes. Th This study describes a case series conducted between 2020 and 2025 on nine captive large felids (Panthera leo and Panthera tigris altaica). Clinical evaluation revealed signs of urinary tract involvement, including dysuria, hematuria, pollakiuria, and lethargy. Urinalysis identified crystalluria in 4/9 animals, with calcium oxalate, struvite, bilirubin, and cystine crystals, along with hyaline and non-hyaline casts. Biochemical analysis showed severe azotemia (mean BUN 162 mg/dL; creatinine 17.1 mg/dL), indicating advanced renal dysfunction. Urinary findings, including high specific gravity (>1.030), alkaline pH, proteinuria, and leukocyturia, suggest altered urinary homeostasis. Although urolith formation appeared infrequent, the presence of renal impairment highlights the clinical significance of urinary disorders in captive wild felids and supports the need for regular monitoring and preventive management strategies.

Key words: *urolithiasis, wild felids, crystalluria.*

INTRODUCTION

Urolithiasis in wild felids is relatively uncommon under natural conditions, but it acquires significant clinical importance in captivity, where environmental, dietary, and behavioral changes favor the development of urinary disorders. This condition is characterized by the formation of uroliths within the urinary tract and has a multifactorial etiopathogenesis comparable to that described in domestic cats, but influenced by the biological and ecological particularities of wild species (Miller & Fowler, 2015).

Wild felids, such as *Panthera leo*, *Panthera tigris*, *Panthera pardus*, and *Lynx lynx*, are strict carnivores adapted to a high-protein diet and an indirect water intake derived mainly from prey consumption. Physiologically, these species exhibit an increased capacity for urine concentration, a typically acidic urinary pH, and a reduced frequency of urination, all of which represent efficient evolutionary adaptations for water conservation. In natural environments, these characteristics do not usually favor urolith formation, as the diet is naturally balanced and physical activity levels are high (Vihol et al., 2017).

However, in captivity, these parameters may be significantly altered. Diets may contain inappropriate mineral levels or differ substantially from natural prey composition, water intake may be insufficient, and reduced physical activity along with chronic stress may influence metabolism and urinary function. These changes lead to increased urinary supersaturation with lithogenic substances, thereby promoting crystallization and urolith formation (Corpa et al., 2003).

The etiology of urolithiasis in wild felids is complex and involves the interaction of dietary, metabolic, and environmental factors. Nutritional imbalances, particularly excess minerals such as calcium, phosphorus, and magnesium, as well as insufficient water intake, contribute to alterations in urine composition. From a metabolic perspective, increased urine concentration capacity, possible disturbances in calcium or purine metabolism, and, in some cases, hepatic disorders may play an important role. Environmental factors, such as chronic stress and restriction of natural behaviors, have also been implicated in the pathogenesis of this condition (Vihol et al., 2017).

The types of uroliths identified in wild felids are similar to those described in domestic cats. Struvite uroliths occur more frequently in captivity and are associated with changes in urinary pH and dietary composition. Calcium oxalate uroliths are commonly reported in large felids, especially in captivity, being correlated with concentrated urine and hypercalciuria, and they cannot be dissolved through medical therapy. Urate uroliths are less common and are mainly associated with hepatic or metabolic dysfunctions. Cystine and silicate uroliths are considered rare and are linked to genetic factors or specific dietary characteristics (Vihol et al., 2017).

Clinical manifestations of urolithiasis in wild felids are difficult to detect, especially in natural environments where direct observation is limited. In captivity, signs such as dysuria, pollakiuria, hematuria, behavioral changes, lethargy, or anorexia may be observed. In severe cases, urinary tract obstruction may occur, representing a medical emergency. In the wild, the condition may remain clinically silent, and diagnosis is often established post mortem (Vihol et al., 2017).

Diagnosis of urolithiasis in these species is challenging and often requires chemical immobilization. Imaging investigations, particularly ultrasonography, represent the methods of choice, complemented by radiography and urinalysis. Biochemical evaluation may provide additional information regarding metabolic status and renal function (Vihol et al., 2017).

Treatment depends on the type of urolith and the severity of the case. Surgical interventions are frequently required, especially in cases involving non-dissolvable uroliths such as calcium oxalate. Dietary management and correction of metabolic imbalances are difficult to implement in captivity but remain essential for preventing recurrence. Fluid therapy and general supportive care are used to stabilize patients (Vihol et al., 2017).

Prevention represents the most effective strategy and involves adapting the diet as closely as possible to natural feeding patterns, ensuring adequate water intake, reducing stress, and periodically monitoring animals in captivity. In this context, proper environmental and nutritional management plays a central role in reducing the incidence of urolithiasis (Tordiffe et al., 2012).

MATERIALS AND METHODS

This descriptive case series was conducted at Târgoviște Zoo and in local households in Dâmbovița County between 2020 and 2025 and included captive wild felids, , with an emphasis on large felines: 5 lions (*Panthera leo*) and 4 Siberian tigers (*Panthera tigris altaica*), aged between 15 and 25 years.

Clinical examination was performed under sedation when the animals were alive and could be handled safely, or during postmortem evaluation after death. Sedation was performed using a combination of medetomidine (10-20 µg/kg, intramuscularly) and ketamine (2-5 mg/kg, intramuscularly), ensuring adequate immobilization and analgesia. In selected cases, butorphanol (0.2-0.4 mg/kg, IM) was added to enhance sedation and provide additional analgesic effects.

Physiological parameters, including heart rate, respiratory rate, mucous membrane color, and oxygen saturation, were continuously monitored throughout the procedure. Reversal of sedation was achieved using atipamezole (50-100 µg/kg, IM) when necessary.

The protocol was selected based on its reliability, safety profile, and suitability for minimizing stress and handling-related risks. in wild or non-cooperative animals, in accordance with current veterinary anesthesia guidelines and animal welfare standards.

Urine samples were analyzed by dipstick and sediment examination with Potchem UA 4210 to detect crystalluria, casts, bacteria, and lipid droplets (Figure 10).

The VetScanner IA system was used to support data analysis and interpretation (Figure 1).

Figure 1. Vet Scanner IA

Figure 2. Biochemical examinations with Arkay Spotchem

RESULTS AND DISCUSSIONS

The clinical examination of the wild felines revealed a cachectic condition, anorexia, marked lethargy, dehydration, and weakness, with reduced responsiveness and poor body condition in all patients (n = 9) (Figures 2-5). The evaluation of the urinary apparatus showed dysuria (n = 5), hematuria (n = 2), pollakiuria (n = 2), and stranguria (n = 1), suggestive of lower urinary tract involvement.

Figure 3. Lion with weight loss and dysuria

Figure 4. Tiger with weight loss and stranguria

Palpation elicited discomfort in the caudal abdomen, and the findings were consistent with a urinary tract disorder requiring further investigation (urinalysis, imaging, and culture where feasible).

Figure 5. Lion with weight loss and chronic kidney disease

Macroscopic examination of the urine samples revealed in a yellow color, no turbidity, and a normal characteristic odor in 7 wild felines and gross hematuria in 2 large cats.

Microscopic examination of urine by VetScanner IA identified the following elements (Figures 6-10):

Crystals: calcium oxalate monohydrate and dihydrate, struvite, bilirubin, cystine;

Cylinders: hyaline and non-hyaline;

Bacteriuria: bacteria-like structures were observed microscopically;

Lipuria: considered compatible with the elimination of marking pheromones;

or more cautiously.

Crystalluria was detected in 4 of the 9 examined animals, whereas overt urolith formation appeared to be infrequent in the studied wild felids.

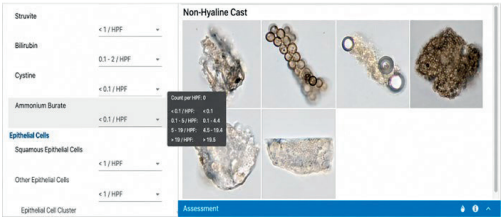

Figure 6. Urine sample with non-hyaline cast (original)

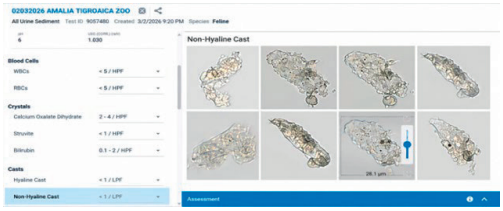

Figure 7. Urine sample with non-hyline cast (original)

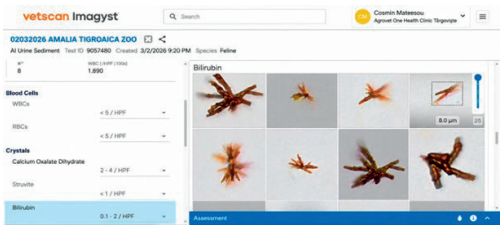

Figure 8. Urine sample with bilirubin cast (original)

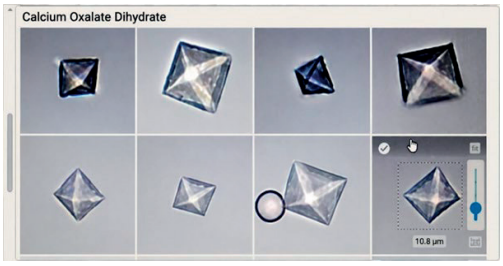

Figure 9. Urine sample with calcium oxalate dihydrate (original)

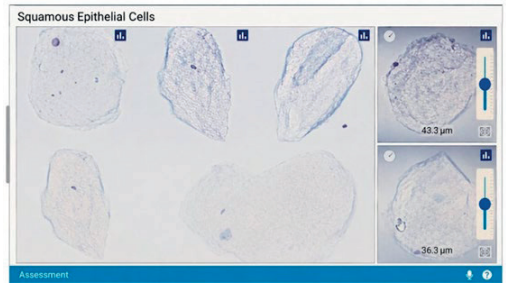

Figure 10. Urine sample with squamous epithelial cells (original)

Severe azotemia was observed across the evaluated animals, with mean BUN of 162 mg/dL and mean creatinine of 17.1 mg/dL, consistent with severe renal dysfunction/uremia. Hematological analysis revealed that total WBC counts were within reference limits, with a normal differential leukocyte count (neutrophils, lymphocytes, monocytes, eosinophils all in range). RBC count was decreased (RBC 5.5 $\times 10^6/\mu L$) with macrocytosis (MCV 64.9 fL, MCH 25.6 pg, MCHC 39.5 g/dL – increased), while HGB (11.6 g/dL) and HCT (26.9%) are at the low end of normal. Marked thrombocytopenia is present (PLT 18 $\times 10^3/\mu L$; PCT 0.016% – low). The analyzer flagged macrocytosis and thrombocytopenia.

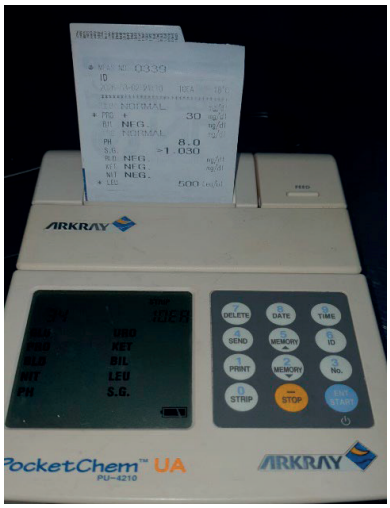

Figure 11. Urine strip examination with PocketChem UA (original)

Table 1. Median urinary dipstick results in the wild felids studied

Parameter	Tiger	Lion
Temperature	18°C	20°C
Glucose (GLU)	Normal	Normal
Protein (PRO)	+ (30 mg/dL)	+++ (300 mg/dL)
Bilirubin (BIL)	Negative	Negative
Urobilinogen (URO)	Normal	Normal
pH	8.0	7.5
Specific gravity (S.G.)	> 1.030	> 1.030
Blood (BLD)	Negative	++ (0.2 mg/dL)
Ketones (KET)	Negative	Negative
Nitrite (NIT)	Negative	Negative
Leukocytes (LEU)	500 Leu/µL	500 Leu/µL

Urolithiasis is well recognized as a major urinary disorder in domestic cats, but it has been reported far less frequently in nondomestic felids and large feline species. Nevertheless, the available literature indicates that this condition

does occur in wild felids, although most reports are limited to isolated case descriptions. Early reports documented struvite urolithiasis in a cheetah (Weber, Raphael, & Boothe, 1984), while later studies described cystine urolithiasis in a caracal (Tordiffe, van der Watt, & Reyers, 2012) and urolithiasis in an Asiatic lion (*Panthera leo persica*) (Vihol et al., 2017). Additional reports include urolithiosis in two lions (Corpa et al., 2003) and hydronephrosis associated with urinary tract pathology in a tiger (Gajendragad et al., 2004). These observations suggest that, although uncommon, urolithiasis should be considered an important differential diagnosis in captive wild felids, particularly in animals showing urinary signs, renal dysfunction, or obstructive complications.

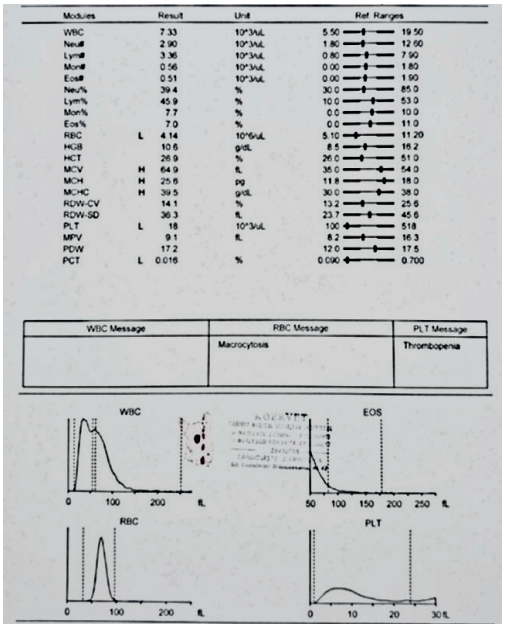

Figure 12. Hematological results of one of the wild felines

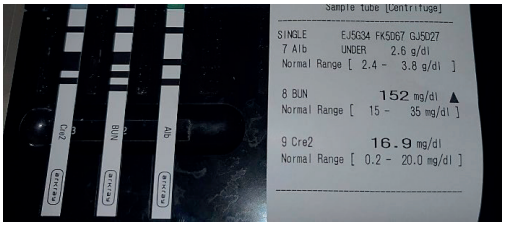

Figure 13. Biochemical results of one of the wild felines

CONCLUSIONS

The present study, conducted at the Târgoviște Zoo between 2020 and 2025, documents clinically relevant urinary findings in captive large felines (*Panthera leo* and *Panthera tigris altaica*) and supports the implementation of a structured surveillance protocol.

Urinalysis performed on free-catch samples, assisted by the VetScanner IA system, demonstrated a heterogeneous crystalluria profile, including calcium oxalate (mono- and dihydrate), struvite, bilirubin and cystine crystals, together with hyaline and non-hyaline casts. The concomitant presence of casts suggests renal tubular involvement in at least part of the evaluated cases and highlights the need to interpret crystalluria not only as a lower urinary tract issue but also in relation to potential renal pathology.

Clinical signs (dysuria, hematuria, pollakiuria, stranguria and caudal abdominal discomfort) were consistent with lower urinary tract disease; however, the marked azotemia (BUN 162 mg/dL; creatinine 17.1 mg/dL) indicates severe renal dysfunction/uremia, highlighting the likelihood of advanced renal impairment and the necessity of integrated assessment (urinalysis, hematology, biochemistry and imaging).

Despite an exclusively carnivorous diet, overt urinary stone formation appeared to be infrequent (4/9).

Hematological findings (macrocytosis with decreased RBC count and marked thrombocytepenia) in the context of severe azotemia emphasize the systemic impact of advanced disease and support the need for comprehensive clinical monitoring, particularly in geriatric zoo felids.

Overall, the results support the implementation of a structured surveillance protocol in captive wild felids, , incorporating periodic urinalysis (with sediment evaluation), biochemical renal profiling and, when feasible, imaging and microbiological culture, in order to facilitate earlier detection of urinary tract disorders and renal disease and to improve clinical outcomes in long-lived, geriatric individuals.

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PANCREATIC INSULINOMA IN A DOG - CASE STUDY

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Abstract

Insulinoma is the most common functional pancreatic endocrine tumor in dogs, characterized by autonomous insulin secretion and persistent hypoglycemia. This case study describes a 9-year-old female Wirehaired Fox Terrier presenting with recurrent neurological episodes. Clinical evaluation revealed severe hypoglycemia (28 mg/dL) and elevated insulin levels (76 µIU/mL). Imaging investigations, including ultrasonography and computed tomography, identified pancreatic masses and lymphadenopathy. Surgical excision was performed, and histopathology confirmed malignant insulinoma with metastases. Despite treatment, the outcome was unfavorable. This case highlights the diagnostic challenges of insulinoma and emphasizes the importance of considering hypoglycemia in dogs with neurological signs for early diagnosis and management.

Key words: *insulinoma, dog, hypoglycemia, pancreatic tumor, case study.*

INTRODUCTION

Insulinoma represents the most common functional pancreatic endocrine tumor in dogs, originating from the neoplastic proliferation of pancreatic β-cells and characterized by autonomous insulin secretion, independent of circulating glucose levels. This inappropriate insulin release leads to persistent or episodic hypoglycemia, which is responsible for the majority of clinical manifestations observed in affected patients (Feldman et al., 2015).

Although insulinomas may initially appear clinically localized, they are considered highly malignant tumors in dogs, with a significant propensity for early metastasis, particularly to regional lymph nodes and the liver (Ettinger et al., 2017). At the time of diagnosis, a substantial proportion of cases already present with metastatic disease, which significantly influences prognosis and therapeutic outcome (Harris et al., 2020).

The clinical presentation is often dominated by neuroglycopenic and adrenergic signs secondary to hypoglycemia. These include tremors, muscle fasciculations, weakness, ataxia, seizures, behavioral changes, and altered levels of consciousness. Due to the intermittent nature of hypoglycemic episodes and the variability of clinical expression, these signs may be misinterpreted as primary neurological disorders, such as idiopathic epilepsy, leading to delays in diagnosis (Nelson & Couto, 2019).

The diagnostic approach to insulinoma requires the integration of clinical findings, laboratory data, and imaging techniques. Persistent hypoglycemia in conjunction with inappropriately normal or elevated insulin levels is considered highly suggestive of the disease. Imaging modalities such as abdominal ultrasonography and computed tomography are essential for tumor localization and staging, although their sensitivity may vary depending on tumor size and operator experience (Goutal et al., 2012).

Therapeutic management includes both medical and surgical approaches. Surgical excision remains the treatment of choice and offers the best chance for prolonged survival, although recurrence is common due to the high metastatic potential of the tumor. Medical management is generally reserved for non-surgical candidates or as an adjunct therapy and focuses on controlling hypoglycemia through dietary modifications and pharmacological intervention (Polton & White, 2007).

The aim of this study is to describe a clinical case of pancreatic insulinoma in a dog,

highlighting the diagnostic challenges, the importance of correlating clinical and paraclinical findings, and the evolution of the disease despite therapeutic intervention.

MATERIALS AND METHODS

The study was conducted on the patient Terry, a 9-year-old neutered female Wirehaired Fox Terrier, presented to the veterinary clinic for recurrent neurological episodes characterized by convulsive manifestations.

Hematology tests were performed using a Esaote My Lab70 automated analyzer, biochemical tests were performed using an ARKRAY Spotchem EZ SP-4430 analyzer, and blood glucose levels were measured using an Accu-Chek glucose meter (Figure 1).

The medical history included trauma resulting from a fight with another dog, for which anti-shock therapy, analgesia, and supportive treatment were administered. The treatment was administered over a period of approximately three days, and the clinical evolution was favorable, with complete recovery within approximately ten days, with no evidence of major residual lesions.This aspect was considered relevant in the differential diagnosis of neurological disorders, particularly seizure activity, as post-traumatic complications could not be initially excluded.

RESULTS AND DISCUSSIONS

During the period 24-28 March 2022, the patient began to present acute neurological manifestations, consisting of muscle contractions, generalized tremor, episodes of empty biting and epileptiform manifestations associated with altered state of consciousness.

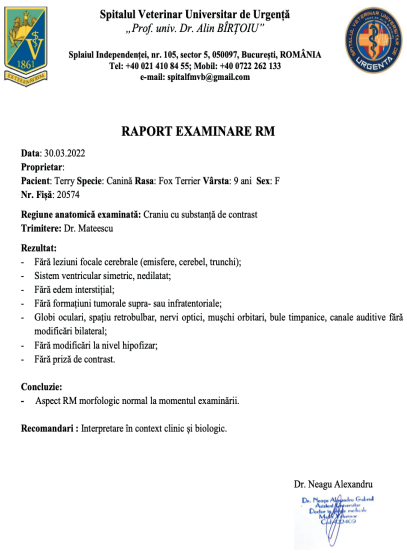
A complete set of hematological and biochemical analyses was performed at the first clinical evaluation. The complete blood count revealed changes in the erythrocyte series, thrombocytopenia and a slight lymphocytosis. Biochemical analyses revealed an increase in alanine aminotransferase (ALT = 167 U/L; reference value < 88 U/L), the other parameters being located within the upper limits of the reference range.

Blood glucose determination revealed markedly low values (28 mg/dL). Initially, hypoglycemia was considered possibly secondary to rehydration infusions, intense muscular effort and reduced food intake. However, hypoglycemia persisted even after intravenous glucose administration (Figure 3).

