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Review article

CURRENT APPROACHES TO MODULATION OF INTESTINAL BARRIER FUNCTIONING IN PIGLETS: A LITERATURE REVIEW

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Abstract

The modulation of the intestinal barrier integrity in piglets during the early postnatal period is pivotal to the restriction of the propagation of enteric diseases agents, digestive efficiency, and performance. This review focuses on the primary morpho-functional changes occurring in the piglets' intestine after birth, including the development of the physical, chemical, and immune barriers. Critical stages in the maturation of intercellular adhesion and its role in keeping epithelial integrity are revised in detail. Particular stress is laid on the regulation of local immunity, including macrophage and dendritic cell involvement, secretory IgA production, and antimicrobial peptide synthesis. The mechanisms of interaction between intestinal microbiota and the immune system are discussed in respect of the stability of microbial community and adaptive immunity regulation. The roles of beneficial bacteria, including *Lactobacillus*, *Bifidobacterium*, and *Clostridia*, in infection resistance and intestinal epithelium metabolism are analyzed. Special emphasis is placed on the impact of weaning on intestinal barrier integrity, given that weaning stress is often accompanied by dysbiosis, disturbed microbial composition, and inflammation. Current approaches to keeping intestinal barrier functioning are reviewed, including the use of probiotics, prebiotics, synbiotics, exogenous enzymes, and antioxidant strategies. The efficacy of biologically active compounds, including short-chain and medium-chain fatty acids, polyphenols, and trace elements that strengthen intercellular junctions, modulate immune responses, and control inflammation, is discussed. The role of

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immunomodulators that activate local immunity, stimulate IgA production, and enhance antimicrobial defense is also considered. This comprehensive literature review highlights the mechanisms of intestinal barrier development in piglets, emphasizing its significance for animal health. The described data can be used to develop new strategies for managing piglet health and optimization of nutrition during weaning.

Keywords: piglets, weaning, intestines, microbial flora, cell communication, immunomodulation, probiotics, antioxidants.

INTRODUCTION

The development and maintenance of the intestinal barrier function is a key aspect of piglet health, resistance to infectious agents and productivity. In the early postnatal period, the intestinal barrier of newborns is immature, which increases the risk of pathogens translocation, the development of inflammatory processes and the impaired nutrient absorption. The weaning period is a crucial period for the maintenance of intestinal homeostasis. The principal change in diet induces an imbalance in the microbiota community and initiates the stress response in weaned piglets. The protective function of the gut is significantly provided by the main components, including physical, secretory and immune barriers. Their integrative interaction determines the effectiveness of the restriction in pathogens' invasion, the regulation of inflammatory response, and the metabolic status of the gut. One of the prospective approaches to maintaining intestinal barrier integrity is the use of immunomodulators, probiotics, prebiotics, short- and medium-chain fatty acids, antioxidants, and plant extracts. These biologically active compounds contribute to the strengthening of intercellular contacts, maintaining optimal microbiota composition, the reduction of oxidative stress and the improvement in local immune response.

This study aimed to analyze the mechanisms of the intestinal barrier regulation in early-aged piglets and to evaluate the efficacy of different approaches that can have a practical value for the increase in both gut health and animal productivity.

MATERIAL AND METHODS

The narrative literature review considers the analysis and generalization of current data regarding mechanisms of formation and modulation of the intestinal barrier function in early-aged piglets. The basis of the study was a critical analysis of peer-reviewed scientific articles, experimental studies, meta-analyses and reviews published in international databases, including PubMed, ScienceDirect, Springerlink, DOAJ, and Google Scholar for the period from 2000 to 2025. The following keywords and their combinations were used: "*piglets*", "*intestinal*

barrier”, “gut microbiota”, “weaning stress”, “probiotics”, “prebiotics”, “postbiotics”, “phytogenic feed additives”.

Several key criteria were used to include a study in the analysis. The inclusion criteria were applied: articles published in peer-reviewed journals; concerned the intestinal barrier integrity; highlighted methods of intestinal health maintenance; contained the results on feed additive supplementation in piglets growing; the reports on microbiome, intestinal epithelium, immunity interplays; concerned innovative approaches to preventing intestinal dysfunctions and modulating gut barrier functioning in piglets. Studies were excluded if they were: conference abstracts; non-English publications; works lacking full-text access or without scientific evidence (no references).

The primary selection was carried out based on both the titles and abstract content analysis. Data synthesis was carried out through qualitative analysis, highlighting key trends and effective approaches to the maintenance of intestinal health in piglets. Besides, the data systematization was constructed using content analysis, comparative analysis of research results and their critical evaluation. The obtained results provide the generalization of current knowledge concerning mechanisms of the intestinal barrier regulation in early-aged piglets and the formulation of a prospective line for further studies in this field.

RESULTS

The modulation of the intestinal barrier function in piglets in the early postnatal period is pivotal to their health and productivity. Understanding the mechanisms of barrier development and the impact of various factors may provide the opportunity to construct an effective strategy for maintenance of the intestinal integrity, which, in turn, contributes to the reduced incidence of diseases and better animal growth.

1. Morphological and functional features of piglet gut in early postnatal ontogenesis

The early postnatal period in piglets is characterized by intense morphological and functional changes in the gastrointestinal tract, which enables the vital process of adaptation to autonomous nutrition. Immediately after birth, the piglet gut is increasingly permeable, due to the immaturity of the epithelial layer and insufficient development of intercellular contacts. The intestinal barrier maturation is a multicomponent process, which includes epithelium differentiation, immune mechanisms promotion, and gradual colonization of the gut with commensal microorganisms. These adaptation changes play a crucial role in the formation of the intestinal barrier function, providing protection against pathogens and optimal nutrient absorption (Lallès et al., 2004).

Similarly to other mammals, the piglet's gut is anatomically divided into the small and large intestines. In newborn piglets, the former makes up approximately 5% of body mass. However, with animal age, this relationship decreases to 1.5%, resulting from the changes in the ratio of organs of the digestive system. The small intestine consists of the duodenum (5%), jejunum (90%), and ileum (5%). Each has specialized morphological and functional organization, which provides effective digestion and nutrients absorption (Lewis and Southern, 2000).

In newborn piglets, the length of the small intestine is approximately 2 m, and its capacity is about 72 ml (Marion et al., 2002). Before the weaning the length of small intestine increases more than three times, and its volume enlargement is more than nine times (Elefson et al., 2021; Wang et al., 2020). Although the surface area of the small intestine does not exceed 0,5 m² in newborn piglets, in 7-month-old animals it achieves about 6 m². This significant increase is dependent on the high nutritional value of the dairy diet, which does not leave a significant amount of undigested residues for fermentation in the large intestine (Greenwood-Van Meerveld et al., 2017). The development of the barrier function depends on the small intestine length, tight junction protein expression, cytokines production, and the morphology of villi and crypts (Dong et al., 2021).

The large intestine of newborn piglets is less differentiated than the small one. It also grows considerably slower (Weström et al., 2020). The length of large intestine reaches approximately 0.8 m, and its volume is about 40 ml (Chin et al., 2017). The large intestine increases in 1.5 times to three weeks of life while its development continues, even after the growth of the small intestine is completed (Elefson et al., 2021). The growth of the large intestine results from the gradual transition of piglets to more fibrous and less energy-rich feed, which stimulates the functional adaptation of the large intestine and the expansion of its enzymatic and absorption capabilities (Greenwood-Van Meerveld et al., 2017). A morphological feature of the pig's large intestine is the absence of villi in the mucosal layer. Meanwhile, it is coated with columnar epithelial cells with microvilli, which form straight tubular crypts. This provides epithelial layer renewal and mucus secretion (Chen et al., 2022). The caecum is relatively short in piglets, which reflects the special features of its digestive system (Wenk, 2001). After weaning, the large intestine grows until reaches 1.5% to 2.5% of the body mass (Hedemann and Knudsen, 2007; Wang et al., 2020; Yang et al., 2016). In adults, the large intestine length measures up to 7,5 m, and its volume 25 l (Barea et al., 2011; Elefson et al., 2021).

The formation of epithelial intercellular contacts in early-aged piglets is a critical mechanism that enables the functioning and the protective properties of the intestinal barrier. The immaturity of the intercellular adhesion in the first days of life promotes the absorption of immunoglobulins but also increases the risk of pathogen transmission (Wang et al., 2016). However, the gradual maturation of intercellular adhesion is controlled by hormonal, microbiological and nutritional

factors. It contributes to stabilizing the gut barrier integrity and controls its permeability (Masiuk et al., 2024a).

Intercellular contacts play a key role in the maturation of intestinal epithelial integrity, barrier regulation, and the selective permeability of piglets' guts in the early postnatal period (Guidoni et al., 2021). Epithelial cell contacts consist of several adhesive protein types, including tight junctions (TJs) and adherens junctions (AJs), which are formed in the first days of life and mature during postnatal development (Zhang et al., 2022).

The establishment of local immunity in the gastrointestinal tract of piglets is a multicomponent process, which includes the development of lymphoid structures, the activation of the mechanisms of cellular and humoral protection, and the interaction with intestinal microbiota (Wen et al., 2021). Piglet's gastrointestinal tract contains an extensive system of lymphoid tissue associated with the gut (GALT), which consists of Peyer's patches, intraepithelial lymphocytes, mesenteric lymph nodes, ileocolic lymph nodes, and lymph nodules in the small intestine (Jørgensen et al., 2022). The formation of all these structures begins in the middle of prenatal development. However, their functional activity depends on postnatal factors, particularly the communication with the intestinal microbiota. Moreover, milk diet components modulate the gut immunity potential.

Humoral immunity in piglets is provided by the colostrum and immunoglobulins of the sow's milk, including IgA, IgG and IgM in the early stages of development. IgA is the main secretory immunoglobulin, which forms immune complexes with pathogens and prevents their adhesion to the intestinal epithelium. IgG is transported across the epithelial barrier of the intestines and provides the systemic immune protection along with IgM antibodies, which participate in the primary immune response. The level of maternal immunoglobulins gradually decreases after weaning, which stimulates the immune system of piglets to synthesize their own antibodies.

Cellular immunity of the gastrointestinal tract is provided by T-lymphocytes, macrophages, and dendritic cells, which play a key role in the pathogen recognition, antigen presentation, and other events of adaptive immune system activation (Schäfer et al., 2022). Macrophages phagocytize microorganisms and produce proinflammatory cytokines, while dendritic cells are responsible for the activation of T-lymphocytes. The main subpopulations of T-lymphocytes differ in both functions and receptors CD4+ and CD8+. Together, these subpopulations regulate the inflammatory processes, the destruction of infected cells, and B-lymphocyte stimulation to produce specific immunoglobulins for long-term protection (Kumar et al., 2022; Maciag et al., 2022).

The postnatal colonization of the gut by microbiota is a crucial step in the development of local immunity (Kuthyar et al., 2023). Commensal bacteria, including *Lactobacillus*, *Bifidobacterium*, and *Clostridia*, are involved in the

regulation of pro-inflammatory and anti-inflammatory processes through the induction of T-regulatory cells, stimulation of IgA secretion and strengthening the intestinal barrier function by increased expression of molecules involved in intercellular contacts. Disbalance in intestinal microbiota is accompanied by various abnormalities including stress, chronic inflammation, increased epithelial permeability and high risk of infectious enteric diseases (Han et al, 2024).

2. Structural and functional organization of the intestinal barrier function

2.1. Physical barrier

The intestinal barrier not only provides selective absorption of nutrients, but is also of utmost importance in the protection against pathogenic microorganisms and toxins (Bao and Wu, 2021). Its structural and functional integrity is an essential feature that maintains homeostasis and reduces pathogens invasion into systemic blood circulation. The main components of the intestinal barrier are physical, secretory, and immune. The physical barrier consists of an intestinal single-layer epithelium, built of enterocytes, connected by tight intercellular contacts, such as TJs, AJs, junctional adhesion molecule (JAM), and desmosomes (Yang et al., 2022). These unique molecular structures provide the epithelial layer integrity, regulate paracellular permeability, and prevent the uncontrolled antigen and microorganism translocation.

The specialized junctions constructed by TJ and AJ proteins provide dynamic regulation depending on individual location and intestinal function, while AJs can trigger intracellular response and TJs structures are responsible for epithelial cell polarity and gut barrier integrity (Hartsock and Nelson, 2008).

TJs are the main structural components of the intestinal barrier, which regulate the paracellular transport and protect the epithelium from pathogen and toxin infiltration. The main proteins, which form these contacts, are claudins, occludin, and zonula occludens proteins (ZO-1, ZO-2, and ZO-3), which interact with the cytoskeletal components of epithelial cells (Chen et al., 2023). TJs are immature structures in the early postnatal life of piglets, which provides a high intestinal permeability in the first days of life. It enables the effective absorption of immunoglobulins from colostrum but also increases the risk of pathogen translocation across the epithelial layer (Inoue and Tsukahara, 2021).

Taking into the account the structure of epithelial cell junctions, occludin and claudins are key molecular markers of TJs and regulate the barrier functioning in the gut (Hartsock and Nelson, 2008). TJs impaired expression can lead to intestinal barrier dysfunction, particularly the development of “Leaky gut” syndrome, which is followed by increased epithelial permeability and chronic inflammatory processes.

Occludin, an integral protein of TJs, participates in the regulation of paracellular transport (Ballabh et al., 2004). Occludin can self-oligomerize or interact with other proteins to form an intercellular adhesion TJ type (Leong and Tong, 2015). Adequate occludin expression is important for intercellular adhesion, which provides the intestinal barrier integrity (Dubreuil, 2017). Claudins also play an essential role in paracellular transport and the regulation of intestinal permeability. A lack of claudin expression and the changes in its spatial distribution can disturb the barrier integrity (Fujita et al., 2006).

Formation and stabilization of TJs are regulated by several hormonal factors, including glucocorticoids and insulin-like growth factor-1. These hormones contribute to TJ protein expression and increase the number of intercellular contacts (Kurz and Seifert, 2021), stimulate colonization of the gut with microbiota, reduce the epithelium permeability (Lim et al., 2023), maintain the balance in protein and lipid nutrition, and minimize the risk of “Leaky gut” syndrome development (Zheng et al., 2024).

AJs are constructed by the association of cadherins (E-cadherin, N-cadherin), catenins (α -catenin, β -catenin), and actin filaments in complex structure, which provide both the strength of intracellular junctions and signaling from intercellular adhesion to intracellular pathways. In early-aged piglets, the adhesive junctions are not completely formed, which determines the increased sensitivity of the gut to both physical and microbial stress. The final maturation of AJs occurs during the first weeks of life and depends on: the mechanical load on gut during the feeding (Yuan et al., 2021); interaction with microbiota, which stimulates E-cadherin and β -catenin expression; the availability of essential amino acids (glutamine, arginine) that are substrates for the formation of cytoskeletal structures of enterocytes (Yin et al., 2022).

Extracellular matrix (ECM) proteins involved in cell-ECM adhesion and modulate physical barrier integrity through various mechanisms (Bontempo et al., 2026; Du et al. 2023; Masiuk et al., 2023a; Masiuk et al., 2024b). Fibronectin (FN) is a multifunctional glycoprotein of the ECM and plays an important role in cellular adhesion, proliferation and migration. FN enhances cell attachment and contributes to the repairing of damage via the interaction of epithelial cells with ECM (Kolachala et al., 2007). In case of malignant tumors, the level of fibronectin is significantly reduced, which indicates its role in the regulation of cellular metabolism (Rick et al., 2019). FN also interacts with collagen matrix and cytoskeletal components, contributing to the support of the tissue structure (Zollinger and Smith, 2017). Monoglyceride blend supplementation resulted in higher concentrations of FN in piglets after weaning, which evidences improved ECM and intestinal barrier (Masiuk et al., 2023b).

2.2. Secretory barrier

The secretory barrier consists of mucins, which form the protective mucosal layer of antimicrobial peptides and immunoglobulins, particularly secretory IgA. These components play a vital role in the pathogen neutralization, preventing their anchoring on the surface of enterocytes and their spreading in the intestinal mucosal layer. Mucins (MUCs) are specialized proteins produced by intestinal epithelial goblet cells, which are the main absorbance component of mucus (Tonetti et al., 2024). Mucin family consists of more than 20 proteins with minor differences in structure and function; in vertebrates MUC2 is predominant in the intestine (Arike and Hansson, 2016). A lack of MUC2 production leads to risk from enteric infections and pro-inflammatory changes, which highlights its importance in gut health (Tadesse et al., 2017).

Mucin content and its secretion are significantly increased in the early postnatal period, which contributes to the strength of the protective barrier (Chen et al., 2022). However, the mucosal layer is relatively thin in the small intestine, which provides the effective transport of nutrients and the interaction with microbiota. Meanwhile, the mucosal layer has one of most tight structure in the large intestine, thus providing the control of the microbial composition and prevent bacterial invasion into the deep layers of mucosa.

The mucosal layer plays a pivotal role in the protection of early-aged piglets' gut, creating physical, chemical and immune barrier between the intestinal content and enterocytes. Mucosal barrier is the first line of protection against pathogenic microorganisms, toxins, and mechanical damages. Furthermore, its structure provides a suitable microenvironment for the beneficial microbiota development (Yang et al., 2023). In newborn piglets, the mucosal barrier structures and their functions are not completely formed, which increases the risk of pathogen translocation across the intestinal epithelium.