Given the persistence of muscle contractions and convulsive episodes, a primary neurological condition was initially suspected.

To evaluate the central nervous system, a cranial magnetic resonance imaging (MRI) with contrast agent was performed. The examination did not reveal structural lesions, tumor processes or focal or diffuse changes in the cerebral parenchyma. It is worth noting that during the imaging investigation the patient presented an epileptiform seizure characterized by muscle contractions, intense vocalizations and episodes of aggression (Figure 2).

Figure 1. ARKRAY Spotchem EZ SP-4430 dry biochemistry analyzer



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RAPORT EXAMINARE RM

Data: 30.03.2022
Proprietar:
Pacient: Terry **Specie**: Canină **Rasa**: Fox Terrier **Vârsta**: 9 ani **Sex**: F
Nr. Fișă: 20574

Regiune anatomică examinată: Craniu cu substanță de contrast
Trimitere: Dr. Mateescu

Rezultat:
- Fără leziuni focale cerebrale (emisfere, cerebel, trunchi);
- Sistem ventricular simetric, nedilatat;
- Fără edem interstițial;
- Fără formațiuni tumorale supra- sau infratentoriale;
- Globi oculari, spațiu retrobulbar, nervi optici, mușchi orbitari, bule timpanice, canale auditive fără modificări bilateral;
- Fără modificări la nivel hipofizar;
- Fără priză de contrast.

Concluzie:
- Aspect RM morfologic normal la momentul examinării.

Recomandari : Interpretare în context clinic și biologic.

Dr. Neagu Alexandru

Figure 2. Result of the MRI examination - normal morphological appearance

Figure 3. Blood glucose - value 28 mg/dL

Subsequently, abdominal ultrasound revealed changes in the pancreas, consisting of the presence of hypoechoic formations in the left pancreatic lobe, suggestive of a tumor process of a neuroendocrine nature (Figure 4).

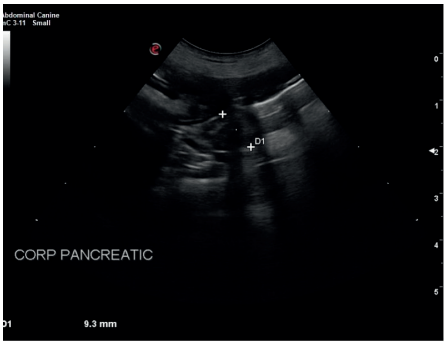


Figure 4. Ultrasound examination - presence of hypoechoic formations in the left pancreatic lobe

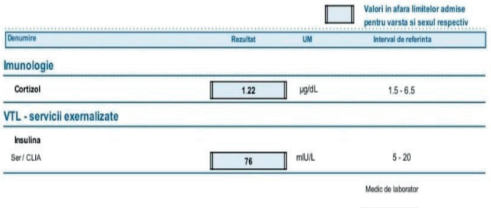
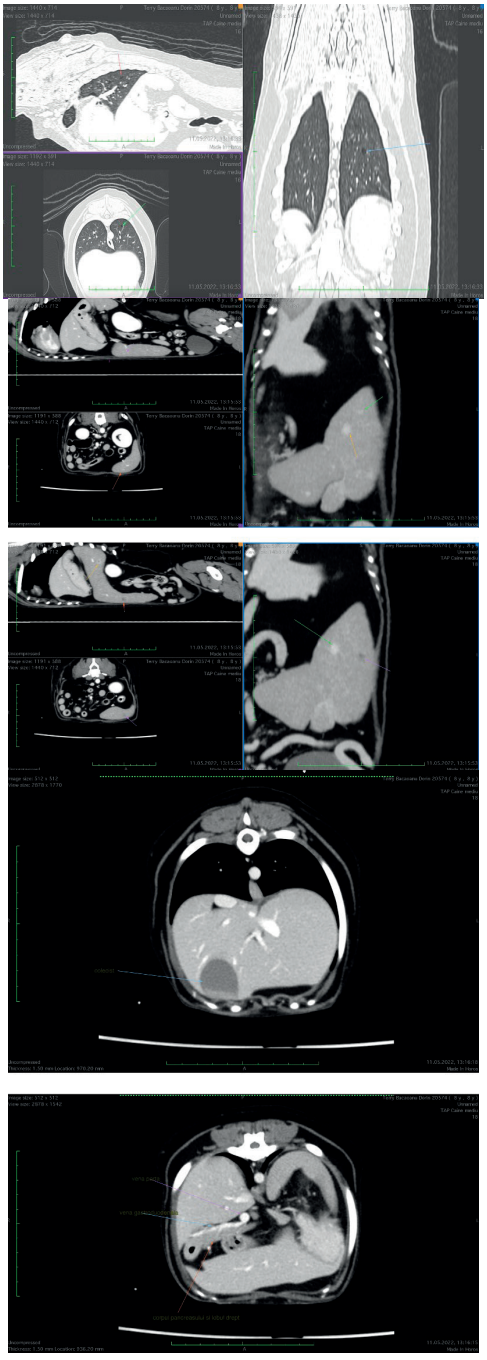


Figure 5. Insulin dosage in insulinoma

The CT scan revealed pulmonary bulla in the terminal portion of the left caudal lobe, slightly enlarged gallbladder with sludge, nonhomogeneous pancreatic parenchyma (small diffuse hyperattenuated areas – minimal contrast enhancement) and deformation of the terminal portion of the left pancreatic lobe (with minimal contrast enhancement), splenic parenchyma with multiple areas of both hyperattenuation (contrast enhancement) and hypoattenuation (cyst), various degrees of osteophytosis (C6→T1; T5→T6; L6–L7–S1) and moderate lymphadenopathy (Figure 6).



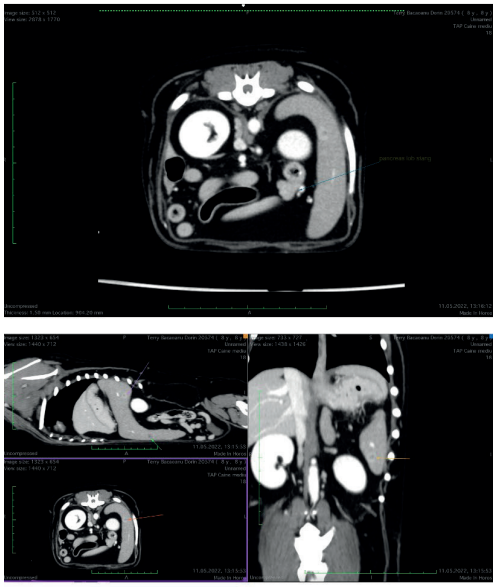


Figure 6. CT Scan - nonhomogeneous pancreatic parenchyma (small diffuse hyperattenuated areas – minimal contrast enhancement) and deformation of the terminal portion of the left pancreatic lobe

Under symptomatic treatment, consisting of anticonvulsants (diazepam administered intrarectally during seizures and phenobarbital administered daily), glucose infusions and supportive therapy (vitamin B complex, antispasmodics and corticosteroids), a transient improvement in symptoms was observed for a period of approximately 3-4 days. However, the convulsive episodes recurred.

On April 23, 2022, the patient was admitted to the Mobile Vet Clinic, where the tests again confirmed persistent hypoglycemia, despite repeated administration of glucose boluses and supportive treatment.

In order to establish a definitive diagnosis, an additional evaluation was recommended at the "Prof. univ. dr. Alin Bîrțoiu" University Hospital of Veterinary Medicine Bucharest. Imaging investigations and laboratory tests were repeated, and the correlation of persistent hypoglycemia with pancreatic changes observed by ultrasound led to the clinical suspicion of pancreatic insulinoma.

Furthermore, the serum insulin concentration was 76 µIU/mL in the presence of hypoglycemia, representing an inappropriately elevated value consistent with insulinoma (Figure 5).

Given the unfavorable clinical evolution and the persistence of hypoglycemia, a surgical excision of the pancreatic masses was elected.

Intraoperatively, two tumor formations located at the pancreatic level were identified and excised. The samples taken were sent for histopathological examination to the LABOKLIN and HISTOVET SRL laboratories, the diagnosis of insulinoma being confirmed after 21 days.

Histopathological examination revealed a pattern consistent with endocrine pancreatic carcinoma (malignant insulinoma), with metastases to the lymph nodes, liver, and spleen.

In the early postoperative period, the patient's clinical condition did not improve. Four days after the surgical intervention, a significant deterioration in the general condition was observed, with the intensification of convulsive seizures.

Given the guarded prognosis and the persistence of severe symptoms, the owner opted for euthanasia of the patient.

The present case illustrates the typical yet diagnostically challenging presentation of canine insulinoma, characterized by recurrent neurological manifestations secondary to persistent hypoglycemia. The initial clinical suspicion of a primary neurological disorder was justified by the predominance of epileptiform episodes, a common feature reported in dogs with insulinoma (Nelson & Couto, 2019). However, the absence of structural abnormalities on MRI redirected the diagnostic approach toward metabolic causes.

Persistent hypoglycemia (28 mg/dL) despite glucose supplementation represented a key diagnostic indicator. In insulinoma, hypoglycemia results from unregulated insulin secretion by neoplastic β-cells, leading to increased peripheral glucose utilization and decreased hepatic glucose production (Feldman et al.,, 2015). The lack of response to glucose therapy observed in this case is consistent with previous reports, where transient correction is followed by rapid recurrence due to continued insulin release (Goutal et al., 2012).

The hematological findings, including thrombocytopenia and mild lymphocytosis, although nonspecific, may reflect systemic stress or paraneoplastic effects. Elevated ALT levels likely indicate hepatic involvement,

which was later confirmed by histopathological evidence of metastasis. Hepatic metastasis is frequently reported in canine insulinoma and is associated with a poorer prognosis (Withrow & Vail, 2013).

Abdominal ultrasonography proved to be a valuable diagnostic tool, identifying hypoechoic pancreatic nodules suggestive of a neuroendocrine tumor. However, it is well documented that ultrasonography may have limited sensitivity in detecting small insulinomas (Polton & White, 2007). In this case, CT examination provided additional information regarding pancreatic architecture and regional lymphadenopathy, supporting the suspicion of malignancy and possible metastatic spread.

Surgical intervention remains the treatment of choice for insulinoma, offering the best chance for temporary clinical improvement and prolonged survival (Tobin et al., 1999). In the present case, the identification of two pancreatic masses and the presence of metastases indicate an advanced stage of disease at the time of diagnosis. This finding aligns with the literature, which reports that most canine insulinomas are malignant at the time of diagnosis, even when clinically localized (Trifonidou et al., 1998).

Despite surgical excision, the postoperative evolution was unfavorable, with worsening neurological signs. This outcome can be explained by several factors, including persistent hypoglycemia due to residual or metastatic tumor tissue, as well as possible postoperative complications. The presence of metastases in the liver, lymph nodes, and spleen significantly worsens the prognosis and limits the effectiveness of surgical management.

An important aspect highlighted by this case is the delay in diagnosis caused by the initial misinterpretation of clinical signs as neurological in origin. This emphasizes the need for routine blood glucose evaluation in dogs presenting with seizures or altered consciousness, especially in middle-aged to older patients. Early identification of hypoglycemia can significantly shorten the diagnostic pathway and improve clinical outcomes.

CONCLUSIONS

Pancreatic insulinoma should be included in the differential diagnosis of older dogs presenting with seizures or other neurological manifestations, especially in the context of persistent hypoglycemia. Establishing the diagnosis requires a multidisciplinary approach based on the correlation of clinical, laboratory and imaging data. Determination of blood glucose, serum insulin dosage and the use of advanced imaging methods, such as MRI or CT, are essential for confirming the diagnosis and establishing the therapeutic course.

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ANIMAL PRODUCTION, PUBLIC HEALTH AND FOOD QUALITY CONTROL

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MICROBIOLOGICAL CONTRIBUTIONS TO THE KNOWLEDGE OF THE MICROFLORA OF SOME VARIETY OF COMMERCIALIZED MEAT

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Abstract

The aim of this research was to investigate the microflora of some meat varieties from young animals sold in different halls in the central market of Chișinau. Classical microbiological research methods were used with preparation and staining according to the Gram method, visualization by immersion microscopy and passage on common and special culture media. At the same time, the microflora on the surfaces and in the air of the sales halls of rabbit, veal and lamb meats was determined. Data were obtained that fall within the category of indices admitted for the delivery of the investigated meats for consumption by insignificant differentiations of indices in veal of 15 microbial cocci cells, compared to indices of 10 and 9 cocci cells in lamb and rabbit meats.

Key words*: meat, young animals, microscopy, microbial colonies, bacteria.*

INTRODUCTION

Meat from young animals represents a significant source with substantial value for some countries, especially France, Italy, some regions of North America. At present, in the conditions of specialized marketing, some consumers increasingly prefer meat varieties of veal, rabbit, lamb, which are often appreciated for their delicate taste, more tender and paler appearance due to finer muscle fibers and a smaller amount of connective tissue (Golban, 2025; Karaman, 2021; Oprean, 2002).

The study of the microflora of meat is important, being essential for predicting shelf life, ensuring safety and developing new preservation methods (such as antimicrobial packaging) to control microbial growth and improve public health.

For these reasons, in some cases microorganisms can cause microbiological deterioration with the intervention of the microbial species *Pseudomonas, Acinetobacter, Salmonella, E. coli, Listeria* and beneficial cultures (lactic acid bacteria), originating from the animal, processing and environment, with a composition that varies depending on the type of meat (fresh, processed) and handling, affecting shelf life and safety (Răpuntean, 2005; Juran, 1986; Golban, 2024).

However, commercial meat is an important vehicle for microbial pathogens responsible for foodborne infections in humans, as its composition and physical characteristics are favorable for the growth of a wide range of microorganisms, including pathogens (Moldovan, 2013; Golban, 2015).

Contamination of meat with foodborne pathogens is a major public health problem. Among bacteria, Salmonella has been frequently associated with gastroenteritis worldwide. Different cooking techniques cannot guarantee the safety of meat if it initially has a high load of pathogenic microorganisms (Golban, 2020; Imre, 2019).

Given some problems related to the consumption of meat sold in markets and elsewhere under inappropriate conditions, it is necessary to control the microbiological quality of the meat sold (Golban, 2023; Tofan, 2004).

The objective of this study was to determine the bacterial load of meat sold in some halls, in order to know the bacterial microflora of some meat varieties from young animals.

MATERIALS AND METHODS

Microbiological research was carried out on meat samples from young animals: lamb, veal, rabbit. Meat samples from 2 halls within the central market of Chișinău were examined.

The following microbiological research methods were used: bacterioscopy, by making microbial preparations and staining according to the Gram method, visualization under a microscope with an immersion objective, and the bacteriological method by cultivation on common and special culture media: agarose, broth, Endo, in order to determine the microbial cultures characteristic of microorganisms isolated in these categories of meat sold within these halls.

RESULTS AND DISCUSSIONS

The indices of Table 1 indicate the number of colonies isolated in the lamb meat assortment in different halls. We note that the number of cocci colonies is higher in the number of 3 microbial colonies in the investigated lamb meat from hall number 1, compared to the number of colonies recorded in the lamb meat from hall number 2, where 1 microbial colony was recorded in the lamb meat samples as a result of the passages performed from the investigated samples.

Table 1. Number of microbial colonies isolated in the lamb meat assortment in the halls

Halls	Lamb meat samples (n/microbial colonies)		
	Agar/Saburo		Endo
	cocic	micettes	*Enterobacteriaceae*
N1	3	0	0
N2	1	0	0

At the same time, passages were performed on special culture media to determine the presence of enteropathogenic fungi and bacteria. As a result of passages on special Saburo culture media to identify the presence of fungi and Endo medium to identify pathogenic species in the bacterial microflora of meat from the genera *Staphylococcus, Salmonella, Escherichia,* etc., the results were negative. The lamb meat samples did not contain these species of microorganisms. Based on the above, it is interesting to read the opinion of some researchers regarding the microbial microflora of meats and the important aspects of the microbial action of microbial species on their quality from some bibliographic studies. Therefore, the most studied microbiological aspects from the point of view of the influence of saprophytic and pathogenic microflora are of interest both directly and indirectly (Carp-Cărare, 2014; Dobrea, 2014).

Table 2 shows the results of the research on the number of isolated colonies in the veal assortment in different halls. We note that the number of cocci colonies is higher in the number of 4 microbial colonies in the investigated veal from hall number 1, and the number of colonies determined in the veal from hall number 2 is 2 microbial colonies in the veal samples, after performing passages on the usual nutrient agar medium. At the same time, it is considered, according to some laboratory research studies, that the action of microorganisms from the meat microflora category has an influence on the immune system and contributes to the prevention of intestinal and respiratory infections (Golban, 2023; Taşbac, 2018).

Table 2. Number of aerobic colonies isolated in the veal assortment in different halls

Halls	Veal meat samples (n/microbial colonies)		
	Agar/Saburo		Endo
	cocic	micettes	*Enterobacteriaceae*
N1	4	0	0
N2	2	0	0

At the same time, cultures were performed on differential media to determine the presence of enteropathogenic fungi and bacteria. As a result of performing passages on special Saburo culture media to identify the presence of fungi and Endo medium to identify pathogenic species from the genera *Salmonella, Escherichia, Staphylococcus,* etc., the results were negative. The veal samples did not contain pathogenic bacterial species. It is worth noting that the mechanisms of action of some microorganisms according to some authors are diverse and complex, especially due to the chemical structure of different meat varieties. These act in most cases on the pathogenic microbial cell membrane by forming pores, or at the level of essential processes of living cells (transcription, translation, replication, biosynthesis of cell wall components) causing changes and contributing to the reduction of pathogenic infections (Dobrea, 2014; Juran, 2000).

As a result of the analyses carried out according to the indicators in Table 3, we observe the indicators of the number of isolated

colonies in the rabbit meat assortment in different halls.

Table 3. Number of aerobic colonies isolated in the rabbit meat assortment in different halls

Halls	Rabbit meat samples (n/microbial colonies)		
	Agar/Saburo		Endo
	cocic	micettes	*Enterobacteriaceae*
N1	3	0	0
N2	1	0	0

A number of cocci colonies in the number of 3 microbial colonies in the rabbit meat investigated in hall number 1 are shown, and the number of colonies revealed in the rabbit meat from hall number 2, constituted 1 microbial colony in the investigated rabbit meat samples, as a result of the inoculations carried out on the usual nutrient agar medium. In this context, based on the analyses presented, we mention the importance of the performance and functionality of the saprophytic bacterial microflora or individual non-pathogenic microorganisms, available in the foods we researched, which represents a normal bacterial microflora, resulting from investigation processes in meat halls for marketing (Fiț, 2015; Guguianu, 2002).

Thus, the need arose to perform passages on special media to determine the presence of fungi and enteropathogenic bacteria. After the analyses performed on the passages on special Saburo culture media to identify the presence of fungi and on Endo medium to identify pathogenic species, the presence of pathogenic bacteria *Salmonella, Escherichia* and *Staphylococcus* were not detected, confirming negative results. These researches in food microbiology are of public interest, an interest that allows us to deduce that the food products of different meat assortments sold in the network are qualitative and correspond to the marketing requirements. They are of importance by appreciable interest according to some authors by correlating the functionality and activity of the microflora and the functionality regarding the requirements of the human body (Josan, 2002; Golban, 2024).

The results of Table 4 confirm the microscopy indices of bacteria isolated from lamb meat samples, where it is observed that the number of cocci bacteria in lamb meat samples under microscopy was 10 cocci bacteria in hall 1, while in hall 2, the number of cocci bacteria was 14 cocci cells under microscopy in lamb meat samples.

Table 4. Microscopy indices of bacteria isolated from lamb meat samples

Halls	Lamb meat samples (microscopic field bacteria)		
	cocic	micettes	*Enterobacteriaceae*
N1	10	0	0
N2	14	0	0

Microorganisms from the category of fungi and pathogenic bacteria under microscopy were not detected.

Table 5 shows the microscopy indices of bacteria isolated from veal meat samples. It is observed that during the veal meat investigations, the number of cocci bacteria under microscopy of veal meat samples under microscopy constituted 15 cocci bacteria in hall 1, compared to hall 2, where the number of cocci bacteria was 11 cocci under microscopy in lamb meat samples.

The results of Table 4 confirm the microscopy indices of bacteria isolated from lamb meat samples, where it is observed that the number of cocci bacteria in lamb meat samples under microscopy was 10 cocci bacteria in hall 1, while in hall 2, the number of cocci bacteria was 14 cocci cells under microscopy in lamb meat samples. Microorganisms from the category of fungi and pathogenic bacteria under microscopy were not detected.

Table 5 shows the microscopy indices of bacteria isolated from veal meat samples. It is observed that during the veal meat investigations, the number of cocci bacteria under microscopy of veal meat samples under microscopy constituted 15 cocci bacteria in hall 1, compared to hall 2, where the number of cocci bacteria was 11 cocci under microscopy in lamb meat samples.

Table 5. Microscopy indices of bacteria isolated from veal meat samples

Halls	Veal meat samples (microscopic field bacteria)		
	cocic	micettes	*Enterobacteriaceae*
N1	15	0	0
N2	11	0	0

Pathogenic microorganisms from the group of

enterobacteria and fungi were not observed. At the same time, as a result of the analysis of Table 6, regarding the microscopy indices of bacteria isolated from rabbit meat samples, the presence of cocci bacteria in rabbit meat samples under microscopy is observed in the number of 9 cocci bacteria as a result of the research carried out in hall 1.

Table 6. Microscopy indices of bacteria isolated from rabbit meat samples

Halls	Rabbit meat samples (microscopic field bacteria)		
	cocic	micettes	*Enterobacteriaceae*
N1	9	0	0
N2	13	0	0

Investigations carried out in hall 2 demonstrated a count of 13 cocci bacteria under microscopy in rabbit meat samples. Microorganisms from the genera of fungi and enteropathogenic bacteria were not detected under microscopy.

CONCLUSIONS

The results of bacteriological analyses of meat samples taken from the investigated market halls revealed that the data obtained in the examined meat assortments fall within the category of indices admitted for delivery for consumption.

As a result of monitoring the data obtained from microscopy of bacteria isolated in samples of lamb, veal and rabbit meat, a number of cocci cells in veal of 15 cocci microbial cells was observed, compared to samples of lamb and rabbit meat where the indices were 10 and 9 cocci cells under microscopy.

In the lamb meat samples, a higher number of colonies was revealed in the investigations in hall 1, in the number of 3 microbial colonies, compared to the veal and rabbit meat assortments where the number of colonies determined in the veal samples a number of 4 colonies and in the rabbit meat a number of 3 colonies. In the veal and rabbit meat samples the number of microbial colonies was 1 colony and 2 microbial colonies.

It is recommended that veterinarians constantly monitor the types of meat sold.

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RECENT ADVANCES IN QPS MICROORGANISMS FOR FOOD AND FEED SAFETY, A PUBLIC HEALTH OVERVIEW

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Abstract

The 2006 EU ban on antibiotic growth promoters (AGPs) in animal feed marked a pivotal shift toward sustainable alternatives like probiotics, regulated under EFSA's Qualified Presumption of Safety (QPS) framework to ensure food and feed safety. Recent 2026 QPS updates expanded approvals for bacteriophages and select microalgae while excluding others due to safety gaps, supporting One Health strategies amid persistent challenges from feed contaminants (mycotoxins, heavy metals), zoonotic risks in novel plant/insect-based feeds, and AMR transmission. This overview synthesizes post-AGP impacts - initial 1-3% growth dips and 4-6% cost rises, offset by probiotics achieving 70-90% AGP efficacy - alongside regulatory evolution, key research gaps in long-term microbiome effects and climate-resilient forages, and practical implications for veterinary public health surveillance from farm to fork.

Key words*: Qualified Presumption of Safety (QPS), antibiotic growth promoters (AGPs), food safety, feed contaminants, One Health.*

INTRODUCTION

Public health faces ongoing uncertainties in how feed contaminants (e.g., mycotoxins, heavy metals) affect food-producing animals and enter the human chain (Sapkota et al., 2007). Questions persist on optimizing sustainable feeds like insect proteins or algae while ensuring nutritional balance across species (Kittitunyapong et al., 2025).

The 2006 EU ban on antibiotic growth promoters (AGPs) in animal feed aimed to curb antimicrobial resistance (AMR) while maintaining livestock productivity. Initial short-term challenges included 1-3% drops in growth rates, 4-6% production cost increases, and rises in enteric diseases like necrotic enteritis in poultry and post-weaning diarrhea in pigs, offset by compensatory therapeutic antibiotic use (up 10-30%). Long-term, strategies such as enhanced biosecurity, vaccines, and alternatives stabilized performance, with overall antibiotic sales declining 25-50% by 2020.

Probiotics emerged as leading AGP replacements, outperforming prebiotics (2-5% FCR gains) and phytogenics (0-8%) *via* competitive exclusion and immunomodulation, achieving 70-90% of AGP efficacy in commercial settings. EU Regulation (EC) No 1831/2003 governs probiotics as "gut flora stabilisers," requiring EFSA FEEDAP approval based on safety, efficacy, and species-specific data. EFSA's Qualified Presumption of Safety (QPS) framework streamlines this for notified taxonomic units meeting unambiguous taxonomy, safety history, and AMR criteria.

Recent 2026 QPS updates notably included bacteriophages at the species level and microalgae like *Microchloropsis gaditana* ("production purposes only", absent viable cells), while excluding filamentous fungi and denying *Bacillus thermoamylovorans* and *Aurantiochytrium acetophilum* due to insufficient knowledge. These advances support One Health-aligned sustainable feed innovations, balancing veterinary public health, food safety, and productivity post-AGP era.

In this context, the paper presents an analysis of the evolution of QPS microorganisms in Europe, in order to highlight recent advances and the results of research made in the last 20 years.

MATERIALS AND METHODS

To ensure a comprehensive understanding of recent advances in Qualified Presumption of Safety (QPS) microorganisms for food and feed safety, a rigorous literature search strategy was employed.

Databases such as PubMed, Scopus, Web of Science, and Google Scholar were utilized, alongside EFSA-specific repositories and veterinary journals *via* CAB Abstracts, to access peer-reviewed studies, regulatory documents, and grey literature.

The search focused on specific keywords and phrases including "Qualified Presumption of Safety" OR "QPS microorganisms", "antibiotic growth promoters" OR "AGPs ban EU", "probiotics animal feed", "feed contaminants" OR "mycotoxins aquaculture", "zoonotic risks novel feeds", "One Health feed safety", and "EFSA QPS updates 2024-2026", combined with Boolean operators (AND/OR/NOT) and filters for publication dates (2003-2026) to capture post-AGP ban developments.

Inclusion criteria prioritized English-language peer-reviewed articles, EFSA opinions, and reviews on veterinary public health implications; exclusions covered non-relevant human-only studies or pre-2003 data. Reference lists of key papers (e.g., EFSA QPS updates) were hand-searched for additional sources, yielding over 150 initial hits refined to 50 core references.

RESULTS AND DISCUSSIONS

The practical implications of unresolved main topics include the long-term effects of antibiotic residues in feed on microbiome resistance in ruminants and poultry, climate-resilient forages (e.g.: How to breed feeds minimizing methane emissions without reducing productivity?) and zoonotic pathogen transmission *via* novel plant-based feeds in aquaculture and livestock (Al-niaeem et al., 2026).

These gaps drive One Health approaches, emphasizing surveillance from farm to fork. For instance, EU regulations highlight needs for better data on dioxins and prions in recycled feeds. Research prioritizes HACCP integration with emerging feeds to close these knowledge voids.

Zoonotic pathogen transmission risk *via* novel plant-based feeds in aquaculture and livestock was recently detected (Al-niaeem et al., 2026).

Novel plant-based feeds in aquaculture and livestock aim to replace traditional fishmeal and soy, but they introduce potential zoonotic risks through contamination and health impacts on animals. Current research highlights indirect rather than direct transmission pathways.

Plant-based aquafeeds, like those from corn, wheat, and soy, are prone to mycotoxin contamination from fungi such as *Aspergillus* species, leading to aflatoxins that impair fish health, immunity, and growth. These toxins increase fish susceptibility to bacterial pathogens like *Aeromonas* and *Klebsiella*, which are zoonotic and can infect humans *via* contaminated seafood. Anti-nutritional factors in feeds may also weaken gut barriers, potentially amplifying pathogen loads (Mauad et al, 2025; Gruber-Dorninger et al., 2025; Glencross et al., 2020).

Shifting to novel plant proteins in livestock feeds carries contamination risks during storage or transport, though direct zoonotic pathogen survival (e.g., viruses like ASFV) is more noted in animal-derived feeds. Plant materials can harbor pathogens *via* fecal or environmental contamination, indirectly boosting zoonotic spillover in intensive systems. Phytogenic additives show promise as antibiotic alternatives but do not eliminate baseline risks from feed quality (Shepon et al., 2023; Holden, 2021; Aminullah et al., 2025; Jones et al., 2029).

Risk assessments emphasize sourcing contaminant-free plants, heat processing feeds, and monitoring mycotoxins. Insect-based novel feeds (e.g., black soldier fly larvae) enhance immunity and disease resistance without evident zoonotic amplification. Adopting One Health surveillance for feed chains can prevent transmission (Kannan et al., 2022; Glencross et al., 2020; Shepon et al., 2023; Mauad et al., 2025).

Unanswered questions on antibiotic use in livestock feeds persist due to gaps in long-term data, regulatory variations, and emerging resistance patterns. These challenges are central to veterinary public health and One Health initiatives (Ghimpețeanu et al., 2022; Zheng et al., 2025).

Key uncertainties include the exact degree to which antibiotic-resistant bacteria (ARB) from medicated feeds transfer to humans *via* meat, milk, or environmental pathways like manure runoff (Enshaie et al., 2025; Myers et al., 2022). Questions remain on whether sub-therapeutic doses in feed select for resistance genes more aggressively in ruminants versus poultry microbiomes (Ghimpețeanu et al., 2022).

It's unclear how effectively non-antibiotic feed additives (e.g., probiotics, essential oils) match growth promotion benefits across diverse livestock species under commercial stress conditions (Zheng et al., 2025). Long-term studies are needed on whether these substitutes reduce overall antimicrobial resistance (AMR) without compromising animal welfare or food productivity (Myers et al., 2022).

Challenges persist in quantifying the crossover-use of human antibiotics in animal feeds, especially in low-resource settings, and its residue impacts on food safety (Myers et al., 2022). Debates continue on optimal withdrawal periods and composting efficacy to eliminate residues like tetracyclines from manure before field application. Harmonizing global surveillance for feed-related AMR genes also lacks standardized protocols (Enshaie et al., 2025).

EU rules on antibiotic use in animal feed strictly prohibit antibiotics as growth promoters in animal feed to curb antimicrobial resistance. This ban, effective since 2006, covers all species including livestock and poultry (CABI News, 2005; EU, 2003).

Regulation (EC) No 1831/2003 bans antibiotics like monensin sodium, salinomycin sodium, avilamycin, and flavophospholipol for non-therapeutic growth promotion in feed (CABI News, 2005). The European Food Safety Authority (EFSA) evaluates additives, authorizing only those proven safe for animals, humans, and the environment with species-specific maximum dosages (EU, 2003).

Regarding their therapeutic and non-therapeutic use, antibiotics remain allowed solely for disease treatment or prevention under veterinary prescription (Regulation (EU) 2019/6), not for yield enhancement (AGRINFO, 2023). Post-2026 import rules extend this to third-country exports, requiring certification against growth promoter use (AGRINFO, 2023; Colli et al., 2025).

Maximum residue limits (MRLs) apply where needed, backed by routine feed and foodstuff testing plus post-market surveillance (CABI News, 2005). Violations trigger market withdrawal, aligning with One Health goals in veterinary public health.

The 2006 EU ban on antibiotic growth promoters (AGPs) had modest, mostly short-term effects on livestock growth rates and farming costs, with studies showing quick adaptation through alternatives like improved hygiene and probiotics (LEI, 2007).

Livestock growth rates dipped initially by 1-3% in poultry and pigs due to higher feed conversion ratios (FCR), but stabilized within 1-2 years as farmers optimized diets and biosecurity (LEI, 2007; Laxminarayan et al., 2015). No long-term productivity losses were widely reported; EU broiler and layer output remained competitive post-ban.

Feed costs rose slightly (2-5%) from increased feed intake and additive prices, contributing to overall production cost hikes of 4-6% for broilers and 11-13% for layers by 2010-2012 (compounded with other regulations like welfare rules) (LEI, 2007). These were offset by market premiums for antibiotic-free meat and efficiency gains (Table 1).

Table 1. Comparative impacts post-2006 AGP ban

Sector	Growth impact	Annual cost impact	Adaptation time
Broilers	-1-2% short-term (LEI, 2007)	+4-6% total (LEI, 2007)	1 year
Pigs	Minimal (~1%) (Laxminarayan et al., 2015)	+1-3% feed (McEwen et al., 2017)	<2 years
Layers	-2% FCR (LEI, 2007)	+11% cumulative (LEI, 2007)	2 years

The 2006 EU ban on antibiotic growth promoters (AGPs) yielded mixed long-term effects on antibiotic resistance, with reductions in specific resistances offset by rises in others due to compensatory therapeutic antibiotic use (Wen et al., 2022; Casewell et al., 2003).

Enterococcal resistance to banned AGPs like avoparcin, virginiamycin, and tylosin declined significantly in animal feces and human carriers within 3-5 years post-ban, as selection pressure lifted. Denmark's monitoring showed sustained drops in macrolide and streptogramin resistance among indicator bacteria by 2010 (Casewell et al., 2003).

Therapeutic antibiotic use rose 10-30% initially in pigs and poultry to manage diseases like necrotic enteritis and post-weaning diarrhea, elevating resistances to tetracyclines, aminoglycosides, and macrolides – key human medicines. Long-term data (to 2020s) indicate persistent higher AMR in *Salmonella* and *Campylobacter* from farms versus pre-ban levels, though overall EU AMR trends stabilized with hygiene improvements (McEwen et al., 2017; McEwen et al., 2018; Karen et al., 2019).

No uniform decline in total AMR occurred; benefits were compound-specific while prophylactic gaps drove broader therapeutic reliance, complicating One Health goals. Recent surveillance suggests net stabilization by 2020, but vigilance continues amid import pressures (Laxminarayan et al., 2015).

Strategies like improved biosecurity, alternative feed additives, and stricter therapeutic antibiotic controls helped mitigate rises in resistance after the EU's 2006 AGP ban (Laxminarayan et al., 2015; McEwen et al., 2017).

Farmers upgraded housing ventilation, all-in-all-out systems, and cleaning protocols, reducing pathogen loads (e.g., *Clostridium perfringens* in poultry) by 20-50%, which curbed the need for prophylactic antibiotics. Denmark's national action plan mandated farm audits, stabilizing overall AMR by 2015 (McEwen et al., 2017).

Feed additives like probiotics, prebiotics, organic acids, and phytogenics partially replaced AGPs, maintaining growth while lowering therapeutic antibiotic use in pigs by 15-25% long-term. Vaccines against key diseases like post-weaning diarrhea and necrotic enteritis further decreased reliance on drugs like tetracyclines (Laxminarayan et al., 2015).

The EU's 2006 AGP ban initially increased therapeutic antibiotic use in pigs and poultry due to heightened disease susceptibility, but long-term trends showed overall declines through adaptive measures.

Therapeutic use spiked short-term (e.g., Denmark saw +20-30% in tetracyclines and macrolides for post-weaning diarrhea in weaners during 2000-2002), as AGPs had masked gut infections like *E. coli* and *Clostridium* (Table 2). By 2010-2020, total use dropped 40-60% across the EU *via* vaccines, zinc oxide (later restricted), and probiotics, stabilizing below pre-ban levels (Casewell et al., 2003; McEwen et al., 2017).

Poultry saw milder initial rises (10-20% in medicated feed for broilers against necrotic enteritis), concentrated in northern Europe, but no sustained increase due to rapid vaccine adoption and biosecurity. EU-wide sales data indicate broiler therapeutic use fell ~25% by 2018, aided by Regulation (EU) 2019/6 benchmarking (McEwen et al., 2018).

Table 2. Trends in short and long term

Species	Short-Term Change	Long-Term Change	Main Drivers (McEwen et al., 2017)
Pigs	+20-116% (2000-02)	-50% (to 2020)	Vaccines, hygiene upgrades
Poultry	+10-20% (2006-08)	-25% (to 2018)	Alternatives, fewer outbreaks

The EU AGP ban did not reduce human vancomycin-resistant enterococci (VRE) infections as reductions in veterinary antimicrobial use, including AGPs, did not consistently translate into immediate, uniform declines in human VRE infections, due to multiple intervening factors such as hospital prescribing practices, infection control, and broader antimicrobial resistance dynamics.

Post-ban assessments (up to 2003) showed declines in VRE carriage among human fecal carriers and in animal indicators like enterococci from pig/poultry feces, linked to avoparcin withdrawal in 1997. However, clinical VRE infections in hospitals did not decrease and even rose in parts of Europe, driven by increased vancomycin use for MRSA treatment rather than animal sources (Casewell, 2003).

Scandinavian data (e.g., Denmark) confirmed rapid drops in animal VRE reservoirs after phased bans, but no parallel reduction in human cases where VRE was already rare (Table 3).

Table 3. Factors of changes

Aspect	Observed change (Casewell, 2003)	Primary driver
Animal VRE carriage	Sharp decline	Ban on avoparcin/virginiamycin
Human fecal carriage	Modest decline	Reduced food chain exposure
Clinical infections	No decline/increase	Hospital vancomycin overuse

Transmission from food animals to humans proved limited, with hospital selection pressures dominating (Casewell, 2003).

After the full 2006 EU AGP ban, animal health issues primarily emerged as short-term increases in specific gut and enteric diseases, though most were managed through alternatives (Huyghebaert et al., 2011; McEwen et al., 2017). Necrotic enteritis caused by *Clostridium perfringens* surged initially in broilers (up to 20-30% higher incidence in some flocks), linked to wet litter and higher feed intake without AGPs suppressing subclinical infections. Subclinical dysbiosis also raised footpad dermatitis and hock burn rates temporarily (Huyghebaert et al., 2011; McEwen et al., 2017).

Post-weaning diarrhea (PWD) from *E. coli* and *Rotavirus* increased markedly in weaners (10-50% more cases in Denmark and Netherlands during 2006-2008), as AGPs had controlled pathogen overgrowth (Table 4). This led to higher mortality (1-3%) and growth setbacks before vaccines and acidifiers took hold (McEwen et al., 2017; Laine et al., 2004).

Table 4. Resolution factors

Species	Main Issue	Duration	Mitigation (Huyghebaert et al., 2011; McEwen et al., 2018)
Poultry	Necrotic enteritis	1-2 years	Vaccines, organic acids
Pigs	Weaning diarrhea	2-3 years	Probiotics, zinc oxide (pre-2022 ban)

Industry-wide, diseases were transient, with no lasting epidemics due to rapid biosecurity upgrades and feed reformulation.

Probiotics played a key supportive role in mitigating animal health issues after the EU's 2006 AGP ban by stabilizing gut microbiota and reducing pathogen loads in pigs and poultry (Huyghebaert et al., 2011; Krysiak et al., 2021). Strains like *Lactobacillus* and *Bacillus* species competed with pathogens such as *Clostridium perfringens* (causing necrotic enteritis) and *E. coli* (post-weaning diarrhea), lowering incidence by 20-40% in trials. They improved feed efficiency and reduced subclinical dysbiosis, partially filling the AGP gap without promoting resistance and inducing gut health stabilization (Krysiak et al., 2021; Sachdeva et al., 2025; McEwen et al., 2018).

In broilers, probiotics cut mortality from enteric diseases by enhancing intestinal barrier function and immune modulation, with meta-analyses showing 5-10% better weight gains versus untreated controls post-ban. Piglet studies confirmed fewer diarrhea days (down 30%) when dosed early, aiding weaning transitions (Sachdeva et al., 2025; Abd El-Hack et al., 2022).

Probiotics worked best alongside vaccines, organic acids, and biosecurity – not as standalone replacements – achieving 70-90% of AGP performance in commercial settings. EFSA-approved multi-strain products proved most effective for sustained use (Krysiak et al., 2021; Abd El-Hack et al., 2022).

Probiotics generally outperform prebiotics and phytogenics as AGP alternatives in livestock, particularly for gut health and growth performance, based on post-EU ban studies (Agustono et al., 2025; Shehata et al., 2022).

Probiotics (live microbes like *Lactobacillus*) directly colonize the gut, reducing pathogens by 20-40% and improving FCR by 5-10% versus controls, often surpassing AGPs in poultry trials. Prebiotics (e.g., MOS, FOS) selectively feed beneficial bacteria, yielding milder gains (2-5% FCR improvement) with less consistency across flocks (Table 5).

Phytogenics (essential oils, oregano) provide antimicrobial effects *via* direct pathogen inhibition but show variable results (0-8% growth boost), limited by volatility and dosage sensitivity (Agustono et al., 2025; Shehata et al., 2022; Applegate et al., 2010; Idowu et al., 2025).

Table 5. Mechanisms and consistency

Alternative	Mechanism (Shehata et al., 2022; Applegate et al., 2010)	Growth benefit	Consistency	Cost-effectiveness
Probiotics	Competitive exclusion, immunomodulation	High (5-10%) (Agustono et al., 2025)	High in pigs/poultry	Moderate
Prebiotics	Substrate for native microbiota	Moderate (2-5%)	Medium (species-dependent)	Low
Phytogenics	Direct antimicrobials, appetite stimulation	Variable (0-8%)	Low (dose/ environment-sensitive)	High short-term

Probiotics excel in multi-strain formulations for sustained effects, while combinations (synbiotics: probiotics + prebiotics + postbiotics) often yield best results in commercial EU settings post-2006 (Sachdeva et al., 2025).

EU probiotics in animal feed are regulated as "gut flora stabilisers" under zootechnical additives *via* Regulation (EC) No 1831/2003. Only EFSA-approved strains with proven safety, efficacy, and quality reach the EU positive list after rigorous dossiers covering

identity, target species, and residue risks (Anadón et al., 2006; Lallemand Animal Nutrition, 2010; Biosafe, 2025; Regulation (EC) No 1831/2003).

Manufacturers submit data to EFSA's FEEDAP Panel, proving no harm to animals, users, consumers, or the environment - often leveraging QPS status for common strains like *Lactobacillus* or *Bacillus* to streamline review. Authorizations specify dosages, species (e.g., pigs, poultry), and claims like performance improvement, with 5-10-year renewals based on post-market monitoring (Anadón et al., 2006; Lallemand Animal Nutrition, 2010, von Wright, 2005).