2.3. Immune barrier

The immune barrier includes cells of innate and adaptive immunity, particularly macrophages, dendritic cells, and B- and T-lymphocytes, which are located in the lamina propria of the mucous membrane (Wiarda and Loving, 2022). These immune cells recognize and eliminate pathogens, and also modulate inflammatory processes and immune tolerance to commensal bacteria (Bemark et al., 2024). Thus, the intestinal barrier is a multicomponent system that provides physical, biochemical (mucosal), and immune protection. The components together maintain the balance between the nutrient absorption and the barrier function against pathogens (Figure 1).

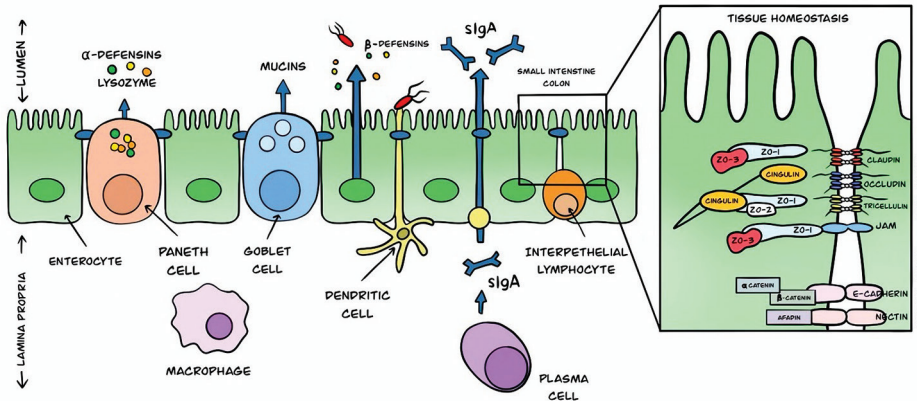


Figure 1. The main components of intestinal barrier in piglets. The physical barrier is constructed by TJ, AJ, and JAM proteins. Secretory (mucosal) barrier consists mucins, defensins, lysozyme, and antimicrobial peptides. Immune barrier consists of secretory IgA, dendritic cells, macrophages, and tissue-resident leukocytes.

Immediately after birth, colostrum consumption is the main means of protection against pathogens due to the unique composition, including high concentrations of immunoglobulins (IgA and IgG), mucins, antimicrobial peptides (defensins, lactoferrin, lysozyme) and a number of other biologically active molecules (Amatucci et al., 2022). Colostral antibodies serve as an “immune filter”, preventing the adhesion of pathogenic bacteria to the intestinal epithelium and neutralizing various antigens.

The intestinal system is the largest immune organ of pigs, given that almost 70% of all immunocompetent cells of the body are in the gut. It plays a key role in protection against pathogenic microorganisms, the regulation of inflammatory processes and support of immune homeostasis (Mörbe et al., 2021). Peyer’s patches contain T- and B-lymphocytes and perform the local immune control by providing antigen recognition and elimination.

Macrophages serve the function of first-line cellular protection in the piglet’s intestine (Yu et al., 2022). Located in the lamina propria of the mucous membrane, they are activated by pathogens’ penetration and/or damaged intestinal barrier integrity. Macrophages phagocytize pathogenic microorganisms, and produce anti-inflammatory and pro-inflammatory cytokines, which regulate the immune response (Li et al., 2023a). In the early postnatal period, the activity of macrophages is not completely developed. Therefore, the immune protection significantly depends on the factors that enter with colostrum. After weaning, the macrophage maturation is activated by metabolites of the intestinal microbiota and direct interaction with commensal bacteria (Tang et al., 2024).

Dendritic cells are the main intestinal antigen-presenting cells, which play an important role in the formation of adaptive immune response (Xia et al., 2022).

They permanently scan the intestinal content, engulf antigens, and transport them to mesenteric lymph nodes to activate immunocompetent T-lymphocytes. Thus, dendritic cells regulate the balance between immune tolerance to commensal bacteria and immune response to pathogens (Saha et al., 2024). In early-aged piglets these mechanisms are not fully developed, which explains their increased sensitivity to infectious agents, particularly during the weaning period (Yang et al., 2024).

Interferons are synthesized by various cell types and play a vital role in antiviral immunity, contributing to macrophage stimulation and the initiation of adaptive immune response. Intestinal macrophages and dendritic cells present antigens and provide the coordination of the immune response. The intestinal immune system is divided into inducible and effector sites. Inducible sites include mesenteric lymph nodes, where the primary activation and differentiation of the immune cells take place (Mowat and Agace, 2014). The effector sites are constructed of the intestinal epithelium, lamina propria, and the stroma of exocrine glands. Their interaction is important for barrier integrity as well as other protective mechanisms against pathogens (Brandtzaeg et al., 2008).

Cytokines are involved in various regulations, including intestinal immunity. Cytokines of the interferon family (IFN) play a central role in antiviral immunity and modulate other immune mechanisms in the gut (Van Winkle et al., 2020). There are three main types of interferons: IFN type I (IFN- α/β), IFN type II (IFN- γ) and IFN type III (IFN- λ) (Mesev et al., 2019). IFN- α/β and IFN- λ are the main mediators of early antiviral response, which contribute to the activation of immune cells and induce the antiviral state in tissues (Ye et al., 2019). IFN- λ receptor is crucial for the protection of the intestinal epithelial barrier by providing the antiviral effect with restricted inflammatory reactivity, which contributes to the maintenance of intestinal homeostasis during viral infections (Casazza and Lazear, 2019).

The balance between cell proliferation and cell death is important for homeostasis. Apoptosis is a widely occurring programmed cell death in all cell types. Caspases are cysteine proteinases that play an important role in apoptotic regulation and inflammatory processes (Brown et al., 2015). Caspase-3 (Casp-3) is a central effector enzyme in the cascade of apoptosis, responsible for the degradation of cellular proteins and cell death initiation (Ghazavi et al., 2022). The caspase-dependent mechanism can be activated by two means that include both exogenic (FasL binding receptors and TNF- α) and endogenic (mitochondrial) factors. The caspases are initiated by DNA damage and/or oxidative stress products, while cytochrome C is released from the mitochondrial membranes, and other pro-apoptotic mitochondria-associated factors (Bax, Bak, Bcl-2, BH3-only), which eventually activate the apoptosis cascades in all vertebrates (Zakariah et al., 2022).

In early-aged piglets, the immune mechanisms, including secretory IgA, macrophages, and dendritic cells, maintain the function of the gut barrier as a part of the total barrier system (Chen et al., 2022). These components play a critical

role in preventing pathogen penetration, and support the microbial balance and the formation of immune tolerance (Ferret-Bernard and Le Huërou-Luron, 2020). The secretory IgA is a key humoral component of local gut immunity (Erbs et al., 2025). Its production begins with the consumption of colostrum, which contains a high concentration of IgA that help to provide the primary passive protection of newborn piglets' intestinal mucosa (Raskova Kafkova et al., 2020). After weaning, the synthesis of IgA is activated by specialized B-lymphocytes located in Peyer's patches and mesenteric lymph nodes (Van der Weken et al., 2021). Secretory IgA builds immune complexes with antigens, preventing their adhesion to gut epithelium, and contributes to neutralization of bacterial toxins.

Taking into account that disturbance in every barrier component can lead to corresponding gut function abnormality, the measurement of respective biomarker content and/or expression is a convenient means to assess intestinal health. Gut health biomarkers are particularly relevant due to current requirements for minimizing invasive sampling in animal health monitoring. The expression and location of the intestinal barrier molecular markers, particularly of adhesive proteins, cytokines, and proteases, are important to assess when discussing the intestinal functional integrity (Du et al., 2023). Monitoring the changes in expression levels enables the creation of effective strategies of the prevention and therapy of diseases concerning intestinal barrier dysfunction (Masiuk et al., 2024b). The prospective application of several molecular markers to assess intestinal barrier integrity and/or disturbance has been described in recent reports (Masiuk et al., 2025; Shatalov and Kirici, 2024; Bontempo et al., 2026).

All described mechanisms build a multicomponent barrier, while each factor can stimulate the others. Thus, orchestral functioning of the intestinal barrier depends on both structural and signaling mechanisms.

3. The current approaches to modulation of the intestinal barrier function in piglets

Given the critical importance of the intestinal barrier integrity for health and productivity of swine farming, different approaches to the maintainance of gut health were proposed in the post-antibiotic era. The use of probiotics, prebiotics and postbiotics, antioxidant strategies, biologically active substances and immunomodulators were assessed and confirmed to be the most promised and prospective.

3.1. Probiotics

Probiotics are live non-pathogenic microorganisms, which, when introduced in sufficient amount, contribute to the improvement of body health (Capurso,

2016). They are widely used to stabilize microbial balance in the gastrointestinal tract, prevent pathogen colonization and to ameliorate optimal intestinal barrier functioning. In the last decade, probiotics have been undoubtedly considered to be a prospective alternative to antibiotics in animal farming. The most widespread probiotics are *Bifidobacterium*, *Lactobacillus*, and certain species of *Enterococcus*, *Streptococcus* and *Bacillus*, which contribute to the support of the healthy intestinal microbiome. Their positive effect is realized through competitive pathogen inhibition, secretion of antimicrobial substances (bacteriocins, organic acids), stimulation of local immunity and improved digestion. The administration of probiotics to sows (during pregnancy and lactation) contributes to improving the animals' health and stimulates piglet development (Pereira et al., 2022). Recent data demonstrated that probiotic supplementation contributes to IgG production in the sows' blood and increases the IgM levels in blood and the ileum mucosa of piglets during weaning. The use of *Bacillus*-derived probiotics has shown a trophic effect on the ileum mucosal barrier and biofilm formation by probiotic bacteria in the large intestines of piglets (Konieczka et al., 2023).

Bifidobacterium is a type of Actinobacteria (earlier Actinomycetes) and is one of the first microorganisms that colonize the gut in newborn animals (Castillo Zuniga et al., 2024; Kavanova et al., 2025). This bacterium plays an important role in the stable microbiome formation, preventing dysbiosis, diarrhea and creating optimal conditions for the development of the immune system. *Bifidobacterium* also increases the tolerance to lactose, decreases cholesterol levels, modulates local immunity and the synthesis of beneficial metabolites (He et al., 2023). For example, dietary supplements containing *Bifidobacterium breve* YIT4064 given to pregnant immunized mice protected their offspring against rotavirus infection better than antiviral immunization alone (Yasui et al., 1995).

Lactobacillus species are lactic acid bacteria, which are gram-positive, rod-shaped, catalase-negative, non-spore-forming, facultatively anaerobic microorganisms. Their main feature is the synthesis of lactic acid, which can acidify the medium, inhibit the growth of pathogens and support the intestinal barrier function (Kavanova et al., 2025). Lactic acid bacteria are widely spread in food products, feed, plant-based substrates, animal and human body, and are one of the key symbionts of the intestinal microbial community (Castillo Zuniga et al., 2024). *Lactobacillus* species have been confirmed to be among the most important probiotic sources relevant to the development of intestinal microbiome. Many studies conducted on pigs have testified that the use of lactic acid bacteria improves productivity, enhances immunity, and reduces the risk of diarrhea (Azevedo et al., 2012; Liu et al., 2013; Mao et al., 2016). The administration of certain strains of *Lactobacillus casei* and *Lactobacillus plantarum* contributes to decreased risk of viral infections and minimizes harmful effects of pathogens (Huang et al., 2021).

Escherichia coli Nissle 1917 (EcN) is a unique gram-negative probiotic that demonstrates an antagonistic effect against a broad spectrum of enteric pathogens.

EcN can regulate the secretion of immune factors that increase immune response (Zhao et al., 2022). EcN administered *per os* to piglets has shown a considerable antiviral effect, improved gut functions and reduced the duration of diarrhea (Michael et al., 2021).

The segmented filamentous bacteria (SFB) are gram-positive microorganisms, related to *Clostridium spp.*; they colonize the apical surface of epithelial cells in the terminal ileum. They play a key role in the protection against enteric infections and contribute to the regulation of immune response, especially to the stimulation of T-helper activity (Th17-cells). SFB are also involved in the prevention of the progress of autoimmune diseases and can modulate the tolerance to food antigens (Flannigan and Denning, 2018).

3.2. Prebiotics

Prebiotics are biologically active substances extracted from probiotics. They are selectively metabolized by commensal microbiome and contribute to body health. They play a key role in supporting optimal microbial balance that stimulate the growth and the activity of probiotics (You et al., 2022). The main groups of prebiotics are functional oligosaccharides, polysaccharides and natural plant-based extracts (You et al., 2022). The main function of prebiotics is the stimulation of the proliferative and metabolic activity of beneficial bacteria in the gastrointestinal tract. The metabolites of these bacteria strengthen the intestinal barrier, prevent the colonization of pathogens, modulate the immune system, activate the synthesis of secretory IgA and cytokines, increase the antioxidant resistance, and decrease the oxidative stress level (Ringseis and Eder, 2022). Prebiotics can reduce the risk of enteric infections and are considered a prospective alternative to antibiotics in animal farming due to various effects including an increase in animal productivity, modulation of barrier integrity, and maintenance of gut functions. The dietary use of pectin-oligosaccharide, lentinan or lactose improves the intestinal microbiome, strengthens the intestinal barrier function, increases the immune response and growth of piglets, and reduces the incidence of diarrhea (Chen et al., 2017; Mao et al., 2017). β -glucan administered orally influenced positively the whole state of animals and contributed to decreased mortality from rotavirus-induced diarrhea (Chethan et al., 2017). The supplementation of milk oligosaccharides or short-chain galacto-oligosaccharide blend and long-chain fructo-oligosaccharides to the diet contributed to the modulation of large-intestinal microbiota content and the increase in immune response (Li et al., 2014).

3.4. Synbiotics

Synbiotics are combinations of probiotics and prebiotics, which positively influence the host, improving survival and the colonization of useful microorganisms in the gastrointestinal tract. They have combined probiotic and prebiotic properties, creating a synergistic effect that helps the survival of probiotics in the intestines (Swanson et al., 2020). The corresponding combination of these two components demonstrates a more pronounced effect. The obtained results demonstrate the prospects of the use of synbiotics as an effective remedy to maintain intestinal health and increase resistance to infections. It has been proven that the oral administration of short-chain galacto-oligosaccharides, long-chain fructo-oligosaccharides and *Bifidobacterium breve* (M-16V) contribute to the decrease in symptoms of rotavirus diarrhea in rats (Rigo-Adrover et al., 2018). The resulting effect of symbiotics has been shown better than that of separate probiotics or prebiotics.

3.5. Postbiotics

Postbiotics are described as “soluble factors secreted by live bacteria, or released after bacterial lysis” (Gurunathan et al., 2023). They are various compounds including microbial cell wall components, peptides, enzymes, organic acids, and their derivatives (Liang and Xing, 2023). The beneficial effects of postbiotics can be mediated by both direct and indirect mechanism. Anti-inflammatory effect of postbiotics considered as one of most promised to maintain gut health (Wang et al., 2024). Furthermore, postbiotics exhibit multiple antimicrobial effects without resistance initiation (Liang and Xing, 2023).

Short-chain fatty acids (SCFA) and monoglycerides contained SCFA have demonstrated as alternative to antibiotics supplementation in animal husbandry (Kovanda et al., 2023). Monoglycerides are capable of maintaining morphological features of intestinal structures and modulate the gut microbiota content. Recent results have showed that the administration of α -glycerol monolaurate to piglets (GML) in a dose of 1 g/kg contributed to the increase of the ratio of the villi height to the crypts depth in duodenum, and stimulated the proliferation of goblet cells (Cui et al., 2020). Furthermore, GML supplementation initiated an increase in the height of villi in the jejunum and ileum, while the depth of the crypts remained unchanged (Li et al., 2022).

Postbiotics containing bacterial and fungal cell wall components deserve special attention due to mild stimulation of gut barrier functioning. Recent results demonstrated that yeast-derived postbiotics improve gut health and reduce pathogen translocation in spite of the absence of intestinal morphological changes (Hung et al., 2025). However, detailed mechanisms of postbiotics on piglet intestine barriers remain unclear and further study is required to explore the impact of various probiotic application on gut barrier integrity and growth performance.

3.6. Plant-derived extracts

Plant-derived extracts are the ancient remedies and are widely used health improvement medicines. Not surprisingly, similar compounds are supplemented for humans and animals. Therefore, several plant-based extracts were confirmed to be beneficial feed additives for farm animals, including pigs. As a rule, derived extracts exhibit multiple effects on metabolic pathways, homeostasis maintenance, and cell functions. Current data confirmed that the biological activity of natural substances is a main cause of the efficacy of plant extracts. The biological activity of many plant compounds were studied, including resveratrol, ginsenosides, saponins, quercetin, loureirin, baicalin, ziyuglycoside II, and genipin. A recent study demonstrated that the effects of these chemicals targeted gut barrier mechanisms. They are capable of modulating intestinal microflora, improving digestion, decreasing inflammation, and supporting the optimal state of the intestinal mucosal barrier (Yang et al., 2024). The use of these plant-based chemicals is considered a prospective approach to boosting animal health and increasing productivity. Particularly, the addition of prebiotics either 20 g/kg of inulin, or 40 g/kg of dried sunchoke to the diet of piglets has been successfully used as feed supplement, improving the qualitative properties of meat (Grela et al., 2021). Despite the long-time use of plant-derived extracts in animal husbandry, the dosage and timing of piglet treatment remain a controversial issue.