Examples include *Pediococcus acidilactici* MA18/5M (Bactocell®) for broilers and *Saccharomyces cerevisiæ* CNCM I-1077 (Levucell® SC) for ruminants and piglets (Table 6). Multi-strain products must demonstrate synergy; non-QPS microbes face full genotoxicity testing (Lallemand Animal Nutrition, 2010; Biosafe, 2025).

Table 6. Important categories

Category	Examples	Target Species
Gut Flora Stabilisers	*Lactobacillus rhamnosus*, *Enterococcus fæcium* (Lallemand Animal Nutrition, 2010)	Poultry, pigs
Zootechnical	*Bacillus subtilis*, *S. cerevisiæ* (Biosafe, 2025)	Ruminants, aquaculture
Conditions	Dosage 10^{8-10} CFU/kg, species-specific (Anadón et al., 2006)	All food animals

EFSA maintains an official register of authorized feed additives, including probiotics classified as "gut flora stabilisers" or zootechnical additives under Regulation (EC) No 1831/2003. No single exhaustive public list exists in one document, but approved strains are detailed in EFSA opinions and commercial databases like the EU Feed Additives Register (EFSA, 2013; EFSA BIOHAZ Panel, 2026; PIGdk, 2024; Regulation (EC) No 1831/2003).

Common EFSA-authorised probiotic strains for animal feed include:

- *Bacillus subtilis* DSM 25841, C-3102 (e.g., in BioPlus YC, GalliPro, Calsporin) for pigs, poultry (PIGdk, 2024).
- *Bacillus amyloliquefaciens* DSM 25840 (e.g., in SolPreme) for pigs (PIGdk, 2024).
- *Bacillus velezensis* (formerly *B. subtilis*) NCIMB 30180 (Calsporin) for multiple species (PIGdk, 2024).
- *Enterococcus fæcium* strains like NCIMB 10415 (Bonvital, Fecinor) for piglets, poultry (PIGdk, 2024).
- *Lactobacillus acidophilus*, *L. helveticus*, *L. bulgaricus*, *L. lactis*, *Streptococcus thermophilus*, *E. fæcium* (Probiotic LACTINA®) for chickens, piglets at $\leq 1\times10^{10}$ CFU/kg (EFSA Journal, 2013).
- *Saccharomyces cerevisiæ* strains like MUCL 39885 (Biosprint), CNCM I-1077 (Levucell SC) for pigs, ruminants (PIGdk, 2024).

The EU Feed Additives Register (*via* EFSA or Commission websites) contains the latest, as approvals specify strains, dosages (often 10^{8}-10^{10} CFU/kg), and target animals. QPS-status microbes (e.g., many *Bacillus, Lactobacillus*) expedite approval if safety data aligns. Multi-strain products require individual efficacy proof (EFSA BIOHAZ Panel, 2026; Biosafe, 2025; EFSA, 2025).

EFSA's 2025 QPS updates expanded the list of qualified presumption of safety (QPS) taxonomic units for probiotic strains, facilitating faster approvals for animal feed use (Nicolle N., 2026; EFSA, 2025, 23:e9169).

Bacteriophages became eligible for QPS status at the species level for the first time, opening doors for phage-based probiotics targeting specific pathogens without disrupting microbiomes. *Microchloropsis gaditana* and *Chlamydomonas reinhardtii* joined the list with "for production purposes only" qualifications, while *Candida oleophila* was added for similar uses. *Lacticaseibacillus huelsenbergensis* and *Serratia plymuthica* received preliminary positive assessments pending full data (Nicolle N., 2026; EFSA , 2025, 23:e9169).

Qualifications were streamlined, extending QPS to certain genetically modified microorganisms (GMMs) if the parental strain qualifies and modifications pose no added risks – crucial for innovative feed probiotics.

Figure 1 illustrates the EFSA Qualified Presumption of Safety (QPS) evaluation process for probiotic microorganisms in animal feed, highlighting decision points that streamline approvals for safe strains like *Lactobacillus* while requiring full assessments for others.

Antimicrobial resistance genes must now be proven non-transferable and intrinsic to the species (Table 7). Denials included *Bacillus thermoamylovorans* (insufficient data) and *Bacillus thuringiensis* (safety concerns) (Nicolle N., 2026; EFSA, 2025, 23:e9169; EFSA, 2026, 24 (1) e9823).

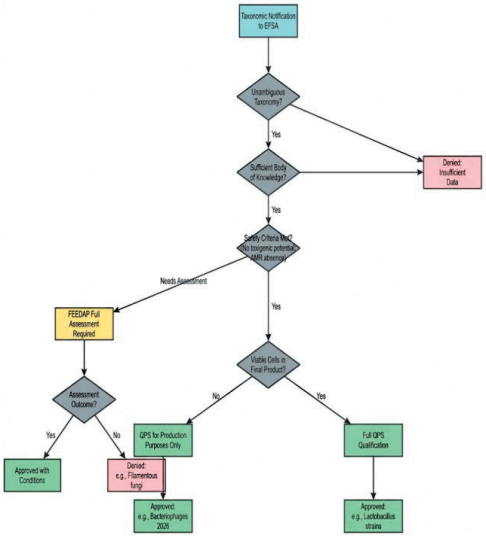

Figure 1. EFSA QPS process for probiotic microorganisms in feed

Microchloropsis gaditana received QPS recommendation with the qualification "for production purposes only," suitable for feed applications where viable cells are absent in the final product. *Bacillus thermoamylovorans* was denied QPS status due to insufficient body of knowledge, lacking extensive safety data and whole-genome sequencing for the type strain (EFSA Journal. 2026;24(1):e9824).

Table 7. Implications for probiotics

Update Category (Nicolle N., 2026; EFSA BIOHAZ Panel, 2026)	Examples	Feed Relevance
New QPS TUs	Bacteriophages, *Microchloropsis gaditana*	Pathogen control, microalgae feeds
GMM Extension	Parental QPS strains with safe edits	Precision probiotics for pigs/poultry
Reassessments	*L. huelsenbergensis* (approved)	Gut stabilisers post-AGP ban

EFSA's BIOHAZ Panel evaluated these as first-time notifications in their January 2026 update (notifications until September 2025). *M. gaditana* (formerly *Nannochloropsis gaditana*), a microalga, met taxonomy, safety history, and AMR criteria for production uses like biomass or enzymes (Table 8). *B. thermoamylovorans* failed on knowledge depth and strain identification, requiring full safety dossiers for feed authorisation (EFSA, 2026; 24(1): e9823; Nicolle, 2026).

Table 8. Comparison between strains concerning the QPS Status

Strain	QPS Status	Qualification/Reasons EFSA, 2026, 24(1):e9824; EFSA, 2026, 24(1): e9823)
Microchloropsis gaditana	Recommended	Production purposes only (safe, known)
Bacillus thermoamylovorans	Not recommended	Lack of body of knowledge, no WGS

"**Production purposes only**" is a key qualification in EFSA's QPS framework for microbial agents used in food/feed where viable cells must be absent from final products.

This qualification applies to taxonomic units (e.g., microalgae like *Microchloropsis gaditana*) employed in biosynthesis of additives such as enzymes, vitamins, or microbial biomass, ensuring no living cells reach consumers or animals. It recognizes safe histories of fermentation products despite limited direct exposure data for the live organisms themselves (Herman et al., 2019; EFSA, 2018, 16(7):e05315).

Applicants must demonstrate through validated methods (e.g., plate counts, viability assays) that viable cells of the production strain are undetectable (<1 CFU/g) in final feed/food items (Table 9). This covers inactivated biomass products too, streamlining approvals under Regulation (EC) No 1831/2003 without full genotoxicity testing (Herman et al., 2019; EFSA, 2022, 20(1):e07045).

Table 9. Examples in practice

Qualified strain/TU (EFSA, 2026, 24(1):e9824)	Use case	Verification needed
Microchloropsis gaditana	DHA-rich biomass	No viable cells post-harvest
Chlamydomonas reinhardtii	Production enzymes	<1 CFU/g in final additive

This supports One Health-aligned sustainable feeds while prioritizing consumer safety in veterinary public health contexts.

"Production purposes only" QPS qualifications apply to microorganisms used in manufacturing processes where no viable cells remain in the final food or feed product (EFSA, 2020, 18(7):e06174; EFSA, 2023, 21(1):7747).

This limits QPS to production of enzymes, additives, or biomass, requiring proof of <1 CFU/g viable cells *via* validated methods like plate counts. It enables safe histories for fermentation aids despite limited live-organism data (EFSA, 2023, 21(1):7747).

Table 10 shows examples of authorized microorganisms used only for production purposes.

- *Microchloropsis gaditana* for DHA biomass production (Herman et al., 2019).
- *Chlamydomonas reinhardtii* enzymes (Herman et al., 2019).
- *Candida oleophila* production uses (Herman et al., 2019).
- *Vibrio natriegens* and *Agrobacterium radiobacter*, recent 2026 additions (Herman et al., 2019).
- *Hæmatococcus lacustris* (pluvialis), astaxanthin (EFSA, 2024, 22(7):e8882).
- *Ogatæa polymorpha*, yeast for enzymes (EFSA, 2024, 22(7):e8882).
- *Bacillus circulans* with cytotoxicity absence (EFSA, 2024, 22(7):e8882).

Corynebacterium ammoniagenes, confirmed status.

Table 10. Examples of authorized microorganisms

Microorganism	EFSA update (EFSA, 2026, 24(1): e9823; EFSA Journal 2023, 21(1):7747)	Typical use
Microchloropsis gaditana	2026	DHA feeds
Chlamydomonas reinhardtii	2026	Enzymes
Hæmatococcus lacustris	Earlier	Pigments
Ogatæa polymorpha	Earlier	Fermentation aids

Certain taxonomic units are categorically excluded from EFSA's QPS evaluations, including "production purposes only" qualifications, due to inherent safety risks and taxonomic ambiguity. EFSA's BIOHAZ Panel excludes entire biological groups from QPS based on historical reviews showing unlikely qualification (EFSA, 2018, 16(7):e05315; EFSA, 2021, 19(1):e06377):

- Filamentous fungi (e.g., *Aspergillus*, *Fusarium*, *Aureobasidium pullulans*): mycotoxin production potential and complex taxonomy prevent presumption of safety (EFSA, 2018, 16(7):e05315; EFSA, 2021, 19(1):e06377).
- Bacteriophages: previously excluded (pre-2026); now eligible at species level but not production-only by default (EFSA, 2018, 16(7):e05315).
- *Enterococcus fæcium*: pathogenicity risks in humans/animals (EFSA, 2021, 19(1):e06377).
- *Escherichia coli* and *Streptomyces* spp.: opportunistic infections, antibiotic production traits (EFSA, 2020, 18(7):e06174).
- *Oomycetes* and *Clostridium butyricum*: toxin and ambiguous positioning concerns (EFSA, 2021, 19(1):e06377).

These groups require case-by-case, strain-level assessments by FEEDAP or other Panels under Regulation (EC) No 1831/2003, bypassing QPS streamlining due to "potentially harmful traits" identified in 2008-2013 updates (Table 11).

Notifications involving them are auto-excluded from biannual QPS lists (EFSA, 2020, 18(7):e06174; EFSA, 2018, 16(7):e05315).

Table 11. Comparison between excluded groups of microorganisms

Excluded group	Reason (EFSA, 2018, 16(7):e05315; EFSA, 2021, 19(1):e06377)	Assessment level
Filamentous fungi	Mycotoxins, taxonomy issues	Strain-by-strain
E. coli	Pathogenicity, AMR genes	Full dossier
Streptomyces spp.	Bioactive/toxin compounds	Case-by-case
Enterococci	Infection risks	Excluded entirely

"For production purposes only" QPS qualification applies to certain microorganisms under EFSA's Qualified Presumption of Safety (QPS) system, meaning they are safe when used solely for producing substances where no viable cells remain in the final product. Examples include:

- *Vibrio natriegens*, recommended for QPS status specifically "for production purposes only" (EFSA, 2025, 23(7): e9510; EFSA, 2026, 24(1): e9823).

- *Bacillus circulans*, granted QPS with the qualification "for production purposes only" (EFSA, 2023, 21(1):7747).
- *Hæmatococcus lacustris* (syn. *Hæmatococcus pluvialis*): QPS status with "for production purposes only" (EFSA, 2023, 21(1):7747).
- *Rhizobium radiobacter* (also noted as *Agrobacterium radiobacter*): Recommended for QPS "for production purposes only" (EFSA, 2026, 24(1): e9823; EFSA, 2024, 22(7):e8882).

This qualification ensures safety by requiring no viable production organisms in the end product, often for enzymes, additives, or biomass in food/feed chains. It's distinct from unqualified QPS, limiting use to non-viable applications (EFSA, 2025, 23(7):e9510).

Vibrio natriegens serves mainly as a fast-growing bacterial host for biotech production, particularly recombinant proteins and metabolites, rather than direct consumer products (Mojica et al., 2024; Hoffart et al., 2017).

Engineered strains like Vmax X2 produce challenging proteins such as cholera toxin (CT), GbpA from *Vibrio cholerœ*, heat-labile enterotoxin (LT) from *E. coli*, and fungal nematotoxin CCTX2 from *Coprinopsis cinerea*. These yield higher amounts (6- to 26-fold over *E. coli*) and simplify purification *via* secretion into media, aiding structure/function studies (Mojica et al., 2024).

Metabolic engineering enables pyruvate, 1,3-propanediol (productivity 2.36 g/L/h), L-alanine, citramalate, poly(3-hydroxybutyrate-co-lactate) [P(3HB-co-LA)], and cadaverine (up to 158 g/L, 14.4 g/L/h) (Wu et al., 2023; Sun et al., 2024; Zheng et al., 2025).

Targets include organic/amino acids under anaerobic conditions for high substrate uptake (Hoffart et al., 2017).

Primarily lab-scale for biotech R & D (e.g., 15N-labeling, perdeuteration for neutron studies), not yet commercial food/feed products due to its "production purposes only" QPS status limiting viable cells in finals. No widespread industrial commodities identified (Mojica et al., 2024; Thoma and Blombach, 2021; Hädrich et al, 2025; Xu et al., 2022).

Vibrio natriegens is primarily used as a recombinant host for producing challenging proteins, including enzymes and toxins, rather than natively producing specific enzymes at scale (Smith et al, 2024; Mojica et al., 2024).

- Cholera toxin (CT) and heat-labile enterotoxin (LT) are secreted *via* Type II secretion system, yielding 6- to 26-fold higher than in E. coli for structural studies (Mojica et al., 2024).
- GbpA (from *Vibrio cholerœ)* presents high-yield production and purification from media (Mojica et al., 2024).
- N-acetyl amino acid racemase, isoprene synthase, and ribonucleoside diphosphate kinase are expressed at 2.6- to 12-fold higher levels than in *E. coli* (Mojica et al., 2024).

Native enzymes are not well-documented for industrial use; focus is on its fast growth for biotech expression of heterologous proteins/toxins, aligning with "production purposes only" QPS. No commercial enzyme products identified beyond research (Smith et al, 2024; Thoma and Blombach, 2021; Hoffart et al., 2017).

The "Production purposes only" qualification for *Agrobacterium radiobacter* (syn. *Rhizobium radiobacter*) in EFSA's QPS list ensures safety by restricting its use to processes where no viable cells remain in the final food/feed product, such as enzyme or additive production (EFSA, 2026, 24(1):e9823).

This limitation addresses the species' body of knowledge (BoK) limitations, including its close relation to pathogenic *Agrobacterium* species (e.g., *A. tumefaciens* causing plant tumors) and opportunistic infections reported in immunocompromised humans, like bacteremia cases. Viable cells pose risks if consumed directly, but non-viable applications (e.g., filtrates) are deemed safe without full case-by-case assessment (EFSA, 2026, 24(1):e9823; EFSA, 2025, 23(7):e9510; EFSA, 2024, 22(7):e8882).

Added in recent 2025-2026 EFSA updates alongside *Vibrio natriegens*; contrasts with unrestricted QPS microbes. Applies to production strains verified absent of virulence factors or toxins (EFSA, 2023, 21(1):7747).

Recent EFSA updates to the QPS list for production microorganisms focus on taxonomic revisions, new inclusions with qualifications for production microorganisms, and safety

verification up to late 2025 (EFSA, 2024, 23(1):e9169; EFSA, 2026,24(1):e9823). These include:

- *Rhizobium radiobacter* is recommended for QPS with "for production purposes only" qualification (EFSA, 2024, 22(7):e8882).
- *Bacillus circulans* is added with "for production purposes only"(EFSA, 2023, 21(1):7747).
- *Hæmatococcus lacustris* (syn. *Hæmatococcus pluvialis*) is recommended for QPS with "for production purposes only"(EFSA, 2023, 21(1):7747).

Qualification changes

- *Bacillus velezensis*: "Absence of aminoglycoside production ability" withdrawn (EFSA, 2023, 21(1):7747).
- *Bacillus paralicheniformis* was updated to "absence of toxigenic activity" and "absence of bacitracin production ability" (EFSA, 2023, 21(1):7747).

No major changes to existing QPS TUs in the April-September 2024 period; list updated for taxonomy accuracy. Ongoing notifications (e.g., 54 in 2024) assessed for feed additives and enzymes, with production-only limits ensuring no viable cells in finals (EFSA, 2024, 23(1):e9169; EFSA, 2026, 24(1):e9823).

CONCLUSIONS

The transition away from antibiotic growth promoters has successfully shifted livestock production toward a more sustainable ecological framework.

By integrating advanced biosecurity and gut flora stabilizers, the industry has balanced animal health with public safety, stabilizing antimicrobial resistance without sacrificing long-term productivity.

Future resilience depends on the continued evolution of safety assessment frameworks, particularly as novel microbial technologies and alternative proteins enter the food chain. Harmonized global surveillance and the exploration of indirect zoonotic pathways will be essential to maintaining a secure, farm-to-fork production system in a changing climate.

The post-AGP era highlights the value of evolving microbial strategies in balancing productivity with food safety and environmental resilience.

Integrated surveillance systems, such as HACCP frameworks, alongside global harmonization efforts, offer pathways to address persistent challenges like indirect zoonotic risks and climate impacts on feed systems.

Prioritizing research into novel feed sources and regulatory alignment for emerging technologies will further advance One Health principles, ensuring sustainable veterinary practices from farm to fork.

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POLYPHENOL–GENTAMICIN SYNERGY AGAINST MULTIDRUG-RESISTANT GRAM-NEGATIVE BACTERIA

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Abstract

Antimicrobial resistance in Gram-negative ESKAPE pathogens demands adjuvant strategies that restore first-line antibiotics. We screened ten natural polyphenols against 32 MDR/XDR clinical isolates of Klebsiella pneumoniae, Acinetobacter baumannii and Pseudomonas aeruginosa, combining broth microdilution, checkerboard FICI, time-kill and crystal-violet biofilm assays. Gallic acid and quercetin displayed the strongest intrinsic activity, while gallic acid- and curcumin-gentamicin pairs produced consistent synergy, reducing gentamicin MICs 4- to 16-fold and resensitizing high-level resistant strains. Synergistic combinations achieved rapid bactericidal killing with durable suppression of regrowth over 24 h and curtailed biofilm formation by up to 82% at sub-inhibitory concentrations. These findings position selected polyphenols as rational aminoglycoside adjuvants and provide a translational basis for polyphenol–antibiotic combinations against critical Gram-negative resistance.

Key words: *antimicrobial resistance; biofilm; gentamicin; polyphenols; synergy.*

INTRODUCTION

The global dissemination of antimicrobial resistance (AMR) among Gram-negative bacteria constitutes a formidable challenge to modern medicine (Javadimarand et al., 2026; Murray et al., 2022). The ESKAPE pathogens, notably *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, are frequently implicated in severe nosocomial infections (Gebeyehu et al., 2026; Sakalauskienė et al., 2025), including ventilator-associated pneumonia, bloodstream infections, and complicated urinary tract infections (De Oliveira et al., 2020). These organisms possess a remarkable repertoire of intrinsic and acquired resistance mechanisms (Pandey et al., 2025), such as the production of extended-spectrum beta-lactamases (Akter et al., 2026) and carbapenemases, overexpression of multidrug efflux pumps (Miftode et al., 2026), and the modification of outer membrane porins (Miller & Arias, 2024). Furthermore, their ability to form robust biofilms on biotic and abiotic surfaces significantly enhances their tolerance to antimicrobial agents and host immune responses (Sharma et al., 2026; Zdarska et al., 2026), leading to chronic and recurrent infections (Sarkar et al., 2024).

Aminoglycosides, such as gentamicin, have historically been a cornerstone in the treatment of severe Gram-negative infections (Erdem & Yilmaz-Tehli, 2026; Franco-Moreno et al., 2026) due to their rapid bactericidal concentration-dependent activity (Qureshi et al., 2025; Krause et al., 2016). However, their clinical utility is increasingly compromised by the widespread emergence of aminoglycoside-modifying enzymes (Alikani et al., 2025) and the up-regulation of efflux systems (Garneau-Tsodikova et al., 2016). Additionally, the narrow therapeutic index of aminoglycosides, associated with nephrotoxicity and ototoxicity, limits the feasibility of simply increasing the administered dose to overcome resistance

(Zadrożniak et al., 2025; Wargo & Edwards, 2014).

In this context, the concept of adjuvant therapy or combination therapy using non-antibiotic compounds to restore the efficacy of existing antibiotics has gained significant traction (Wright, 2016). Natural polyphenols, a diverse class of plant secondary metabolites, have emerged as promising candidates (Shahnawaz et al., 2026). These compounds exhibit a wide range of biological activities, including intrinsic antimicrobial properties (Talha & Roque-Borda, 2026) and the ability to modulate bacterial resistance mechanisms (Manso et al., 2021). Polyphenols can perturb the bacterial outer membrane (Hamza et al., 2026), inhibit efflux pumps (Nappa et al., 2026), interfere with quorum sensing systems (Lima et al., 2025) and generate reactive oxygen species (Rao & Zheng, 2025), thereby sensitizing bacteria to conventional antibiotics (De Rossi et al., 2025).

This study aims to systematically evaluate the intrinsic antimicrobial activity of a diverse library of ten natural polyphenols and to rigorously quantify their synergistic interactions with gentamicin against a well-characterized collection of clinical MDR and XDR strains of *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa.*

MATERIALS AND METHODS

The research was conducted between May 2024 and February 2026 in the Microbiology Laboratory of the Faculty of Biology, University of Bucharest.

A total of 32 clinical strains (12 *K. pneumoniae*, 10 *A. baumannii*, 10 *P. aeruginosa*) were isolated from patients with severe nosocomial infections. The strains were characterized as MDR or XDR based on standard clinical susceptibility testing. Bacteria were routinely cultured in Tryptic Soy Broth or on Mueller-Hinton Agar at 37°C.

Gentamicin sulfate and ten natural polyphenols (gallic acid, curcumin, quercetin, resveratrol, epicatechin, kaempferol, caffeic acid, ferulic acid, vanillic acid, and p-coumaric acid) of analytical grade (≥95% purity) were utilized. Stock solutions were prepared in appropriate solvents and diluted in culture media prior to use, ensuring that the final solvent concentration did not exceed 1% (v/v) to avoid solvent-induced toxicity.

The intrinsic antimicrobial activity of the polyphenols and gentamicin was evaluated by determining the MIC using the broth microdilution method in 96-well microtiter plates, according to the Clinical and Laboratory Standards Institute guidelines. The MIC was defined as the lowest concentration of the compound that completely inhibited visible bacterial growth after 18-24 h of incubation at 37°C.

The synergistic interactions between the most active polyphenols (gallic acid, quercetin, curcumin, and resveratrol) and gentamicin were evaluated using the checkerboard microdilution method. Serial two-fold dilutions of the polyphenol and gentamicin were combined in a 96-well plate to create a matrix of varying concentration combinations. The fractional inhibitory concentration index was calculated as FICI = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination / MIC of drug B alone). Synergy was defined as FICI $\leq$ 0.5, additivity as 0.5 < FICI $\leq$ 1.0, indifference as 1.0 < FICI $\leq$ 4.0, and antagonism as FICI > 4.0.

Time-kill assays were performed to evaluate the dynamic bactericidal activity of the synergistic combinations over 24 h. Bacterial cultures (initial inoculum approximately 10^6 CFU/mL) in cation-adjusted Mueller-Hinton broth were exposed to gentamicin alone, polyphenol alone, and their combination at specific sub-inhibitory concentrations (e.g., MIC/2). Aliquots were removed at predetermined time points (0, 4, 8, 12, 24 h), serially diluted, and plated on Mueller-Hinton Agar for colony counting. A bactericidal effect was defined as a ≥3 log10 CFU/mL reduction compared to the initial inoculum.

The effect of the treatments on biofilm formation was evaluated using the crystal violet assay. Briefly, bacterial suspensions were incubated in 96-well plates in the presence of sub-inhibitory concentrations of the agents alone or in combination. After 24 h, planktonic cells were removed, and adherent biofilm biomass was stained with 0.1% crystal violet, solubilized in 33% acetic acid, and quantified by measuring the optical density at 595 nm.

All experiments were performed in triplicate. Data are presented as mean ± standard deviation. Statistical significance was determined using one-way analysis of variance followed by Tukey's post hoc test, with $p < 0.05$ considered statistically significant. The overall experimental workflow and analytical structure of the study are summarized in Figure 1.

RESULTS AND DISCUSSIONS

The screening of the ten polyphenols revealed structure-dependent variations in antimicrobial efficacy. Gallic acid demonstrated the most potent and consistent activity across all three species, with median MIC values of 128 µg/mL for *K. pneumoniae,* 64 µg/mL for *A. baumannii,* and 256 µg/mL for *P. aeruginosa.* Quercetin also exhibited notable activity, with median MIC values ranging from 128 to 512 µg/mL across the examined species. *A. baumannii* was generally the most susceptible species to the tested polyphenols, whereas *P. aeruginosa* exhibited the highest intrinsic resistance. A consolidated cross-species synopsis of the principal quantitative findings is provided in Table 1, while the most relevant numerical trends are visualized in Figure 1.

Table 1. Intrinsic antimicrobial activity and synergy profile of selected polyphenols combined with gentamicin against MDR/XDR Gram-negative clinical isolates

Polyphenol or Combination	Biological Context	Reported Quantitative Finding	Interpretive Note
Gallic acid	*K. pneumoniae*	Median MIC = 128 µg/mL	Most active polyphenol against this species among the tested library
Gallic acid	*A. baumannii*	Median MIC = 64 µg/mL	Lowest reported gallic acid MIC among the three species; highest susceptibility
Gallic acid	*P. aeruginosa*	Median MIC = 256 µg/mL	Highest intrinsic resistance to gallic acid among the three species
Quercetin	Species panel	Median MICs = 128–512 µg/mL	Notable intrinsic activity, but less consistently potent than gallic acid
Gallic acid + gentamicin	Tested strains	Synergy in 87.5% of strains; 4- to 16-fold gentamicin MIC reduction	Most robust combinational effect reported in the study
Curcumin + gentamicin	Tested strains	Synergy reported in the majority of tested strains	Promising adjuvant effect supporting aminoglycoside rescue
Quercetin or epicatechin + gentamicin	Tested strains	Significant synergistic or additive effects	Adjunctive activity was present, but less extensively quantified than for gallic acid
Gallic acid/gentamicin combination	XDR K. pneumoniae KP-04	>3 log10 CFU/mL reduction within 6 h; eradication by 12 h; no regrowth to 24 h	Rapid and sustained bactericidal activity in the representative time-kill experiment
Sub-inhibitory combination	*K. pneumoniae* biofilm	82% biofilm inhibition versus 25-35% for single agents	Marked enhancement of antibiofilm performance under combination exposure

The checkerboard assay revealed synergistic interactions between specific polyphenols and gentamicin. Gallic acid exhibited the strongest synergy, with FICI values ≤ 0.5 observed in 87.5% of the tested strains. The combination of gallic acid and gentamicin resulted in a 4- to 16-fold reduction in the MIC of the antibiotic. Quercetin and epicatechin also demonstrated significant synergistic or additive effects. The synergistic effect was most pronounced in strains exhibiting high-level baseline resistance to gentamicin (MIC > 16 µg/mL), suggesting

that the polyphenols effectively neutralize acquired resistance mechanisms. The frequency and quantitative scale of the most robust gallic acid–gentamicin interaction are summarized in Figure 2.

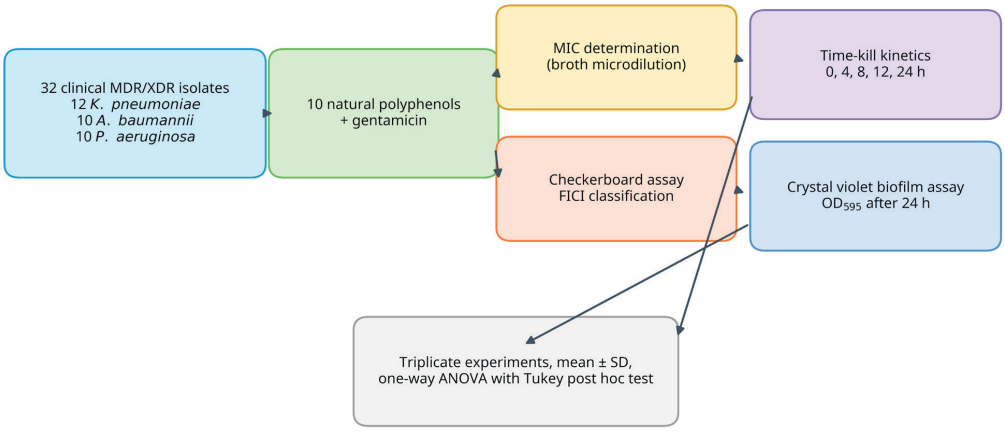

Figure 1. Experimental workflow and analytical structure of the study. The scheme summarizes isolate selection, the tested compound library, MIC determination, checkerboard-based FICI analysis, time-kill kinetics, antibiofilm testing, and the final statistical workflow

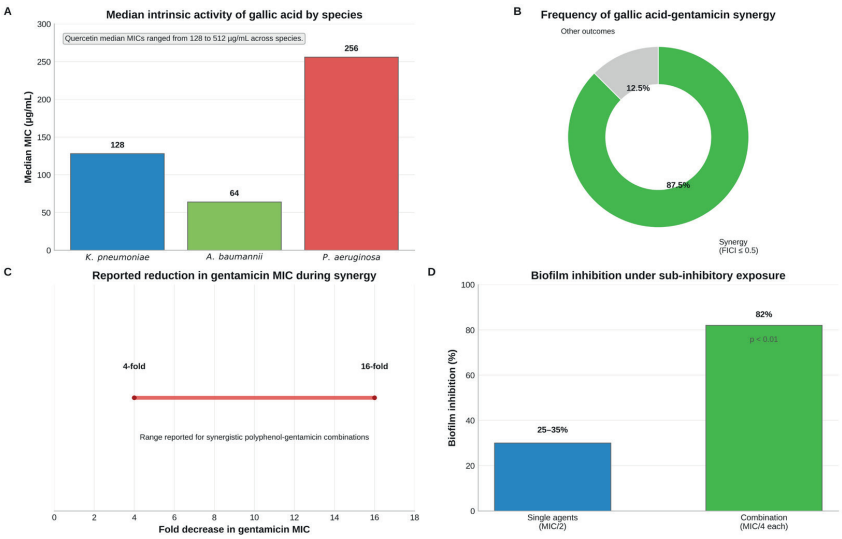

Figure 2. Quantitative summary of the leading polyphenol-gentamicin findings reported in the manuscript. Panel A shows species-resolved gallic acid MIC values. Panel B summarizes the frequency of gallic acid–gentamicin synergy. Panel C shows the reported range of gentamicin MIC reduction in synergistic combinations. Panel D compares single-agent and combination antibiofilm performance

Time-kill assays confirmed the rapid and sustained bactericidal activity of the synergistic combinations. Against the XDR *K. pneumoniae* strain KP-04, gentamicin alone at MIC/2 exhibited only a transient bacteriostatic effect followed by rapid regrowth. In contrast, the combination of gentamicin and gallic acid, both used at MIC/2, achieved a bactericidal effect exceeding a 3 log10 reduction within 6 h and completely eradicated the bacterial population below the limit of detection by 12 h, with no recrudescence observed up to 24 h. A time-resolved schematic summary of this representative experiment is presented in Figure 3.

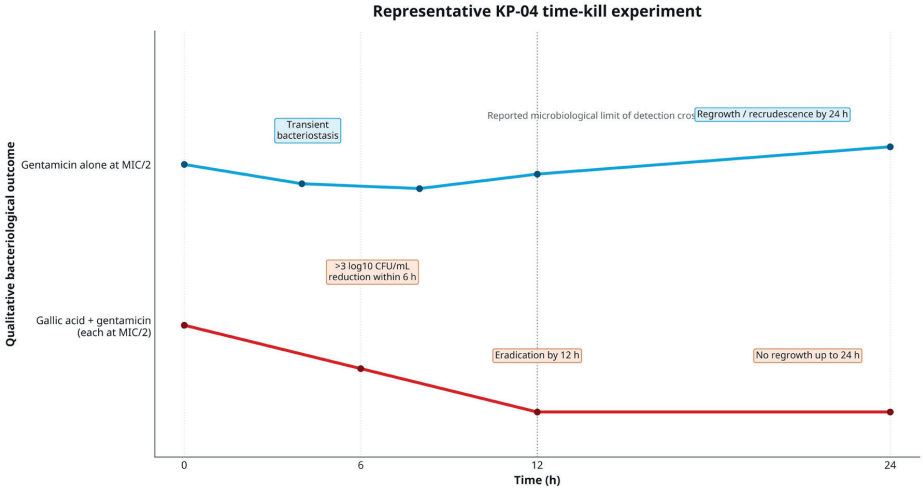

Figure 3. Time-resolved outcome of the representative KP-04 time-kill experiment. Gentamicin alone at MIC/2 produced only transient bacteriostasis followed by regrowth, whereas the gallic acid–gentamicin combination achieved a bactericidal effect within 6 h, eradication by 12 h, and no regrowth up to 24 h. This figure is a data-faithful schematic reconstruction of the reported outcome and does not infer unreported intermediate colony counts

The synergistic combinations also demonstrated significant anti-biofilm properties. While individual treatments with gallic acid or gentamicin at MIC/2 inhibited biofilm formation by 25-35%, their combination at lower concentrations (MIC/4 each) resulted in an 82% inhibition of biofilm formation in *K. pneumoniae* ($p < 0.01$). This finding suggests that the polyphenols not only facilitate antibiotic entry into planktonic cells but also interfere with the early stages of biofilm development, possibly through quorum-quenching mechanism. The numerical contrast between single-agent and combination exposure is also captured in Figure 1.

The findings of this study provide evidence for the utility of natural polyphenols as adjuvants capable of restoring the efficacy of aminoglycoside antibiotics against MDR Gram-negative pathogens. The synergism observed, particularly with gallic acid and quercetin, highlights the potential of these compounds to overcome the resistance barriers of critical ESKAPE-group organisms. A mechanistic interpretation consistent with the observed microbiological outcomes and the cited literature is summarized in Figure 4.

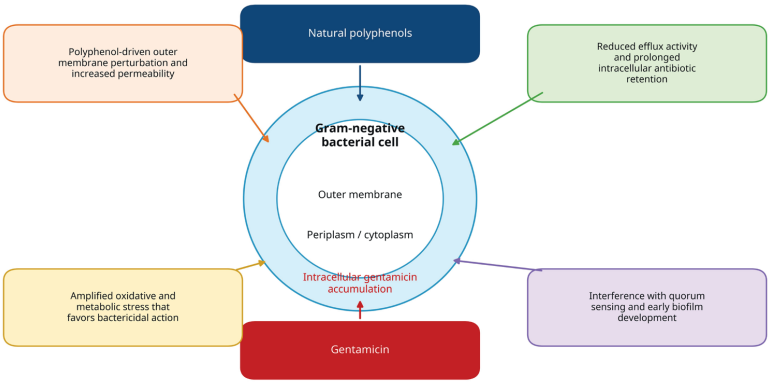

Figure 4. Proposed mechanisms underlying the potentiation of gentamicin by natural polyphenols in Gram-negative pathogens. The model integrates membrane permeabilization, reduced efflux-mediated extrusion, amplification of oxidative stress, and interference with quorum sensing and early biofilm development, in line with the study findings and the cited literature (Duda-Madej et al., 2025; Liang et al, 2026)

The mechanism underlying this synergism is likely multifactorial. Polyphenols, particularly those containing catechol or gallol moieties (Bolaños-Cardet, 2026) can interact with the bacterial outer membrane, destabilizing the lipopolysaccharide layer (Wang et al., 2025) and increasing permeability (Xie et al., 2017). This perturbation can facilitate gentamicin influx by partially bypassing the restricted porin barrier (Hashim et al., 2025). Furthermore, polyphenols have been shown to act as competitive inhibitors of resistance-nodulation-division-type efflux pumps, thereby preventing active antibiotic extrusion and maintaining lethal intracellular concentrations (Ramata-Stunda et al., 2022). In parallel, their capacity to enhance oxidative stress and interfere with quorum sensing may reinforce both bactericidal and antibiofilm outcomes (Ormeanu et al., 2025).

The ability of the synergistic combinations to rapidly eradicate bacterial populations (Fu et al., 2025) and prevent the selection of resistant mutants (Gan et al., 2025), as demonstrated by the time-kill assay, is of direct translational interest (Nath & Rodríguez-Rojas, 2026). Moreover, the anti-biofilm activity suggests that these combinations could be effective in chronic or device-associated infections where conventional monotherapy frequently fails. Collectively, the integrated visual synthesis developed for the present manuscript strengthens the interpretation that polyphenol-assisted aminoglycoside rescue merits further preclinical and mechanistic investigation.

CONCLUSIONS

This study demonstrates that natural polyphenols, specifically gallic acid and quercetin, exhibit synergistic interactions with gentamicin against clinical MDR and XDR strains of *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*. The combination therapy effectively reduces the required antibiotic dose, restores susceptibility in highly resistant isolates, and exhibits rapid bactericidal and anti-biofilm activity.

These findings support the development of polyphenol-antibiotic combination therapies as a viable strategy to combat the growing threat of antimicrobial resistance.

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ROLE OF EUBIOTICS IN ANIMAL HEALTH AND PERFORMANCE: A REVIEW

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Abstract

The escalating restrictions on antibiotic growth promoters (AGPs) in livestock production have catalyzed the search for sustainable alternatives to safeguard animal health and productivity. Eubiotics - a comprehensive category of feed additives encompassing probiotics, prebiotics, synbiotics, organic acids, phytogenic compounds, enzymes, and postbiotics -have emerged as potent tools for enhancing gastrointestinal health, nutrient utilization, immune competence, and zootechnical performance. This review synthesizes current knowledge regarding the mechanisms of action, applications, and systemic benefits of eubiotics in animal nutrition. A structured literature review (2018–2025) was conducted across major databases, including PubMed, Web of Science, Scopus, and ScienceDirect. Evidence indicates that eubiotics maintain intestinal homeostasis by modulating microbial populations, reinforcing gut barrier integrity, and mitigating pathogen colonization. While numerous studies report significant improvements in growth rates, feed conversion ratios (FCR), and animal welfare, inconsistencies persist due to variations in experimental design, dosage, and environmental factors. Further research is imperative to standardize methodologies and elucidate the complex interactions between eubiotics, host physiology, and the microbiome.

Key words: *eubiotics, gut microbiota, animal health, feed additive, performance.*

INTRODUCTION

The surging global demand for animal-derived proteins has intensified the shift toward sustainable production systems that prioritize animal welfare and productivity while minimizing environmental footprints. For decades, antibiotic growth promoters (AGPs) were foundational in livestock production for enhancing performance and controlling subclinical infections. However, the rise of antimicrobial resistance (AMR) has triggered global legislative bans on routine antibiotic use in feed (Alayande et al., 2020; Chowdhury et al., 2025). Consequently, scientific inquiry has shifted toward identifying nutritional strategies that sustain performance without reliance on traditional antimicrobials.

Among the most promising alternatives are eubiotics, a diverse group of feed additives designed to foster microbial symbiosis and gastrointestinal resilience (Jha et al., 2021; Yue et al., 2025). The term "eubiotic" originates from the Greek words “eu” (good) and “bios” (life) and refers to substances that contribute to the maintenance of microbial homeostasis. Eubiotics include a broad spectrum of functional additives, from probiotics and prebiotics to more recent innovations such as postbiotics and phytogenics (Broom, 2022; Azad et al., 2020).

The gastrointestinal tract (GIT) plays a central role in animal performance; beyond its digestive function, it serves as the primary immunological interface between the host and its environment. A balanced microbiota is essential for nutrient metabolism, pathogen exclusion, and epithelial integrity. Conversely, dysbiosis - the disruption of this microbial balance - impairs nutrient absorption and increases susceptibility to disease. Recent advances in metagenomics have highlighted how eubiotics influence these pathways by modulating microbial diversity and improving gut integrity (Cho et al., 2024; Śliżewska et al., 2020).

The application of eubiotics has been extensively investigated in poultry, swine, ruminants, and aquaculture systems. Improvements in growth performance, feed efficiency, nutrient digestibility, and disease resistance have been widely documented (Krysiak et al., 2021; Liao & Nyachoti, 2022; Gheorghe-Irimia et al., 2023). In addition,

eubiotics contribute to mitigating pathogen shedding and improving food safety (Abdel-Latif et al., 2018; Abdel-Wareth et al., 2019).

Despite the growing body of evidence, several challenges remain. The efficacy of these additives is subject to variables such as physiological stage, dietary matrix, and management practices. Furthermore, the mechanisms underlying the interactions between eubiotics and host metabolism are not yet fully elucidated (Duarte et al., 2020; Elghandour et al., 2022). This review aims to summarize the current state of knowledge regarding eubiotics, focusing on their mechanisms of action and their role in optimizing performance in modern livestock systems.

MATERIALS AND METHODS

Study Design

This study was conducted as a narrative review based on a structured search strategy and a qualitative synthesis of peer-reviewed evidence. The scope encompassed the effects of various eubiotics (probiotics, prebiotics, organic acids, enzymes, etc.) on gut modulation, nutrient utilization, and productive performance across major livestock species (Nguyen et al., 2020; Jha et al., 2021).

Data Sources and Search Strategy

A comprehensive search was performed using PubMed, Web of Science, Scopus, and ScienceDirect, supplemented by Google Scholar for citation verification. Search strings utilized Boolean operators (AND, OR) and focused on terms such as “eubiotics”, “gut microbiota”, “livestock performance”, and “feed additives”. The temporal scope was restricted to 2018-2025 to ensure an up-to-date perspective.