3.7. Exogenous enzymes

The addition of exogenous enzymes to the diet results in more effective conversion of food in the small intestine, that ameliorates definite compounds availability for microbial fermentation. For instance, proteases and carbohydrases effectively cleave proteins resistant to endogenous secretory enzymes and viscous non-starched polysaccharides accordingly. Both of them reduce the digestive viscosity and improve the nutrients absorption (Kim et al., 2022; Valente Junior et al., 2024). The beneficial effect of exogenous enzymes on the intestine functioning has been confirmed in recent reports. The use of enzymatic supplements in feeding improves digestion, contributes to optimal utilization of nutrients and supports the balance of intestinal microbiota (Rao et al., 2023). The key aspects of their activity is the capability to decrease the viscosity of intestinal content, to prevent the anti-nutritional factors and to reduce the level of substrates that can contribute to the excessive development of pathogenic microflora (Long et al., 2021).

Given that enzymes' effect on the animal health and productivity is dependent on their potential to improve the gut functioning, enzymes supplementation could be beneficial when the conditions of maintenance are far from optimal (Rao et al., 2023). For instance, in cases of increased microbial load or insufficient feed level enzymatic supplements can substantially reduce the risk of intestinal dysfunction,

contributing to the strengthening of the intestinal barrier function and the improvement of general animal health (Liu et al., 2023).

In the last decade, significant progress in the study of enzymes application in pig farming was achieved. Among the most effective enzymes administered to pigs several main groups were reported: phytases, xylanases, beta-glucanases, proteases, amylases, lactases, and cellulases (Dziurva et al., 2023; Edison et al., 2022; Kim et al., 2022; Moita and Kim, 2022; Lee et al., 2019; Valente Junior et al., 2024; Zhu et al., 2022). However, some aspects of enzyme supplementation remain unclear, including dosage, mixing with other feed additives, and optimal timing of administration.

3.8. Antioxidant strategies that support intestinal barrier

Antioxidants have long been used to reduce various oxidative damages. The support of early-aged piglets intestinal barrier function significantly depends on the effective control of oxidative stress, which occurs as a result of intensive growth, alterations in the diet and influence of external stress factors, particularly weaning (Blavi et al., 2021; Zhang et al., 2015). Antioxidant strategies are destined for the decrease the level of reactive oxygen species, cellular homeostasis maintenance, and the increase in antioxidant defense system activity. One of the key mechanisms of antioxidant defense is the use of natural antioxidants, including vitamins and other biologically active compounds. Vitamin E (α -tocopherol) and vitamin C (ascorbic acid) play an important role in the stabilization of cell membranes of enterocytes (Orengo et al., 2021). Vitamin A contributes to the regeneration of intestinal epithelium and supports the expression of TJ proteins (Wu et al., 2023). Polyphenols, particularly quercetin, catechins of green tea, resveratrol, and curcumin exhibit powerful antioxidant effect and proinflammatory activity that modulate signaling pathways linked with oxidative stress, decrease the level of proinflammatory cytokines' production, and contribute to the strengthening of intercellular adhesion in the gut epithelial layer (Pistol et al., 2023).

Several microelements, especially selenium and zinc, play an important role in antioxidant strategies due to direct regulation of antioxidant enzymes' activity. Selenium, present in glutathione peroxidase, protects enterocytes from oxidative damage, and zinc stabilizes the structure of TJ proteins and contributes to the intestinal epithelium regeneration (Zhou et al., 2024).

Prebiotics and probiotics also take a part in the antioxidant defense of the gut, particularly certain strains of *Lactobacillus* and *Bifidobacterium* that produce enzymes, which neutralize the active forms of oxygen. Prebiotics, including fructo-oligosaccharides and galacto-oligosaccharides, contribute to the growth of useful microflora that supports the balance of antioxidant processes (Pang et al., 2022). SCFA play a special role in the support of barrier function, particularly butyrate

enhances the expression of antioxidant enzymes and the TJ proteins (Liu et al., 2021).

The transmission of antioxidant capability between the sow and its fetuses is realized through the axis gut-placenta, as demonstrated in a recent report. Dietary glycerol monolaurate (GML) application exhibited a protective effect in sows during pregnancy (Zhang et al., 2024). Monoglycerides, particularly GML, are recognized as a prospective feed additives capable of activating the antioxidant system in pigs. Another study has shown a significant increase in both IL-10 level and glutathione peroxidase activity in weaned piglets (Li et al., 2022). Furthermore, glycerol monolactate addition to the diet decreased the concentrations of malonic dialdehyde and hydrogen peroxide in the ileum of pigs and stimulated the activity of superoxide dismutase and catalase in both the small and large intestines, which reflects its potent antioxidant properties (Li et al., 2023a).

Given that current data evidence that the results of antioxidant supplementation can be modulated by various external factors, further research is required to optimize antioxidant-containing diets.

3.9. Modulation of intestinal barrier integrity in piglets by biologically active compounds

Biologically active compounds are various natural chemicals that exhibit multiple effects. They can modulate the intestinal barrier by integrative strengthening of the intercellular contacts, immune response, and microbiota balance, and reduction of inflammation. The main biologically active compounds, which have proven themselves successful in practice, are polyphenols (quercetin, resveratrol, catechins, curcumin), SCFA (butyrate, propionate, acetate), prebiotics (fructooligosaccharides, galactooligosaccharides, inulin), peptides (lactoferrin, immunoglobulins IgA, defensins, casein peptides), microelements (Zink, Selenium), and medium chain fatty acids (MCFA) (caprylic, capric, lauric acids). All of them contribute to strengthening the intestinal barrier in piglets, decreasing inflammatory processes and supporting microbiome balance.

Some of the most important biologically active compounds are polyphenols, which have potent antioxidant and proinflammatory properties (Mao et al., 2024). Quercetin, resveratrol, catechins, and curcumin contribute to the decrease in proinflammatory cytokines (IL-6, TNF- α), stimulate the expression of TJ proteins (occludin, claudins, ZO-1) and maintain the balance in enterocyte recycling (Pistol et al., 2023).

MCFA, including caprylic, capric, and lauric acids, exhibit antimicrobial properties, contribute to the balance of intestinal microflora, decrease the levels of pathogenic bacteria (*Escherichia coli*, *Clostridium spp.*) and strengthen the intestinal barrier function. Besides, the MCFA as energy substrates ameliorate gut morphology, stimulate growth, increase immunity and can be supplemented as a

prospective alternative to antibiotics (Dahmer et al., 2020; Hanczakowska et al., 2016). Recent results have shown the high bacteriostatic activity of MCFA against a wide spectrum of pathogens, including gram-positive and gram-negative bacteria, viruses, fungi, and protozoa (Kovanda et al., 2023; Jackman et al., 2020). During weaning, piglets are stressed, which worsens nutrient absorption and disturbs gut energy balance. MCFA applied to the diet improve digestion, lipid metabolism, and provide optimal development and growth of weaned piglets (Cui et al., 2022).

SCFA play a key role in the intestinal barrier functioning (Ślupecka-Ziemilska et al., 2022). Butyrate is the source of metabolic energy for enterocytes. Furthermore, butyrate stimulates the secretion of mucins by goblet cells, increases TJ proteins' expression, ameliorates intestinal barrier integrity, and decreases pathogens' invasion (Cui et al., 2020; Li et al., 2023b; Li et al., 2022; Thomas et al., 2020). The addition of GML to the sows' diet improves the digestibility of crude fat and stimulates the average daily gain of piglets during weaning (Li et al., 2023c). Besides, GML supplementation improves the digestibility of various nutrients, decreases the pro-inflammatory processes, reduces intestinal permeability, and stabilizes the microbiota community in weaned piglets (Wei et al., 2023).

The improvement of intestinal barrier functions by immunomodulators is accompanied by local immunity strengthening, improved intercellular adhesion, and reduced inflammatory process in piglets (Masiuk et al., 2025). The application of immunomodulators, including β -glucans, lipopolysaccharides, nucleotides, cytokines (IL-10, IFN- γ), probiotics, and plant-based extracts, has been confirmed to be prospective feed additives that stimulate innate immunity, ameliorate both secretory IgA and antimicrobial peptides production (Chang et al., 2023; Saha et al., 2024). The supplementation of piglets' diet with β -glucans improves the barrier integrity via enhanced TJ proteins' (occludin, claudin-1, ZO-1) expression and stimulation of macrophage activity.

Thus, immunomodulators are prospective instruments for maintaining the intestinal barrier in piglets, decreasing the risk of infectious diseases, modulating immune response and providing optimal gastrointestinal tract adaptation to weaning stress.

CONCLUSION

This data analysis proves that intestinal barrier modulation in piglets is multifactorial and includes physical, secretory, and immune barrier regulation. The maturation of intercellular junctions in the first weeks of life strengthens the physical barrier and restricts its permeability. Secretory barrier development is accompanied by increased production of mucins, defensins, antimicrobial peptides, and lysozyme. The immune barrier stimulation depends on communication with the gut microbiota.

Weaning is a critical period for gut functioning in piglets. It is accompanied by acute changes in diet, stress, and dysbiosis, which together can cause post-weaning diarrhea, inflammation, and the disruption of the intestinal barrier. Thus, current approaches to maintaining intestinal health include the use of probiotics, prebiotics, synbiotics, exogenous enzymes, antioxidant compounds, and immunomodulators. All these provide the intestinal barrier integrity, reduce inflammation, and boost the immune response.

New solutions for an integrated approach to intestinal barrier preservation are promising areas for future research. Further research should concentrate on diet optimization and genetic elements of the intestinal barrier functioning in addition to the use of recognized agents. When taken as a whole, the idea of intestinal barrier preservation serves as the cornerstone of the strategy for enhancing animal health and pig farming profitability.

Authors' contribution

DM conceived the concept, study framework and took part in literature data analysis, OS analyzed and grouped literature data, DM and OS equally contributed to the writing of the manuscript draft, VN was involved in conceptualization, proofreading, and final revisions.

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Ethical statement

This study did not involve experimental research on live animals. All procedures were conducted in accordance with applicable institutional and ethical standards. ARRIVE guidelines are not applicable to this study.

Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no conflict of interest regarding the present work.

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SAVREMENI PRISTUPI MODULACIJI FUNKCIJE INTESTINALNE BARIJERE KOD PRASADI: PREGLED LITERATURE

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Kratak sadržaj

Modulacija integriteta crevne barijere kod prasadi tokom ranog postnatalnog perioda ključna je za ograničavanje razmnožavanja uzročnika crevnih bolesti, održavanje efikasnosti varenja i obezbeđivanje performansi. Ovaj pregled se fokusira na primarne morfo-funkcionalne promene koje se dešavaju u crevima prasadi nakon rođenja, uključujući razvoj fizičkih, hemijskih i imunih barijera. Kritične faze u sazrevanju međucelijske adhezije i njena uloga u očuvanju integriteta epitela su detaljno razmotrene. Poseban naglasak je stavljen na regulaciju lokalnog imuniteta, uključujući učešće makrofaga i dendritskih ćelija, proizvodnju sekretornih IgA i sintezu antimikrobnih peptida. Mehanizmi interakcije između crevnog mikrobioma i imunog sistema razmotreni su u pogledu stabilnosti mikrobne zajednice i regulacije adaptivnog imuniteta. Analizirane se uloge korisnih bakterija, uključujući vrste rodova *Lactobacillus*, *Bifidobacterium* i *Clostridia*, u otpornosti na infekcije i metabolizam crevnog epitela. Poseban naglasak je stavljen na uticaj odbijanja prasadi na integritet crevne barijere, s obzirom na to da je stres kod odbijanja često praćen disbiozom, poremećajem mikrobnog sastava i zapaljenjem. Razmatrani se aktuelni pristupi održavanju funkcije crevne barijere, uključujući primenu probiotika, prebiotika, sinbiotika, egzogenih enzima i antioksidativnih strategija. Razmatrana je efikasnost biološki aktivnih jedinjenja, uključujući masne kiseline kratkog i srednje dugog lanca, polifenole i elemente u tragovima, koji jačaju međucelijske veze, moduliraju imune odgovore i kontrolišu zapaljenje. Takođe je razmatrana uloga imunomodulatora koji aktiviraju lokalni imunitet, stimulišu proizvodnju IgA i poboljšavaju antimikrobnu odbranu. Ovaj sveobuhvatni pregled literature ističe mehanizme razvoja crevne barijere kod prasadi, naglašavajući njen značaj za zdravlje životinja. Opisani podaci mogu se koristiti za razvoj novih strategija za upravljanje zdravljem prasadi i optimizaciju ishrane tokom perioda odbijanja.

Ključne reči: prasadi, zalučenje, creva, mikrobna flora, ćelijska komunikacija, imunomodulacija, probiotici, antioksidansi.

Original research article

EPIDEMIOLOGY OF HAEMORRHAGIC FEVER WITH RENAL SYNDROME IN THE AUTONOMOUS PROVINCE OF VOJVODINA, SERBIA, 1992–2023

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Abstract

Although Serbia is recognized as endemic for a haemorrhagic fever with renal syndrome, long-term epidemiological data from subnational regions remain limited. This study aimed to describe temporal, spatial, seasonal and demographic patterns of this disease in the Autonomous Province of Vojvodina, northern Serbia, over 32 years (1992-2023), and to assess potential associations between disease occurrence and selected climatic parameters. A retrospective descriptive study was conducted based on routine surveillance data. Annual crude and age- and gender-specific incidence rates were calculated. Geographic clustering was assessed at the district and municipality levels. Correlations between disease incidence and temperature, precipitation, relative humidity and number of rainy days were evaluated using Spearman's rank correlation coefficient. Over the study period, a total of 82 cases were reported (average annual incidence: 0.13/100,000; 95% CI: 0.10-0.16), characterized by sporadic occurrence and substantial interannual variability, with two deaths recorded. The highest annual incidences were recorded in 2012 (0.41/100,000) and 2014 (0.52/100,000). Incidence exhibited marked spatial heterogeneity, with the highest long-term rates in South Bačka and Srem districts, and municipal hotspots (Beočin, Irig, Sremski Karlovci). Males aged 30–39 years experienced the highest incidence

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rate (0.56/100,000), whereas cases were rare among children. A pronounced seasonal peak was observed in June, accounting for 24.4% of all reported cases. No statistically significant associations were found between annual incidence and climatic parameters. Monthly analysis revealed a positive correlation between number of cases and temperature ($\rho = 0.606$, $p = 0.037$) and a negative correlation with relative humidity ($\rho = -0.621$, $p = 0.031$). Our findings indicate that haemorrhagic fever with renal syndrome remains a low-incidence but endemic zoonosis in AP Vojvodina, characterised by distinct geographic hotspots, well-defined demographic patterns, and stable early-summer seasonality. Enhanced integrated surveillance, improved diagnostic coverage and integration of ecological and environmental data are needed to better understand localized risk and support timely public health interventions.

Key words: Hantavirus, zoonoses, epidemiology, morbidity, geographical distribution, rodents, climatic factors; seasons

INTRODUCTION

Hantaviruses are important rodent-borne pathogens that cause haemorrhagic fever with renal syndrome (HFRS) in Europe and Asia, and hantavirus cardiopulmonary syndrome in the Americas (Kruger et al., 2015; Avšič-Županc et al., 2019). Human infection typically occurs through inhalation of aerosolised rodent excreta, most commonly from species belonging to the Murinae or Arvicolinae subfamilies (Kruger et al., 2015; Avšič-Županc et al., 2019; Lupuşoru et al., 2023). European HFRS is predominantly caused by *Puumala orthohantavirus* (PUUV) and *Dobrava–Belgrade orthohantavirus* (DOBV), which differ in virulence, epidemiology, and rodent reservoirs: PUUV is associated with *Myodes glareolus* and typically causes nephropathia epidemica, a mild form of HFRS, whereas DOBV—carried by several *Apodemus* species—can result in more severe disease and higher case fatality rates (Heyman et al., 2007; Klempa, 2009; Khalil et al., 2019).

Within Europe, HFRS exhibits marked spatial and temporal variations driven by rodent population dynamics, seasonal patterns, and climatic factors that influence rodent abundance and human exposure (Rose et al., 2003; Vapalahti et al., 2003; Linard et al., 2007). Endemic circulation and periodic peaks have been reported in Finland, Sweden, Germany, Belgium, Slovenia, and other countries of central and south-eastern Europe (Klempa, 2009; Avšič Županc et al., 2014; Douglas et al., 2021). Climatic fluctuations, particularly in temperature and precipitation, are recognised as important ecological drivers modulating rodent population cycles and subsequent viral transmission to humans (Klempa, 2009; Avšič Županc et al., 2014; Stamenković et al., 2015; Douglas et al., 2021).

The Republic of Serbia (Serbia) is part of the Balkan hantavirus endemic zone, with confirmed circulation of PUUV and multiple DOBV lineages in both rodent hosts and human populations. Previous national studies have reported sporadic

cases and occasional outbreaks, predominantly in forested, agricultural, and peri-urban regions of northern Serbia (Papa et al., 2006; Stanojevic et al., 2019; Štrbac et al., 2022).

This study provides a 32-year (1992–2023) epidemiological assessment of HFRS in the Autonomous Province (AP) of Vojvodina, examining temporal trends, geographic heterogeneity, age- and gender-specific patterns, seasonal distribution, and associations between HFRS incidence and meteorological variables.