Inclusion and Exclusion Criteria

Primary research and systematic reviews published in English within the specified timeframe were included. Criteria required studies to provide clear methodological frameworks and report outcomes related to intestinal health or zootechnical parameters. Studies on probiotics, prebiotics, synbiotics, organic acids, and enzyme-based additives were prioritized (Azad et al., 2020; Chukwudi et al., 2024). Excluded were studies focusing on human nutrition, non-peer-reviewed editorials, and conference abstracts lacking full-text data.

Data extraction focused on species-specific responses, dosage strategies, and mechanisms of action related to gut microbiota modulation and performance outcomes.

RESULTS AND DISCUSSIONS

Types of Eubiotics and Their Mechanisms of Action

Eubiotics encompass a diverse array of additives with the shared goal of optimizing gastrointestinal homeostasis.

Probiotics, including Lactobacillus, Bacillus, and yeast strains, enhance microbial balance through competitive exclusion and antimicrobial metabolite production (Krysiak et al., 2021; Liao & Nyachoti, 2022).

Prebiotics such as fructo-oligosaccharides and mannan-oligosaccharides selectively stimulate beneficial bacteria, increasing short-chain fatty acid production and improving gut energy metabolism (Azad et al., 2020; Broom, 2022).

Synbiotics, combining probiotics and prebiotics, exert synergistic effects that enhance microbial colonization and intestinal health (Duarte et al., 2020; Shah et al., 2023).

Organic acids such as butyric and citric acid reduce intestinal pH and inhibit pathogenic bacteria, improving enzymatic activity and nutrient digestibility (Nguyen et al., 2020; Chukwudi et al., 2024).

Postbiotics and enzyme-based additives improve gut functionality through direct modulation of immune responses and nutrient availability (Elghandour et al., 2022; Cho et al., 2024).

Effects on Gut Microbiota and Intestinal Health

Modulation of the gut microbiome is a central mechanism of eubiotic action. Supplementation consistently increases beneficial bacterial populations while reducing pathogens such as Escherichia coli and Salmonella spp. (Śliżewska et al., 2020; Abdel-Latif et al., 2023, Șonea et al., 2024).

Eubiotics also improve intestinal morphology, increasing villus height and villus-to-crypt ratio, thereby enhancing nutrient absorption capacity (Cho et al., 2024; Abdel-Wareth et al., 2019).

Nutrient Digestibility and Feed Efficiency

Organic acids improve enzymatic digestion and nutrient absorption efficiency, while exogenous enzymes enhance degradation of anti-nutritional factors (Nguyen et al., 2020; Elghandour et al., 2022).

Integrated strategies combining probiotics, prebiotics, and enzymes show the most consistent improvements in feed conversion ratio and nutrient retention (Jha et al., 2021; Maina et al., 2022).

Immunomodulatory and Performance Outcomes

The gastrointestinal tract contains a major portion of the immune system, and eubiotics enhance immune regulation by strengthening epithelial barriers and modulating cytokine expression (Duarte et al., 2020; Chowdhury et al., 2025).

These effects translate into improved growth performance, feed efficiency, and disease resistance in poultry, swine, and ruminants (Abd El-Latif & Omar, 2023; Murshed et al., 2023; Șonea et al.,2023).

Current Challenges and Knowledge Gaps

Despite strong evidence, variability in outcomes remains due to differences in species, diet composition, and environmental conditions (Alayande et al., 2020; Yue et al., 2025, Gheorghe-Irimia et al., 2024).

The mechanisms governing host-microbiota-eubiotic interactions are still not fully understood, and further omics-based approaches are needed to standardize dosage and optimize combinations (Elghandour et al., 2022; Chowdhury et al., 2025).

CONCLUSIONS

Collectively, the available evidence supports the use of eubiotics as promising nutritional strategies for enhancing gut health, strengthening host resilience, and improving productive outcomes in livestock species. Probiotics, prebiotics, synbiotics, organic acids, enzymes, and postbiotics contribute to the maintenance of gastrointestinal homeostasis through modulation of gut microbiota, enhancement of intestinal barrier integrity, stimulation of immune responses, and improvement of nutrient utilization.

Numerous studies have reported positive effects of eubiotic supplementation on growth performance, feed efficiency, disease resistance, and animal welfare. Improvements in intestinal morphology, beneficial microbial populations, and digestive efficiency appear to be among the most consistent findings across poultry, swine, and ruminant production systems. In addition, growing evidence supports the use of eubiotics as sustainable alternatives to antibiotic growth promoters, contributing to efforts aimed at reducing antimicrobial resistance in animal agriculture.

Despite these benefits, the efficacy of eubiotics may vary according to animal species, physiological stage, dietary composition, environmental conditions, and the specific additives used. Differences in experimental methodologies and supplementation protocols continue to limit direct comparisons among studies.

Future research should focus on the development of standardized evaluation protocols, identification of optimal combinations of eubiotic compounds, and a deeper understanding of host–microbiota interactions using modern molecular and microbiome-based approaches. Further investigation is also required to assess the long-term economic and health impacts of eubiotic supplementation under commercial production conditions.

Overall, eubiotics represent a promising and scientifically supported strategy for promoting sustainable livestock production by simultaneously improving animal health, productivity, and food production efficiency while reducing dependence on antibiotic growth promoters.

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ANIMAL MODELS OF SKIN INFECTIONS AND TREATMENT WITH ESSENTIAL OILS - REVIEW

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Abstract

Polymicrobial wound infections represent a major clinical challenge, stimulating interest in alternative therapeutic solutions such as essential oils (EOs). The aim of the study was to perform a synthesis analysis on the efficacy of EOs in the in vivo treatment of these infections, including antimicrobial mechanisms, anti-inflammatory and healing effects. Articles were identified using PubMed, Web of Science, Scopus, Science Direct and Google Scholar, published between 2014-2025 and written in English. In vivo evaluations predominantly used murine animal models, on which wounds infected with aerobic and/or anaerobic bacterial strains were induced. The results showed that EOs (lavender, oregano, thyme and clove) significantly reduce bacterial load, disrupt biofilms and enhance antibiotic efficacy. Certain EOs reduce proinflammatory cytokine levels and accelerate wound closure, an aspect confirmed by histological analyses and standardized statistical tests, thus underlining the potential of this approach as an adjuvant therapy even though limitations related to chemical composition variability, irritation risks, lack of regulation and the need for large-scale standardized studies are also highlighted. Evidence suggests a promising role for EOs in the treatment of polymicrobial infections, requiring, however, further clinical validation.

Key words: *antimicrobial, essential oils, in vivo studies, polymicrobial infections, wound healing.*

INTRODUCTION

Skin and soft tissue infections (SSTI) represent a major public health and veterinary problem, with a high incidence and significant impact on morbidity, quality of life, and treatment-related costs. These infections range from superficial, self-limiting conditions to severe disorders with systemic potential, especially when multidrug-resistant bacterial strains are involved. An important aggravating factor in SSTI pathogenesis is the ability of certain bacteria to form biofilms - organized structures that provide protection against the host immune response and markedly reduce the effectiveness of conventional antibiotics. In this context, methicillin-resistant *Staphylococcus aureus* (MRSA) stands out as one of the most problematic etiological agents, being frequently involved in both community-acquired and nosocomial infections (Yin et al., 2024; Cășaru et al., 2024).

Wound infections, in particular, are a major cause of delayed healing and local and systemic complications, especially in chronic wounds. In these settings, persistent colonization and microbial biofilm formation dramatically reduce the efficacy of antimicrobial therapy, maintain chronic inflammation, and prevent normal progression of tissue regeneration (Kennewell et al., 2019). This clinical reality highlights the need for therapeutic approaches that simultaneously target infection control, inflammation modulation, and the support of healing.

Over the last decade, scientific interest in essential oils (EOs) has grown exponentially, driven by the need to identify effective therapeutic alternatives amid the global antimicrobial resistance crisis.

The biological activity of essential oils is mainly attributed to their major bioactive components, such as carvacrol, thymol, and cinnamaldehyde. These compounds exert antibacterial effects via complex mechanisms, including bacterial membrane disruption, perturbation of ionic homeostasis, and inhibition of virulence factor expression. Unlike classical antibiotics, which

often target a single molecular site, essential oils act simultaneously on multiple cellular structures and processes, thereby reducing the probability of resistance development (Chouhan et al., 2017). Nevertheless, their direct clinical application is limited by disadvantages such as high volatility, poor water solubility, and potential irritant effects on injured tissues (Bakkali et al., 2008).

To overcome these limitations, recent research has focused on developing advanced delivery systems for essential oils to improve stability, bioavailability, and safety. Among the most studied platforms are hydrogels, nanofibers, and nanoemulsions, which enable controlled release of active compounds directly at the wound site (Lin et al., 2021; Cai et al., 2023; Mohsen et al., 2023; Shafiq et al., 2025). Validation of these systems in animal models is essential to demonstrate *in vivo* efficacy and to understand the pharmacodynamic behavior of essential oils in a living organism (Bhatia et al., 2022).

A key component in evaluating new therapeutic strategies for SSTI is the use of experimental animal models, which allow investigation of infection dynamics and healing processes in a complex biological context. Murine models are the most frequently used due to low costs, reproducibility, and the availability of genetically modified strains. They are suitable for studying infections caused by *Staphylococcus aureus*, including MRSA, and for analyzing host immune responses. These models enable assessment of EO-based topical treatments, including nanostructured formulations, under controlled conditions, providing relevant data on bacterial load reduction, inflammation modulation, and acceleration of wound healing (Yin et al., 2024; Bhatia et al., 2022; Parker, 2017).

In addition to murine models, other animal species – such as rabbits and pigs – are used to more faithfully reproduce the structure and physiology of human skin. Porcine models, in particular, are considered the gold standard in experimental dermatology due to anatomical and functional similarities to human skin, including epidermal thickness, vascularization, and scarring processes (Shiff et al., 2023; Lindblad, 2008). These models are especially relevant for translational validation of EO delivery systems and for assessing local safety, cutaneous tolerability, and therapeutic efficacy in complex infections, including those associated with bacterial biofilms (Bhatia et al., 2022; Trøstrup et al., 2013; Shiff et al., 2023; Jamin et al., 2020).

Moreover, bacteria belonging to the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp.) play a central role in resistant infections and are responsible for most difficult-to-treat nosocomial infections. Their capacity to develop complex resistance mechanisms means that available therapeutic options are increasingly limited, emphasizing the need for alternative infection control strategies (Arias & Murray, 2024; Preda & Popa, 2021).

In this context, essential oils – secondary metabolites of plants – represent a valuable source of bioactive compounds with high therapeutic potential. Their chemical complexity, often consisting of dozens or even hundreds of volatile constituents, provides a major advantage in preventing resistance, as it is more difficult for bacteria to evolve effective adaptive mechanisms through single-point mutations compared with synthetic single-molecule antibiotics (Scandorieiro et al., 2016; Herman & Herman, 2015).

The aim of this review is to synthesize the available *in vivo* evidence on the efficacy of essential oils and their constituents for the treatment of cutaneous infections over the period 2014-2025, with emphasis on experimental models, proposed mechanisms of action, advanced formulations, and safety. This approach allows a perspective on the translational potential of essential oils in managing cutaneous infections in both human and veterinary medicine.

MATERIALS AND METHODS

Study design and methodological framework

This work is a narrative synthesis with systematic elements, conducted using an iterative search and selection strategy in accordance with PRISMA 2020 reporting principles (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) (Page et al., 2021). The methodological objective was to identify, analyze, and synthesize *in vivo*

experimental studies that evaluated the efficacy of essential oils and their bioactive constituents in the treatment of cutaneous infections using animal models.

Studies published between 2014 and 2025 were analyzed, investigating topical EO-based interventions administered either as neat oils or in advanced delivery formulations (hydrogels, nanoemulsions, nanostructured lipid carriers (NLC), nanoparticles, nanofibers, or vesicular systems) applied in experimental models of infected cutaneous wounds.

Literature search strategy

Last search (all databases): 19 January 2026. Coverage period: 1 January 2014-31 December 2025. Search strategies were adapted to the syntax of each database while preserving the main concepts: (1) essential oils; (2) skin infections/infected wounds; (3) *in vivo*/animal model; (4) topical administration/formulations.

The literature search was systematically performed in the following databases: PubMed, Web of Science, Scopus, Science Direct and Google Scholar (as a supplementary source). The search covered the period from January 1, 2014, to December 31, 2025, and was restricted to original research articles published in English.

The exact search string used was: (“essential oil” OR “phytochemical”) AND (“infected wound” OR “skin infection” OR “MRSA” OR “*Pseudomonas aeruginosa*”) AND (“*in vivo*” OR “animal model” OR “mouse” OR “rat” OR “porcine”).

To ensure reproducibility, the search was performed independently by two reviewers. The Boolean operators AND/OR were applied to combine terms related to the intervention (essential oils), the condition (skin infections), and the study design (animal models). We manually screened the reference lists of all included studies and relevant reviews to identify additional records. For each database, the search was conducted across titles, abstracts, and keywords without applying initial filters for animal sex or breed to maintain a broad coverage of the preclinical evidence.

Only original articles published in peer-reviewed journals and reporting quantifiable experimental data relevant to the study objectives were considered.

Studies were included based on strict cumulative criteria to ensure relevance and comparability with the main objective focused on SSTI. Eligible studies used infected wound or cutaneous lesion models with experimentally induced or confirmed bacterial or fungal infection; non-infected wounds used solely for healing evaluation were excluded to reduce heterogeneity. Only mammalian *in vivo* studies were included, primarily in mice and rats and, where relevant, rabbits or pigs. Established experimental models were required (e.g., contaminated excisional wounds, subcutaneous abscesses, biofilm-associated wound models). Infections were induced using well-documented pathogens, predominantly *Staphylococcus aureus* (including MRSA) and *Pseudomonas aeruginosa*, as well as other clinically important bacteria or fungi. Interventions consisted of topical administration of an essential oil, a bioactive constituent, or an advanced EO-based formulation, with appropriate control groups (vehicle or standard treatments such as mupirocin or vancomycin).

For the purpose of this review, the term “essential oils” also includes major bioactive constituents when these were evaluated as isolated compounds within *in vivo* models. Eligible studies reported quantifiable outcomes including microbial burden reduction, wound closure/contracture rates, histopathology and/or inflammatory and oxidative stress markers.

Studies limited to *in vitro* or *ex vivo* investigations, non-mammalian models, reviews/meta-analyses/book chapters, or reports lacking original experimental data were excluded. Studies that did not clearly describe infection induction, EO dose/concentration, or the treatment protocol were also excluded.

Study selection was performed by two independent reviewers through screening of titles/abstracts and then full texts. The screening process was conducted independently by the two reviewers using a standardized approach based on predefined eligibility criteria. No automation tools were used during the screening process. Discrepancies were resolved by discussions until consensus. Selection followed PRISMA recommendations to ensure transparency.

Essential information extracted from eligible studies included the experimental model, wound and infection type, EO intervention, comparator,

and main reported outcomes (bacterial load reduction, wound healing progression, histological and inflammatory parameters). Data were synthesized narratively and comparatively. The search syntax was adapted to each database to account for differences in indexing and search functionalities. Detailed search strategies for each database are available upon request to ensure full reproducibility.

Risk of bias assessment

The methodological quality of the included studies was evaluated using SYRCLE's RoB tool for animal studies by two independent reviewers, with any disagreements resolved by consensus. Each study was systematically assessed across ten key domains to identify potential biases: (1) sequence generation (selection bias); (2) baseline characteristics; (3) allocation concealment; (4) random housing; (5) blinding of caregivers and investigators (performance bias); (6) random outcome selection; (7) blinding of outcome assessors (detection bias); (8) incomplete outcome data (attrition bias); (9) selective outcome reporting (reporting bias); and (10) other sources of bias. For each domain, the risk was strictly categorized as low, high, or unclear to provide a robust overview of the preclinical evidence's strength and the results were centralized to provide an overview of the robustness of the preclinical evidence.

The systematic search initially identified 55 records from electronic databases (PubMed, Web of Science, Scopus, Science Direct and Google Scholar) and manual searching. After the removal of 6 duplicates, 49 records were screened based on title and abstract. Following this initial screening, 27 records were excluded for being *in vitro*/*ex vivo* studies, using non-mammalian models, or lacking relevant original data. The full texts of the remaining 22 reports were assessed for eligibility. At this stage, 10 reports were excluded due to the absence of essential oil/active constituent interventions, lack of quantifiable outcomes, or insufficient data for comparison. Ultimately, 12 studies met all inclusion criteria and were included in the final synthesis (Figure 1).

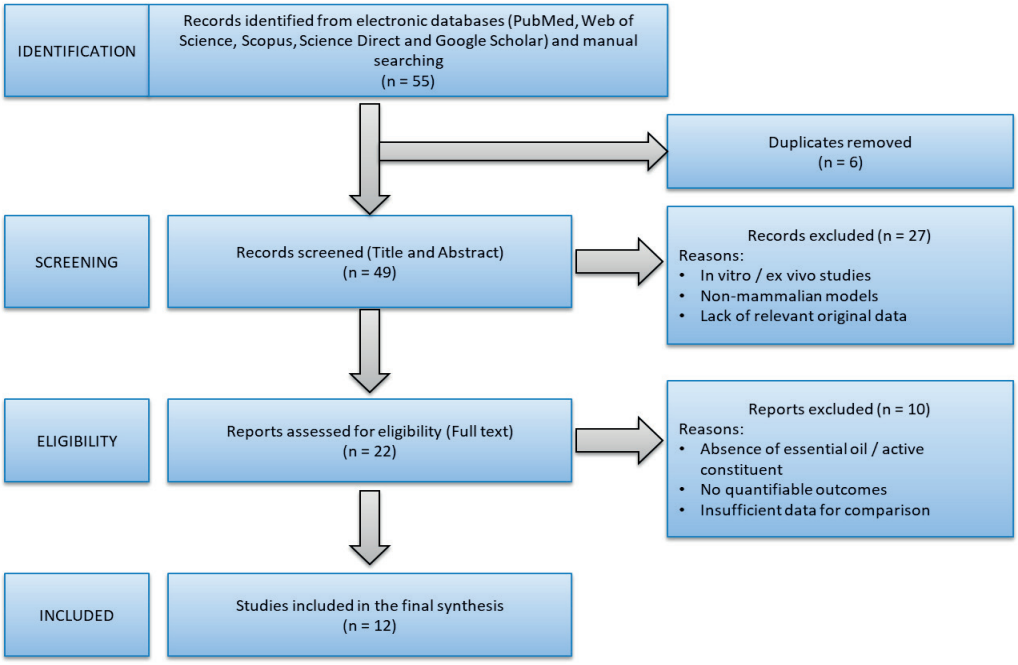

Figure 1. PRISMA flow diagram of study selection (original)

RESULTS AND DISCUSSIONS

The literature search and selection process, summarized in the PRISMA flow diagram (Figure 1), resulted in the inclusion of 12 original studies published between 2014 and 2025. These studies demonstrate a high degree of methodological diversity but converge on the significant therapeutic potential of essential oils in managing infected wounds

The analysis of the literature published over the last decade highlights a marked increase in scientific interest in using essential oils in regenerative medicine, experimental dermatology, and infected wound management. The included studies show substantial methodological diversity yet converge on consistent conclusions regarding the therapeutic potential of essential oils. The results are structured according to experimental model type, wound induction procedures, molecular mechanisms involved, antimicrobial efficacy, impact on tissue healing, pharmaceutical formulation innovations, and safety profile (Table 1).

Table 1. *In vivo* studies evaluating essential oils in cutaneous infections (selected from the bibliography).