MATERIAL AND METHODS

Study design and setting

A retrospective, population-based epidemiological study on all HFRS cases reported in the AP of Vojvodina, Serbia, from 1 January 1992 to 31 December 2023 was conducted. AP of Vojvodina is located in northern Serbia and consists of seven administrative districts and 45 municipalities, with an estimated population of approximately 1.8 million in 2022 (RZS, 2025).

Data sources and case definitions

Data on HFRS cases were obtained from official surveillance records of the Institute of Public Health of Vojvodina (IPHV), the regional reference institution for communicable disease surveillance. Case notification forms were collected at local public health departments and subsequently forwarded to the IPHV.

Case definitions followed national reporting standards (Štrbac et al., 2022) and were aligned with the World Health Organization (WHO) criteria for hantavirus infections (WHO, 2025). A confirmed case was defined as a reported HFRS case with laboratory evidence of hantavirus infection demonstrated by one or more of the following: detection of hantavirus-specific IgM antibodies; a fourfold or greater increase in IgG antibody titres or hantavirus-specific IgG seroconversion; a positive reverse transcriptase–polymerase chain reaction (RT-PCR) result for hantavirus RNA; positive immunohistochemical detection of hantavirus antigens.

Population denominators for calculating incidence rates were obtained from the Statistical Office of the Republic of Serbia based on census years (1991, 2002, 2011, 2022) (RZS, 2025).

Variables and analytical approach

Extracted variables included the year and month of diagnosis, district and municipality of residence, age, gender, and outcome (survival or death). Average gender- and age-specific incidence rates were calculated based on 8 age groups (0–

4, 5–9, 10–19, 20–29, 30–39, 40–49, 50–59, ≥ 60 years). In addition, spatial analyses included district- and municipality-level annual average incidence rates over the 32-year study period.

Meteorological data

Monthly and annual meteorological parameters (the average temperature in °C, average rainfall in mm, average relative humidity in %, and average number of rainy days ≥ 0.1 mm) were obtained from the Meteorological Service of the Republic Hydrometeorological Institute of Serbia (RHMZ, 2025). Data were matched to case months and aggregated for the study period.

Statistical analysis

Descriptive statistics were used to summarise incidence, demographic characteristics, temporal trends, and spatial distribution. The average incidence rate and its 95% confidence interval were calculated assuming a Poisson distribution of case counts, with person-years at risk as the denominator.

Spearman correlation coefficients (ρ) were used to assess associations between monthly HFRS case counts and climatic parameters. Statistical significance was defined as $p < 0.05$ (two-tailed).

All statistical analyses were conducted using IBM SPSS Statistics software, version 21 (IBM Corp., Armonk, NY, USA). A spatial distribution map was created using QGIS (version 3.42.1).

Ethics statement

The data from this retrospective study are based on the results of HFRS surveillance conducted between 1992 and 2023 as part of the routine surveillance at the Center for Disease Control and Prevention IPHV ; therefore, approval from the Ethics Committee was not required. All analyses were conducted using anonymized and aggregated surveillance data.

RESULTS

Figure 1 summarizes the reported cases, incidence rates, and mortality of HFRS in AP of Vojvodina from 1992 to 2023. Over these 32 years, 82 cases were registered, corresponding to an overall average annual incidence rate of 0.13 per 100,000 population (95% CI: 0.10–0.16).

Case occurrence was sporadic, with most years reporting between 0 and 8 cases. The highest annual counts and incidence rates were recorded in 2012 (8

cases; 0.41/100,000) and 2014 (10 cases; 0.52/100,000), followed by 2023 (7 cases; 0.40/100,000). Several years—particularly between 2019 and 2022—reported zero cases, indicating periods of minimal or absent detected transmission.

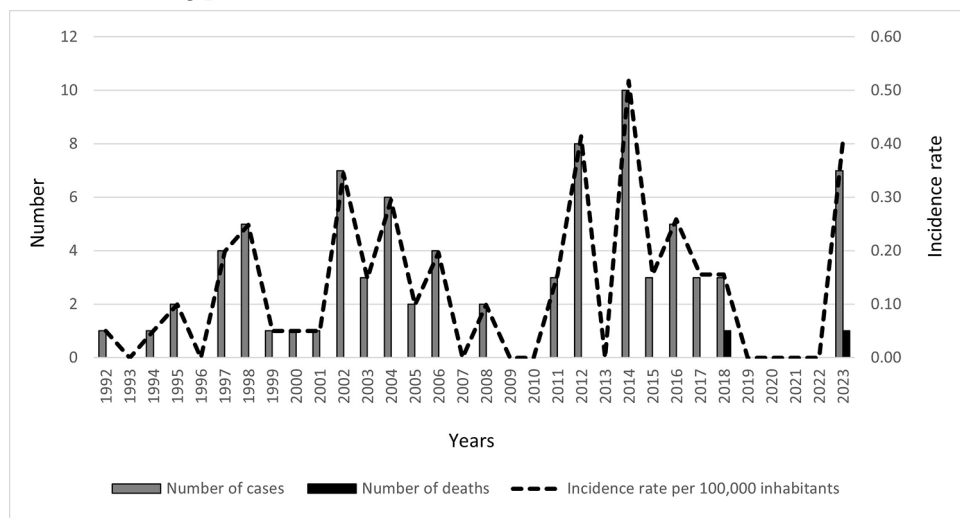


Figure 1. Reported Cases, Incidence Rates per 100,000 population, and Deaths from Haemorrhagic Fever with Renal Syndrome in AP of Vojvodina, 1992–2023.

Throughout the study period, HFRS-associated mortality remained very low, with only two deaths documented: one in 2018 and one in 2023. The latter occurred in a patient who had stayed in a neighboring country during the maximum incubation period, following rodent exposure while camping.

The average incidence rate (between 1992 and 2023) of HFRS in AP of Vojvodina shows pronounced geographical variability across administrative districts. The highest average incidence was recorded in South Bačka (0.27 per 100,000 population, 95% CI: 0.14–0.39), followed by Srem (0.22 per 100,000, 95% CI: 0.12–0.31). Moderate incidence levels were observed in Central Banat (0.06 per 100,000, 95% CI: 0.00–0.12) and South Banat (0.05 per 100,000, 95% CI: -0.01–0.10). By contrast, considerably lower rates were documented in North Bačka (0.02 per 100,000, 95% CI: -0.02–0.06) and West Bačka (0.01 per 100,000, 95% CI: -0.02–0.04). North Banat reported no registered cases during the observed period (Figure 2).

The average incidence of HFRS across municipalities of AP of Vojvodina during the period 1992–2023 showed pronounced spatial heterogeneity. Among municipalities with reported incidence, Beočin (1.99/100,000, 95% CI: 0.63–3.36) and Irig (1.34/100,000, 95% CI: -0.02–2.70) demonstrated the highest long-term average rates, indicating localized areas of increased epidemiological activity. Elevated averages were also recorded in Sremski Karlovci (0.69/100,000, 95% CI: -0.29–1.67), Šid (0.58/100,000, 95% CI: 0.06–1.10), and Temerin (0.46/100,000, 95% CI: -0.10–1.02).

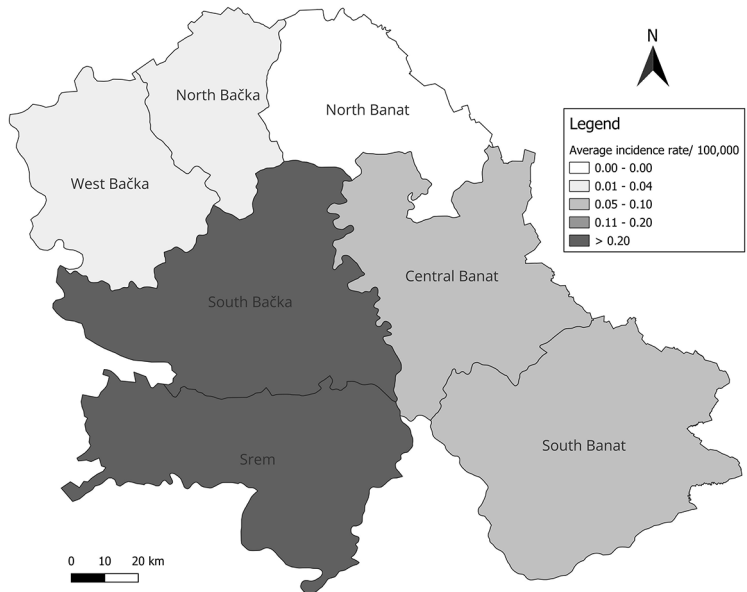


Figure 2. Average Incidence Rate of Haemorrhagic Fever with Renal Syndrome per 100,000 population by District in AP of Vojvodina, 1992–2023.