Study	Animal model	Wound type	Infectious agent	Essential oil/ active compound	Formulation	Dose/ concentration	Main outcomes
Modarresi et al., 2019	Mouse	Infected excisional wound	*Staphylococcus aureus*	*Mentha piperita*	Topical essential oil	10 µL/day	Reduced bacterial burden; enhanced epithelialization and collagen deposition
Manzuoerha et al., 2019	Rat	MRSA-infected excisional wound	MRSA	*Anethum graveolens*	Topical ointment	2% (w/w)	Significant MRSA reduction; increased fibroblast proliferation and collagen synthesis
Farahpour et al., 2020	Rat	Infected excisional wound	*Staphylococcus aureus*	*Zataria multiflora*	Topical essential oil	1%	Anti-inflammatory effect; increased angiogenesis and collagen synthesis
Ghodrati & Farahpour, 2018	Rat	Infected wound	*Staphylococcus aureus*	*Mentha piperita*	Nanostructured lipid carriers (NLC)	0.50%	Faster wound closure and reduced bacterial load
Khezri et al., 2019	Rat	Infected wound	*Staphylococcus aureus*	*Rosmarinus officinalis*	EO encapsulated in NLC	0.50%	Increased collagen deposition; reduced inflammatory infiltrate
Nejati et al., 2015	Rat	Fungal-infected wound	*Candida albicans*	*Rosmarinus officinalis*	Topical essential oil	1%	Antifungal activity and accelerated wound healing
Ahmadi et al., 2022	Rat	Burn wound	*Pseudomonas aeruginosa*	*Zataria multiflora*	Topical essential oil	1%	Reduced burn area and bacterial load
Vila Nova et al., 2024	Rat	Infected wound	*Staphylococcus aureus*	*Melaleuca alternifolia*	Hydrocolloid gel with EO	1%	Antimicrobial and anti-inflammatory effects; faster wound closure
Teymouri et al., 2024	Rat	Infected burn wound	*Staphylococcus aureus*	*Lavandula officinalis*	Nanoemulsion gel	0.50%	Improved healing rate; reduced bacterial load; improved histology
El-Sakhawy et al., 2023	Rat	Fungal-infected wound	*Candida* spp.	*Eucalyptus globulus*	Topical essential oil	1%	Anticandidal activity; improved re-epithelialization
Alanazi et al., 2022	Rat	MRSA-infected wound	MRSA	*Syzygium aromaticum* (clove)	Topical EO ± imipenem	1%	Synergistic antibacterial activity and improved healing
Mohsen et al., 2023	Rat	Infected wound	*Staphylococcus aureus*	Thymol	Cationic nanoparticle-based hydrogel	0.30%	Reduced bacterial load and accelerated wound contraction

Risk of Bias (RoB) Analysis

The systematic application of the SYRCLE tool revealed that while 100% of the 12 studies reported low risk for selective outcome reporting, significant gaps remain in other areas. Specifically, 83% of the studies (n=10) provided

insufficient details regarding allocation concealment, leading to an unclear risk classification. Furthermore, blinding of outcome assessors was explicitly mentioned in only 25% of the reports (n=3). Most studies (approx. 70%) failed to describe random housing conditions adequately. Despite these reporting gaps, the baseline characteristics (weight, age, and strain) were consistently well-defined across 92% of the analyzed literature, providing a relatively robust foundation for the observed therapeutic efficacy of essential oils. This lack of transparency in animal experimental design underscores the need for rigorous adoption of the ARRIVE guidelines in future research

Animal models and wound induction procedures

Animal models play a crucial and indispensable role in biomedical research, especially in studying infected wounds, cutaneous lesions, and their healing mechanisms, because they allow controlled simulation of pathological conditions and investigation of therapeutic interventions in complex biological contexts. They facilitate understanding of host-pathogen interactions and of inflammatory, regenerative, and immunological processes involved in wound healing, providing essential information for the development and validation of therapeutic strategies (Lindblad, 2008). The use of different animal species is justified by distinct anatomical and physiological features that influence tissue repair mechanisms and the translational relevance of findings (Abdullahi et al., 2014).

Murine models (mouse and rat)

Most *in vivo* studies analyzed used rodents as primary biological models, representing more than 80% of investigations.

Murine models are widely used owing to broad availability of specific antibodies and advanced molecular analysis techniques. Major advantages include the possibility of robust statistical analyses and the use of genetically modified strains, such as diabetic mice, which reproduce delayed healing conditions observed clinically.

Recent literature indicates that most studies evaluating EO efficacy in cutaneous infections use mice (often BALB/c) or Wistar/Sprague–Dawley rats due to standardized skin wound induction protocols (Manzuoerha et al., 2019; Khezri et al., 2019; Teymouri et al., 2024).

A major limitation is the presence of the *panniculus carnosus*, a subcutaneous muscle layer that promotes rapid closure by contraction, a mechanism that differs from predominantly re-epithelialization-driven healing in humans. To reduce this discrepancy, many studies used silicone splints that limit contraction and force healing by granulation and re-epithelialization, increasing translational relevance (Trøstrup et al., 2013). Although murine skin shares the fundamental layers of human skin, there are profound histological, mechanical, and metabolic differences. Mouse skin is characterized by thin epidermis and dermis, a flat dermo-epidermal interface, high hair density, and accelerated regenerative cycles (Dahiya, 2009; Wong et al., 2011). Similarly, rats are classified as “loose-skin” animals due to increased elasticity and reduced adhesion to underlying structures (Abdullahi et al., 2014; Dahiya, 2009).

The porcine model

Porcine models are considered the gold standard in experimental dermatology due to remarkable similarities between porcine and human skin, including comparable epidermal and dermal thickness, collagen/elastin ratio, hair follicle density, and healing mechanisms. They are particularly valuable for evaluating skin penetration, local toxicity, and performance of dressings and topical formulations (Herman & Herman, 2015; Kopecki et al., 2017). Limitations include high costs, logistical challenges, and strict ethical requirements. Biochemically, pigs share with human collagen composition, structural proteins (e.g., keratin, fibronectin, vimentin), and immune cell profiles. A key anatomical difference is the *panniculus carnosus*, which is adherent to deep fascia and separated from adipose tissue; this promotes healing predominantly via granulation tissue formation and re-epithelialization – similar to humans – rather than contraction as in rodents (Ancuța et al., 2026; Roy et al., 2016).

Methodological considerations

Inducing cutaneous lesions in animal models is crucial in dermatological research, enabling

investigation of wound healing processes and therapeutic interventions. Various techniques have been used to create experimental skin injuries, each with distinct characteristics and implications. Skin injury models can be classified by wound type, including incisional and excisional wounds, abrasions, burns, lacerations, and open fractures (Maslova et al., 2020). Once wounds are created, infection can be induced by introducing microbial agents. Wound contamination with microorganisms is almost universal, and not all microorganisms lead to adverse effects (Kupper & Fuhlbrigge, 2004). Therefore, it is essential to evaluate the specific pathogens involved to draw clinically relevant conclusions.

Uniformity in wound creation is essential to reduce experimental variability. Controlled wound production ensures consistent size and depth, which is critical for reproducibility. Moreover, understanding immune responses to surgical interventions and factors influencing surgical site infections can inform better therapeutic strategies (Dai et al., 2011; Liu et al., 2024).

Based on the reviewed literature, three major procedures were identified:

Excisional wound model

The excisional wound model is one of the most widely used *in vivo* experimental models for evaluating cutaneous healing and is considered a reference standard in studies investigating EO capacity to accelerate re-epithelialization, stimulate tissue regeneration, and prevent or control infections in open wounds (Manzuoerha et al., 2019; Khezri et al., 2019; Teymouri et al., 2024). It allows detailed analysis of key healing stages, including inflammation, granulation tissue formation, angiogenesis, and extracellular matrix remodeling.

Methodologically, a standardized circular portion of skin (typically 4-8 mm in diameter) is surgically removed on the dorsal region. The excision is full-thickness and performed with a biopsy punch to ensure reproducibility. The wound is left open or covered with specific dressings depending on study goals, and topical treatments are applied directly for a defined period.

Using this model, Teymouri et al. (2024) demonstrated that topical Lavandula angustifolia (lavender) EO significantly accelerates wound healing by stimulating granulation tissue formation and inducing expression of transforming growth factor beta (TGF-β), a key mediator of tissue regeneration and collagen synthesis. In a similar *S. aureus*-infected excisional wound model, Modarresi et al. (2019) showed that Mentha piperita EO significantly reduces local bacterial burden and inflammatory response, facilitating transition to the proliferative phase and promoting collagen deposition.

Infected burn model

The infected burn model is relevant for studying severe cutaneous infections, as thermal injury creates a favorable environment for colonization and proliferation of opportunistic pathogens, especially *Pseudomonas aeruginosa*, a common burn pathogen known for high antimicrobial resistance. Such lesions are useful for testing oils with strong antibacterial activity.

In this model, thermal injury is induced by applying a preheated metal device (approximately 100°C) to the dorsal region for a controlled duration (4-10 seconds), followed by local inoculation with a bacterial suspension to achieve reproducible infection (Teymouri et al., 2024; Lu et al., 2022).

For example, Ahmadi et al. (2022) demonstrated in a *P. aeruginosa*-infected burn model that topical *Zataria multiflora* EO significantly reduced bacterial load and burn area, accelerated healing, and suppressed systemic inflammation, protecting animals from sepsis. These findings highlight the potential of essential oils as alternative therapeutics in burn wound infection management.

Subcutaneous abscess and biofilm model

This model is used to test the ability of advanced formulations to penetrate biological barriers (the abscess capsule or biofilm matrix) (Ding et al., 2018). The procedure involves subcutaneous injection of a bacterial suspension, most commonly *Staphylococcus aureus* or MRSA, leading to formation of a well-delimited abscess characterized by intense local inflammation and purulent accumulation (Ding et al., 2018). Alternatively, for biofilm studies, a superficial skin lesion can be maintained under an occlusive dressing to promote development of a mature biofilm before treatment initiation (Youn, 2020).

These models are particularly relevant for assessing nanostructured delivery systems. Recent studies indicate that nanoformulations – unlike unformulated Eos – can overcome the physical barriers of abscesses and bacterial biofilms (Albano et al., 2025; Lin et al., 2021). Nanoemulsions and other nanosystems loaded with phytochemicals or EOs show superior biofilm penetration and disruption, including against resistant pathogens (Albano et al., 2025; Lin et al., 2021). For example, cinnamon EO nanoemulsions exhibit significantly improved antibacterial and antibiofilm activity compared with free oil and demonstrate increased efficacy against MRSA biofilms (Kwon et al., 2021). Similarly, polymeric nanofibers or nanoparticles loaded with EOs in chronic wound models reduced bacterial burden and supported complete epidermal regeneration, underscoring their potential as alternative strategies for biofilm-associated chronic cutaneous infections (Lin et al., 2021).

Mechanisms of action of essential oils in cutaneous infections

The main mechanisms of EO action are schematized in Figure 2 and include direct antimicrobial activity, antibiofilm effects, and modulation of the wound microenvironment.

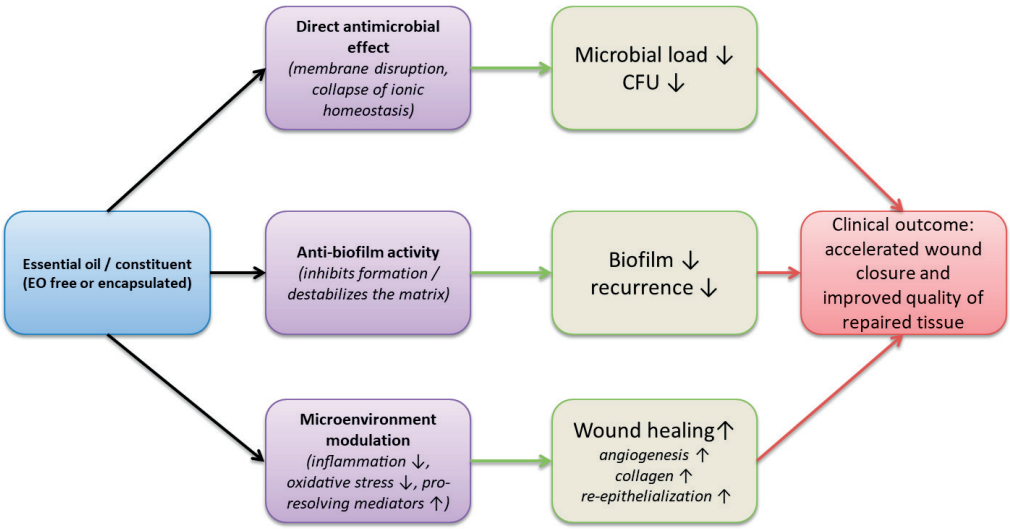

Figure 2: Mechanisms of action of essential oils in skin infections (original)

Direct antimicrobial activity: from membrane to biofilm

Although most mechanistic perspectives originate from *in vitro* studies, the *in vivo* evidence analysed supports these mechanisms indirectly through consistent reductions in bacterial load, improved wound healing, and modulation of inflammatory responses (Yin et al., 2024; Diniz et al., 2022; Manzuoerha et al., 2019).

In vivo evidence indicates that EO antimicrobial activity is central in controlling cutaneous infections, especially with antibiotic-resistant Gram-positive and Gram-negative pathogens. Unlike conventional antimicrobials targeting a single molecular site, EOs act through multifactorial mechanisms, reducing the likelihood of rapid resistance selection (Kennewell et al., 2019; Roshni & Rekha, 2024).

In vivo studies show that topical EOs or their constituents significantly reduce microbial burden quantified by CFU in wound tissue. Such effects have been reported in models infected with *S. aureus, MRSA, P. aeruginosa* (including MDR strains), carbapenem-resistant *K. pneumoniae*, and *Candida albicans* (Manzuoerha et al., 2019; Farahpour et al., 2020; Lu et al., 2022; Alturaiki et al., 2023; Nejati et al., 2015).

Mechanistically, major EO constituents (e.g., thymol, carvacrol, eugenol, linalool, terpinen-4-

ol) are lipophilic and interact directly with microbial cell membranes, increasing permeability, collapsing transmembrane potential, causing leakage of ions and essential metabolites, and ultimately cell death. *In vivo*, these mechanisms are reflected by rapid CFU reduction and limitation of infection spread into adjacent tissues.

A particularly relevant aspect is antibiofilm activity. Biofilm is a major barrier in chronic infected wounds, and several included studies indicate that EO-based formulations can reduce biofilm density and prevent re-formation (Yin et al., 2024; Kwon et al., 2021; Lin et al., 2021; Roshni & Rekha, 2024). For instance, eucalyptus oil nanoemulsions incorporated into hydrogels demonstrated antibiofilm activity associated with accelerated wound healing, suggesting that biofilm disruption directly contributes to restoration of physiological healing (Cai et al., 2023; Orchard, A., & van Vuuren, S. 2017).

Immunomodulation and control of local inflammation

Excessive and persistent inflammation is a major determinant of chronic infected wounds. In this respect, the ability of EOs to modulate local inflammation complements their direct antimicrobial activity.

Multiple *in vivo* studies reported reduced expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 in EO-treated tissues, accompanied by increased anti-inflammatory and pro-resolution mediators including IL-10 and TGF-β, suggesting re-calibration of the wound inflammatory microenvironment (Modarresi et al., 2019; Farahpour et al., 2020; Cai et al., 2023).

Biologically, these changes indicate accelerated transition from the inflammatory to the proliferative phase, with a reduced and more organized inflammatory infiltrate and limited extracellular matrix destruction. This immunomodulation is particularly relevant in MRSA and *P. aeruginosa* infections where inflammation contributes significantly to delayed healing and tissue damage (Ahmadi et al., 2022; Farahpour et al., 2020).

Antioxidant activity and stimulation of tissue regeneration

Oxidative stress is recognized as a key factor in infected wound pathogenesis, being associated with damage to membranes, proteins, and DNA. Several studies show that topical EO treatment improves local antioxidant status, reflected by increased total antioxidant capacity (TAC) and decreased lipid oxidation markers such as malondialdehyde (MDA) (Farahpour et al., 2020; Seyed Ahmadi et al., 2019).

These antioxidant effects correlate with stimulation of growth factors involved in angiogenesis and regeneration, including VEGF, FGF-2, IGF-1, and EGF. Histologically, this translates into enhanced angiogenesis, more organized collagen deposition, and accelerated re-epithelialization, indicating that EOs not only control infection but actively support cutaneous regeneration (Table 2).

Table 2. Relationship between essential oil/constituent, dominant mechanism and *in vivo* therapeutic effect

EO/Constituent	Dominant mechanisms	*In vivo* effects	Supporting studies
Tea tree (Melaleuca)	Membrane disruption; immunomodulation	Antimicrobial + anti-inflammatory	Vila Nova et al., 2024
Clove (eugenol)	Antimicrobial; synergy with antibiotics	MRSA control; adjuvant potential	Alanazi et al., 2022
Cinnamon (cinnamaldehyde)	Anti-biofilm; membrane disruption; ↓ oxidative stress	↑TAC/↓MDA; biofilm inhibition	Seyed Ahmadi et al., 2019; Yin et al., 2024
Zataria multiflora	Antimicrobial + anti-inflammatory + pro-angiogenic	↓ microbial load; ↑ angiogenesis/collagen	Farahpour et al., 2020; Ahmadi et al., 2022
Thymol	Membrane effects; anti-inflammatory; delivery-dependent	Higher efficacy in advanced systems	Mohsen et al., 2023; Zohdy et al., 2025

To the best of our knowledge, this is one of the first reviews focusing specifically on *in vivo* mammalian models of infected wounds treated with essential oil-based formulations within the 2014-2025 timeframe. The evidence synthesized in this review consistently supports that essential oils and their bioactive constituents exert a dual therapeutic effect in animal models

of cutaneous infections: effective antimicrobial control, including against resistant pathogens and biofilms, and optimization of healing through reduction of persistent inflammation, limitation of oxidative stress, and stimulation of tissue regeneration. This combination is particularly relevant in infected wounds, where pathogen elimination must be accompanied by restoration of a pro-healing microenvironment to enable transition to proliferative and remodeling phases (Tong et al., 2015; Kennewell et al., 2019; Roshni & Rekha, 2024).

The included studies indicate that EOs act through pleiotropic mechanisms, including direct antimicrobial activity, antibiofilm effects, immunomodulation, and antioxidant properties, contributing synergistically to bacterial burden reduction and accelerated healing (Modarresi et al., 2019; Kennewell et al., 2019; Farahpour et al., 2020; Cai et al., 2023).

Unlike conventional antibiotics, which usually target a single molecular site, EOs and major constituents exert multi-target antimicrobial activity, predominantly via membrane perturbation, altered ionic permeability, and inhibition of virulence factors. This complex action may reduce the likelihood of rapid resistance selection (Chouhan et al., 2017; Bakkali et al., 2008; Kennewell et al., 2019).

Biofilm formation is a critical factor in chronic infected wounds and a major cause of therapeutic failure (Trøstrup et al., 2013). Advanced EO formulations (nanoemulsions, loaded hydrogels, NLC) improve penetration of active compounds into biofilm matrices, reduce bacterial density, and support accelerated re-epithelialization (Albano et al., 2025; Lin et al., 2021; Cai et al., 2023).

Excessive inflammation and oxidative stress are central drivers of delayed healing. Several included studies report that topical EOs reduce pro-inflammatory markers (TNF-α, IL-1β, IL-6) and increase pro-resolution mediators (IL-10, TGF-β), facilitating the transition from inflammation to proliferation (Modarresi et al., 2019; Farahpour et al., 2020; Cai et al., 2023).

In parallel, documented antioxidant effects (↑TAC and ↓MDA) correlate with increased keratin synthesis, organized collagen deposition, and enhanced angiogenesis, confirmed histologically by H&E and Masson staining (Farahpour et al., 2020; Seyed Ahmadi et al., 2019).

Most included studies used rodent models due to reproducibility and access to advanced molecular tools (Lindblad, 2008; Kopecki et al., 2017; Abdullahi et al., 2014; Dahiya, 2009). However, rodent healing is dominated by contraction, potentially overestimating closure speed compared with humans. Silicone splints mitigate this limitation and improve translational relevance (Wong et al., 2011).

Porcine models remain the reference standard for validating skin penetration, tolerability, and efficacy of topical formulations and are essential for translation to clinical applications (Shiff et al., 2023; Herman & Herman, 2015; Ancuța et al., 2026; Roy et al., 2016).

Limitations of unformulated EOs (volatility, instability, irritation) have been partially addressed through encapsulation into nanoemulsions, NLC, hydrogels, and polymeric nanoparticles, providing improved stability, controlled release, and better penetration into infected tissue (Herman & Herman, 2015; Cai et al., 2023; Teymouri et al., 2024; Ghodrati & Farahpour, 2018; Khezri et al., 2019).

Combined strategies pairing EOs with antibiotics or physical modalities (blue light, photodynamic therapy) have shown synergistic effects against MDR pathogens, but require rigorous safety assessment (Lu et al., 2022; Alanazi et al., 2022; Abo-Neima et al., 2023).

Interpretation is limited by heterogeneity in models, wound induction protocols, EO chemotypes, doses, vehicles, and treatment durations. Incomplete reporting of randomization, blinding, and toxicity evaluations also limits comparability and prevents robust quantitative meta-analysis (Roshni & Rekha, 2024; Page et al., 2021). Predominance of unclear risk-of-bias domains should be considered when interpreting the strength of preclinical evidence.

Future research should focus on standardizing experimental models, aligning reporting with PRISMA 2020, and directly comparing EOs to standard antimicrobials, especially in clinically relevant porcine models. Systematic exploration of EO–antibiotic combinations and long-term safety evaluation will be essential for translation toward clinical practice (Wang et al., 2022; Zohdy et al., 2025; Page et al., 2021).

CONCLUSIONS

The *in vivo* evidence synthesized in this review supports that essential oils and their major bioactive constituents are promising therapeutic candidates for managing bacterial and fungal cutaneous infections in animal models. Between 2014 and 2025, research evolved from antimicrobial screening to complex mechanistic investigations, demonstrating pleiotropic EO mechanisms combining antimicrobial and antibiofilm activity with anti-inflammatory, antioxidant, and pro-regenerative effects that support efficient transition toward proliferative wound healing phases.

Through this functional combination, EOs can overcome limitations of conventional approaches that focus exclusively on infection control by concurrently restoring the cutaneous microenvironment required for effective healing.

Murine and porcine models provide complementary insights for developing EO-based topical therapies. Rodents support rapid, reproducible, mechanistic evaluations but contraction-driven closure limits direct extrapolation. Porcine models, due to structural and functional similarity to human skin, remain critical for validating advanced formulations, local safety, and clinical potential.

Beyond relevance for human wound care, these findings have important implications for veterinary medicine, where infected wounds are frequent and challenging. EO-based nanoformulations may serve as alternative or adjunct strategies for post-surgical, traumatic, or chronic wounds in companion and farm animals. However, species-specific differences in skin structure, immune response, and healing dynamics require dedicated studies to optimize formulation composition, dosing schemes, and safety profiles across veterinary contexts.

Interpretation of this review is limited by incomplete reporting of key methodological aspects (randomization and blinding) in many included studies, leading to predominance of unclear risk-of-bias domains that should be considered when evaluating robustness and strength of available preclinical evidence.

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HUMAN-DOG INTERACTION IN EMOTION REGULATION: ANIMAL-ASSISTED INTERVENTIONS VS. CLASSICAL PSYCHOTHERAPEUTIC INTERVENTIONS

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Abstract

Animal-assisted therapies, particularly those based on human-dog interaction, have attracted increasing scientific interest due to their potential to influence emotional processes through specific psychophysiological mechanisms. Emotion regulation is a fundamental process for mental health and individual adaptation, and a major transdiagnostic factor in anxiety and affective disorders. The present experimental study compared the effectiveness of dog-assisted therapy (AAT) with cognitive-behavioural therapy (CBT) in a sample of 60 university students (aged 19-25) with moderate anxiety. Participants were randomly assigned to six weekly sessions of either AAT or CBT. Assessments included psychological measures of emotion regulation, anxiety, and affect, as well as psychophysiological markers: salivary oxytocin and cortisol, heart rate, and heart rate variability. Results showed significant increases in oxytocin and heart rate variability, and greater reductions in heart rate and cortisol in the AAT group compared to the CBT group. Oxytocin was positively correlated with heart rate variability and negatively with difficulties in emotion regulation. Limitations include the short-term design, small sample size, lack of blinding, and possible uncontrolled expectancy effects.

Key words*: human-dog interaction, oxytocin, emotional regulation, animal-assisted therapies, heart rate variability.*

INTRODUCTION

Emotion regulation is an essential psychological process for an individual's adaptation to environmental demands, being closely linked to mental health and optimal social functioning, and constituting a central transdiagnostic mechanism in anxiety disorders, depression, and vulnerability to stress (Gross, 2015).

Many contemporary models of psychopathology conceptualize emotion regulation difficulties as a key factor in the development and maintenance of anxiety and affective disorders (Thayer & Lane, 2009), although the strength and direction of these relationships may vary across contexts and populations. Anxiety is conceptualized as the result of sustained hyperactivation of stress response systems, associated with difficulties in processing and accepting emotions (Spielberger et al., 1983).

The relationship between anxiety and emotional dysregulation is bidirectional: high levels of anxiety increase the use of maladaptive strategies, while deficient regulation maintains symptomatology (Gross, 2015).

Interaction with animals is associated with stress reduction and the activation of social affiliation systems (Friedmann & Son, 2009).

In this context, human-dog interaction has attracted considerable scientific interest, being regarded as a particular form of human-animal interaction with high psychotherapeutic potential (Rusu, 2012; Papuc et al., 2013). Contact with dogs leads to increased oxytocin levels and reduced cortisol levels (Odendaal & Meintjes, 2003; Uvnäs-Moberg et al., 2015). Beetz et al. (2012) suggest that the psychophysiological effects of human-animal interaction are mediated through oxytocinergic mechanisms, contributing to emotion regulation and anxiety reduction.

Dogs are frequently used in animal-assisted interventions due to their ability to respond to human emotional signals and to facilitate secure attachment relationships (Beetz et al., 2012). Difficulties in emotional regulation are associated with persistent negative affect, increased autonomic activation, and inefficient

use of cognitive strategies (Brosschot et al., 2006).

While dog-assisted interventions are typically evaluated in terms of human psychological outcomes, increasing attention has been directed toward the welfare and physiological responses of the animals involved, highlighting the importance of considering human-animal interaction as a bidirectional process rather than a unidirectional therapeutic tool. Factors such as the dog's temperament, training, and stress levels may influence both the effectiveness and ethical implementation of such interventions. Although previous research suggests beneficial effects of human-dog interaction on stress-related outcomes, there is a lack of controlled experimental studies directly comparing dog-assisted interventions with established psychotherapeutic approaches such as cognitive-behavioural therapy, particularly using combined psychological and psychophysiological measures (e.g., oxytocin, heart rate variability) within the same design. (Barker & Wolen, 2008; Rodriguez et al., 2020). Cognitive-behavioural therapy is a form of psychotherapy that aims to identify and modify dysfunctional thoughts and behaviors in order to improve emotional well-being and individual adaptation (Beck, 2011). Animal-assisted therapy is a planned therapeutic intervention in which animals are used as an integral part of the treatment process to support individuals' physical, emotional, or psychological recovery (Fine, 2010). The present study aimed to compare the effects of cognitive-behavioural therapy (CBT) and dog-assisted therapy on emotional, cognitive, and physiological outcomes in students with moderate anxiety. Based on this objective, it was hypothesized that dog-assisted therapy would be associated with higher oxytocin levels, lower heart rate, and increased heart rate variability compared to CBT.

MATERIALS AND METHODS

The study was approved by the Ethics Committee of USAMV Cluj-Napoca (Decision no. 552, March 10, 2026) in accordance with national and European legislation on the ethics of scientific research.

All participants provided written informed consent prior to participation. Participants were recruited from the university student population.

Inclusion criteria included age between 19 and 25 years, moderate anxiety (scores between 40-59 as measured by STAI), university student status, and the availability to participate in therapy sessions.

Exclusion criteria also comprised medical or psychological conditions that could interfere with participation or bias the results, such as neurological disorders (e.g., epilepsy, traumatic brain injury), severe or unstable medical conditions (e.g., uncontrolled diabetes, significant cardiovascular disease), substance use disorders, severe depression, psychotic symptoms, or cognitive impairments affecting comprehension and engagement in therapy. Participants were randomly assigned to one of two groups (dog-assisted therapy vs. cognitive-behavioural therapy). During the study period, participants did not receive any other psychological treatments or psychotropic medications**.**

The study included 60 students (N = 60) of both genders, aged between 19 and 25 years (M = 21.8, SD = 1.9), diagnosed with moderate levels of anxiety. The participants were randomly assigned to two groups of 30 students each. One group received dog-assisted therapy (AAT) (n = 30), while the other group received cognitive-behavioural therapy (CBT) (n = 30).

The research instruments were selected in accordance with the study objectives. Emotion regulation was assessed using the Difficulties in Emotion Regulation Scale (DERS) ($\alpha = .91$), an instrument with high validity and reliability in both clinical and non-clinical populations (Gratz & Roemer, 2004).

The DERS is a 36-item self-report measure designed to assess multiple dimensions of emotion regulation difficulties. Respondents are asked to indicate the extent to which each statement applies to them "in general," using a 5-point Likert scale (1 = almost never; 5 = almost always). In this study, the DERS was administered in a self-report format under standardized conditions, ensuring anonymity and informed consent. The average completion time was approximately 7-10 minutes.

Trait anxiety was assessed using the trait subscale (Form Y-2) of the State-Trait Anxiety Inventory (STAI) (α = .88) (Spielberger et al., 1983). The STAI-T is a 20-item self-report instrument designed to measure relatively stable individual differences in proneness to anxiety. Participants indicate how they generally feel in relation to environmental stress using a 4-point Likert scale (1 = almost never; 4 = almost always). After reverse-coded items are recorded, a total score is calculated by counting score responses, with higher scores indicating higher levels of trait anxiety.

The possible score range for each answer is set between 20 and 80 points. Positive and negative affect were assessed using the Positive and Negative Affect Schedule (PANAS) (α = .86) (Watson et al., 1988). The PANAS is a 20-item self-report instrument measuring two distinct affective dimensions: positive affect and negative affect, each subscale containing 10 items.

Participants rate the extent to which they have experienced each affective state using a 5-point Likert scale (1 = very slightly or not at all; 5 = extremely), with responses referring to a specified time frame (e.g., "in general," "during the past week," or "at this moment").

Subscale scores are obtained by the sum of relevant items, with higher values indicating higher levels of positive or negative affect, respectively.

The two dimensions are conceptualized as relatively independent.

Statistical analyses were performed using SPSS v27 (IBM Corp.). Data were initially screened for missing values, outliers, and normality using the Shapiro-Wilk test, as well as visual inspection of histograms and skewness and kurtosis indices.

Between-group differences over time were analysed using a repeated-measures ANOVA (2 × 2: Group × Time) to examine main and interaction effects. To assess pre-post changes within each group, pairwise comparisons were conducted using paired-sample t-tests.

Effect sizes were calculated using Cohen's d for pairwise comparisons and partial eta squared (η^2) for ANOVA. The level of statistical significance was set at $p < .05$. To control for Type I error in the case of multiple comparisons, Bonferroni corrections were applied.

To explore HRV as the potential mechanisms underlying the intervention effects, a mediation analysis was conducted using linear regression and a bootstrap approach (5,000 samples) to estimate indirect effects and their 95% confidence intervals. Group was the independent variable while the dependent variable was the change in DERS scores. Heart Rate and Heart Rate Variability (HRV) Heart rate (HR) and heart rate variability (HRV) were recorded via electrocardiography (ECG) and analysed according to international standards (Task Force of the European Society of Cardiology, 1996; Shaffer & Ginsberg, 2017) ECG signals were acquired using a 3-lead system (limb leads) with a sampling rate of 1,000 Hz and electrodes placed on standard limb positions. Signal preprocessing included band-pass filtering (0.5-40 Hz), artifact correction, and visual inspection RR intervals were extracted for HRV computation.

Time-domain HRV parameters measured were SDNN (standard deviation of NN intervals), RMSSD (root mean square of successive differences), and pNN50 (percentage of successive NN intervals differing by >50 ms) Frequency-domain parameters included LF (low-frequency power, 0.04-0.15 Hz), HF (high-frequency power, 0.15-0.40 Hz), and the LF/HF ratio HRV analysis was performed using Kubios HRV Premium v3.5 (Kubios Oy, Finland) with standard settings for artifact correction and detrending.

Salivary oxytocin was measured using a commercial ELISA kit (Cayman Chemical, Ann Arbor, MI, USA; catalog no. 500440) with an analytical sensitivity of 15 pg/mL, intra-assay variation of 6,5%, and inter-assay variation of 7,8%, following the manufacturer's instructions and current methodological recommendations (MacLean et al., 2019).

Salivary cortisol was measured using a commercial ELISA kit (Salimetrics, State College, PA, USA; catalog no. 1-3002) with an analytical sensitivity of 0.003 µg/dL (≈0.03ng/mL), intra-assay variation of 3.5%, and inter-assay variation of 5.1% (Hennessy et al., 2009).

All saliva samples were collected at 08:00 a.m. to control for diurnal variation using passive drool into sterile tubes and stored at –80°C until analysis.

Pre-intervention assessments were conducted one day before therapy, and post-intervention assessments one day after therapy completion.
Therapy lasted 6 weeks, consisting of one 60-minute session per week Participants were randomly assigned to dog-assisted therapy (AAT) or cognitive-behavioural therapy (CBT)

RESULTS AND DISCUSSIONS

Both interventions led to significant improvements across psychological and physiological outcomes.
DERS scores at pre-test and post-test for both CBT and AAT are presented in Table 1 and Figure 1.

Table. 1. Effects of CBT and AAT on Emotion Regulation (DERS)

Measure	Group	Pretest M (SD)	Posttest M (SD)
Biomarker (pg/mL)	CBT	12.4 (3.5)	13.8 (3.2)
Biomarker (pg/mL)	AAT	12.1 (3.7)	18.9 (3.5)
Resting HR (bpm)	CBT	78.5 (6.2)	74.2 (5.7)
Resting HR (bpm)	AAT	79.1 (6.5)	70.3 (5.1)

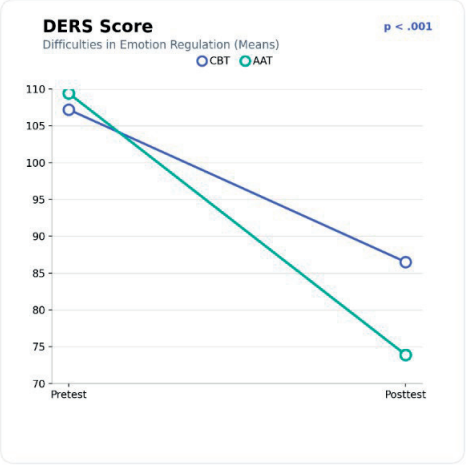

Figure 1. Group changes in emotional regulation

Both CBT and AAT were associated with significant reductions in emotion regulation difficulties, with large effect sizes (CBT: d = 1.63; AAT: d = 2.21). A repeated-measures ANOVA revealed a significant Group × Time interaction, F (1.58) = 10.87, p = .002, η^2 = .16, indicating that changes over time differed between groups, with a greater reduction observed in the AAT condition.
A significant Group × Time interaction was observed for anxiety scores, F (1.58) = 6.44, p = .014, indicating differential reductions across interventions, with larger decreases in the AAT group.
For negative affect, the Group × Time interaction was significant, F (1.58) = 8.01, p = .006, with greater reductions observed in the AAT group.
For positive affect, a significant interaction effect was also found, F (1.58) = 6.21, p = .015, with greater increases in positive affect in the AAT group compared to CBT.
Heart rate variability (HRV) and heart rate values are presented in Table 2 and Figure 2.

Table 2. Heart rate variability (HRV) and heart rate values

Group	Pretest M (SD)	Posttest M (SD)	t (df)	p	d
CBT	107.2 (14.2)	86.5 (12.4)	8.91 (29)	< .001	1.63
AAT	109.4 (15.0)	73.9 (10.8)	12.14 (29)	< .001	2.21

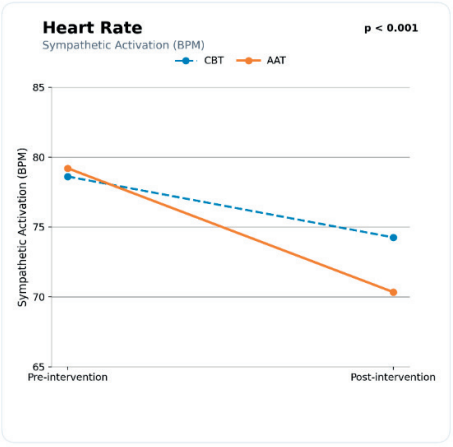

Figure 2. Heart Rate in CBT and AAT

Heart rate variability (HRV) increased in both groups, with a larger increase in the AAT group (24%) compared to CBT (10%), F (1.58) = 9.32, p = .003, η^2 = .14.
Resting heart rate decreased in both groups (CBT: t = 4.03, p < .001, d = 0.74; AAT: t = 8.76, p < .001, d = 1.61), with larger reductions in the AAT group.

Biomarker levels (pg/mL) increased in both groups, with a modest increase in CBT (t = 2.17, p = .038, d = 0.41) and a larger increase in AAT (t = 9.12, p < .001, d = 1.66).
Salivary oxytocin values are presented in Table 3 and Figure 3.

Table 3. Salivary oxytocin levels and heart rate pre- and post-intervention

Group	Pre-intervention (pg/mL)	Post-intervention (pg/mL)
CBT	12.4 (SD = 3.5)	13.8 (SD = 3.2)
AAT	12.1 (SD = 3.7)	18.9 (SD = 3.5)

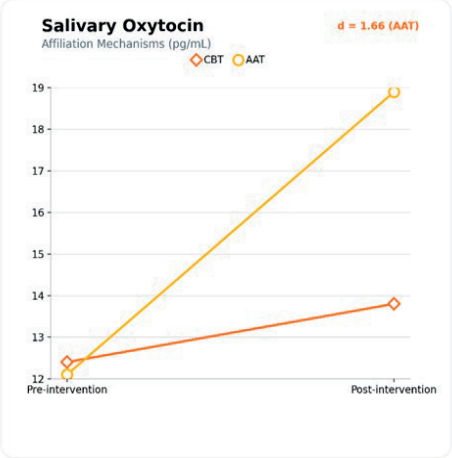

Figure 3. Salivary Oxytocin Levels in CBT and AAT

A mediation analysis (bootstrapped, 5,000 samples) examined whether HRV mediated the relationship between intervention type and changes in emotion regulation (DERS). The model is presented in Figure 5.
A significant indirect effect was observed, β = -. Salivary oxytocin increased significantly in both groups (t =2.17, p = .038, d = .41 for CBT, t = 9.12, p <.001, d = 1.66 for AAT), with a larger increase observed in the AAT group.
Salivary cortisol values are presented in Table 4 and Figure 4.
Both interventions were associated with reductions in salivary cortisol (CBT: t = 2.72, p = .008, d = .70, AAT: t =, p =, d =), with a greater decrease observed in the AAT group.
29, 95% CI [-.48, -.09], indicating partial mediation.

Significant correlations were observed between physiological markers and emotion regulation outcomes: oxytocin–DERS (r = -.54, p < .001), heart rate–DERS (r = .49, p < .001), and HRV–DERS (r = -.61, p < .001).

Table 4. Salivary cortisol changes pre- and post-intervention

Group	Pre-intervention	Post-intervention	% Change
CBT	18.5 (SD = 4.1) pg/mL	15.5 (SD = 3.8) pg/mL	-16%
AAT	18.7 (SD = 4.3) pg/mL	12.3 (SD = 3.5) pg/mL	-34%

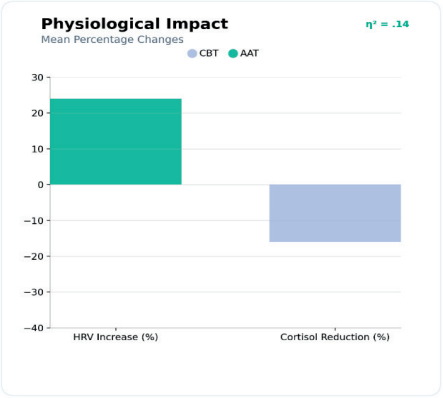

Figure 4. Salivary Cortisol Levels in CBT and AAT

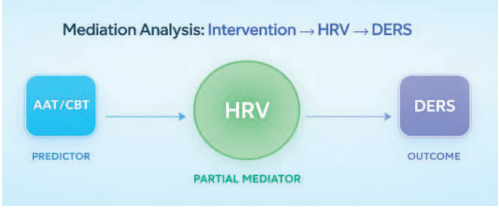

Figure 5. Mediation analysis between the two types of intervention

The present study examined the comparative effects of cognitive-behavioural therapy (CBT) and animal-assisted therapy (AAT) on emotional, affective, and physiological outcomes in students with moderate anxiety. Both interventions were associated with significant improvements across all domains, supporting the view that emotion regulation is a modifiable process through structured psychological interventions.

Across outcomes, AAT was associated with larger changes compared to CBT, including greater reductions in emotion regulation difficulties, anxiety, and negative affect, as well as greater increases in positive affect and heart rate variability. These findings are consistent with previous research suggesting that human-animal interaction may facilitate stress reduction and emotional adjustment (Beetz et al., 2012; Uvnäs-Moberg et al., 2015).

The observed increase in HRV in the AAT group is consistent with models linking vagal regulation to emotional flexibility and adaptive responding (Thayer et al., 2012; Porges, 2007). Higher HRV has been associated with improved regulatory capacity and reduced vulnerability to anxiety (McCraty & Shaffer, 2015), suggesting that autonomic regulation may represent one pathway through which AAT exerts its effects.

Similarly, the increase in salivary oxytocin and the reduction in cortisol levels observed in the AAT group align with evidence indicating that human-dog interaction may influence affiliative and stress-related neuroendocrine systems (Odendaal & Meintjes, 2003; Hennessy et al., 2009). These physiological changes are consistent with the hypothesis that AAT engages bottom-up regulatory processes related to stress attenuation and social bonding.

In contrast, CBT is typically conceptualized as operating through top-down mechanisms, including cognitive restructuring and reappraisal processes associated with prefrontal regulation of emotional responses (Gross, 2015; Thayer et al., 2012). The present findings suggest that these approaches may rely on partially distinct mechanisms, with CBT emphasizing cognitive control and AAT engaging autonomic and neuroendocrine pathways.

The mediation analysis provides preliminary support for the role of HRV as a potential mechanism linking intervention type to improvements in emotion regulation. However, given the study design, these findings should be interpreted cautiously and do not establish causal mediation.

From a theoretical perspective, the results support integrative models of emotion regulation that incorporate both cognitive and physiological processes. From a clinical perspective, they suggest that AAT may represent a useful complementary intervention, particularly in contexts where experiential or relational engagement may enhance treatment response.

Several limitations should be acknowledged. The sample consisted exclusively of students, limiting generalizability. The study employed a short-term pre-post design without follow-up, preventing conclusions about long-term effects. The absence of blinding introduces the possibility of expectancy and therapist effects, and the novelty of interacting with a dog may have influenced responses. In addition, only a limited set of physiological markers was assessed, and potential confounders such as prior experience with animals were not controlled.

Future research should include larger and more diverse samples, incorporate longitudinal designs, and employ more rigorous control of potential confounding variables. The inclusion of additional physiological and behavioural measures may further clarify the mechanisms underlying the observed effects. Given the number of outcomes assessed, future studies may also benefit from formal correction for multiple comparisons.

CONCLUSIONS

Dog-assisted therapy (AAT) was associated with greater improvements in emotional regulation among students compared to cognitive-behavioural therapy (CBT), as reflected by a reduction in DERS scores ($d = 2.21$). The results suggest that heart rate variability (HRV) may serve as a relevant biomarker of emotional regulation, and that increases in HRV could partially mediate reductions in emotional dysregulation through optimization of vagal control and attenuation of sympathetic activation. Psychological effects were accompanied by more pronounced positive neurophysiological changes. These findings support the potential of an integrative therapeutic model, in which multimodal interventions might produce synergistic effects. However, further controlled studies with larger samples are needed to confirm these associations and to establish the clinical and preventive relevance of AAT for students with moderate anxiety.

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