The City of Novi Sad, the largest urban center in AP of Vojvodina, recorded a long-term average incidence of 0.22/100,000 (95% CI: 0.11–0.33), reflecting sporadic but continuous case occurrence over the three-decade period (Figure 3).

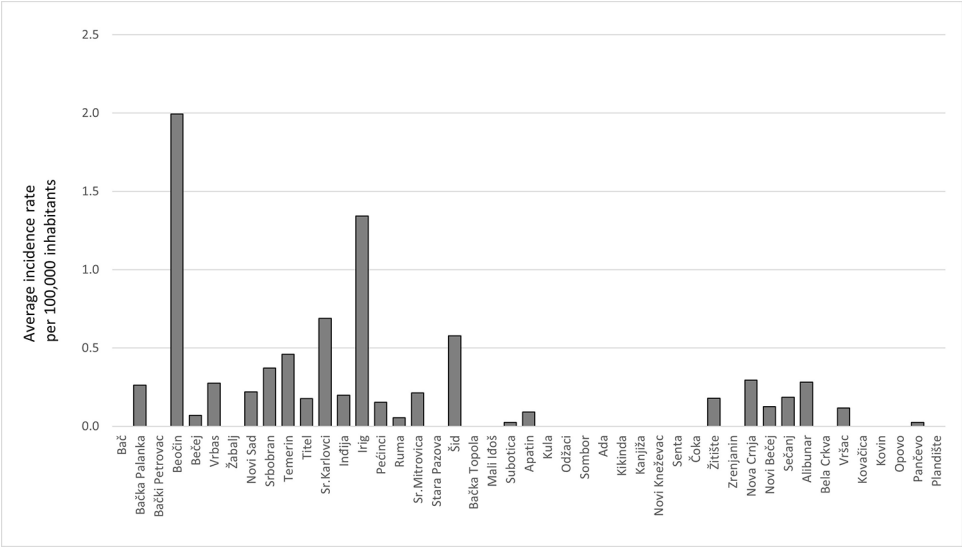


Figure 3. Average Incidence Rate of Haemorrhagic Fever with Renal Syndrome per 100,000 population by Municipality in AP of Vojvodina, 1992–2023.

The analysis of age- and gender-specific average incidence rates of HFRS in AP of Vojvodina in the period 1992–2023 demonstrates clear demographic differences in disease occurrence. Males consistently exhibited higher incidence rates across nearly all age groups, indicating a strong sex-related pattern in HFRS epidemiology.

Among males, the incidence increased from adolescence and peaked in the 30–39 age group (0.56 per 100,000), followed by moderately elevated rates in the 20–29 (0.49) and 40–49 (0.24) age groups. Incidence declined after the age of 50. No cases were recorded in boys aged 0–9 years.

In females, the incidence rates were substantially lower across all age categories. The highest values were observed in the 20–29 age group (0.08), followed by the 50–59 (0.06), and 30–39 (0.05) age groups, whereas most others age groups showed minimal or no recorded incidence. Notably, the 5–9 age group (0.07) was the only childhood group with measurable incidence in females (Figure 4).

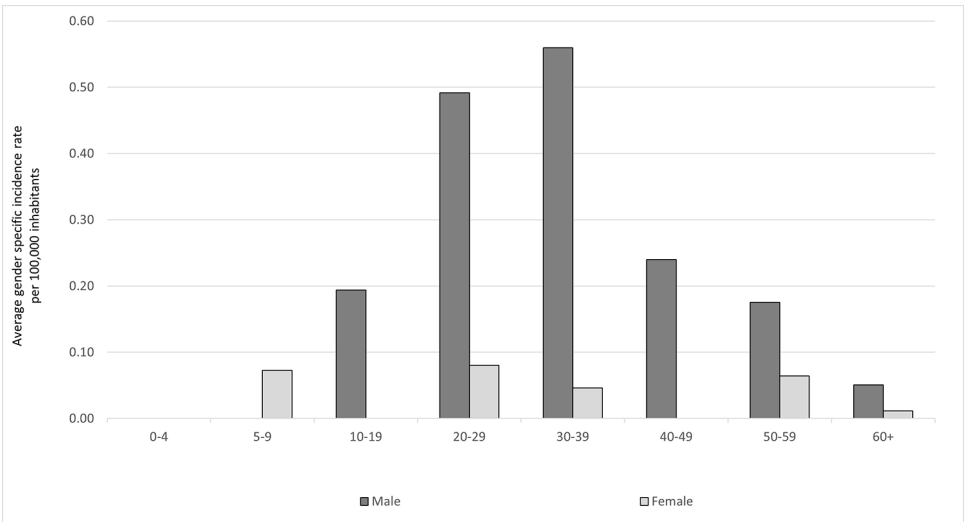


Figure 4. Average Age- and Gender-Specific Incidence Rate of Haemorrhagic Fever with Renal Syndrome per 100,000 population in AP of Vojvodina, 1992–2023.

The monthly distribution of HFRS cases in AP of Vojvodina from 1992 to 2023 demonstrates a clear seasonal pattern, with cases recorded throughout the year but markedly concentrated in late spring and early summer. The highest proportion of cases occurred in June (24.39%), representing the primary seasonal peak. A secondary elevation was observed in April and July (each 13.41%), followed by May and October (each 8.54%), as well as August and November (each 7.32%) (Figure 5).

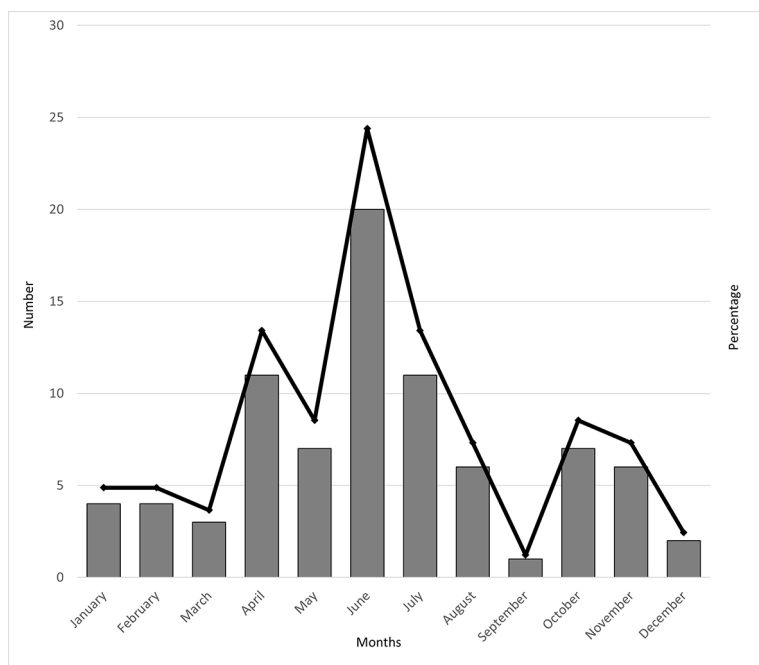


Figure 5. Monthly Distribution of Haemorrhagic Fever with Renal Syndrome Cases in AP of Vojvodina, 1992–2023.

The long-term annual dataset for AP of Vojvodina (1992–2023) illustrates considerable interannual variability in the incidence of HFRS and its potential association with key meteorological parameters. The annual HFRS incidence rate fluctuated between 0 and 0.52 per 100,000 inhabitants. Many years have recorded no cases, indicating low but episodic transmission patterns.

Average annual temperature in AP of Vojvodina ranged from 10.5 to 13.6 °C, demonstrating a gradual warming trend toward the end of the study period. Precipitation levels showed substantial variability, with annual averages spanning 24.4 mm (2000) to 75.7 mm (2010). Relative humidity remained relatively stable over the period, typically between 66% and 78%, with the highest values generally coinciding with wetter years.

The average number of rainy days (defined as days with precipitation ≥ 0.1 mm) varied moderately, ranging from 8 to 12 days per year, with no clear long-term trend but notable interannual differences consistent with precipitation variability (Figure 6). There was no statistically significant association between the incidence rate of HFRS and any of the four observed climatic parameters ($p > 0.05$).

Table 1 presents the monthly distribution of HFRS cases in AP of Vojvodina over the period 1992–2023, alongside key climatic variables, including average temperature (°C), average rainfall (mm), average relative humidity (%), and the average number of rainy days with precipitation ≥ 0.1 mm.

Correlation analysis revealed a statistically significant positive correlation between the number of HFRS cases and average temperature ($\rho = 0.606$, $p = 0.037$), suggesting higher incidence during warmer months. By contrast, a significant negative correlation was observed with average relative humidity ($\rho = -0.621$, $p = 0.031$), indicating lower case numbers during periods of higher humidity. No significant associations were found between HFRS incidence and either average rainfall ($\rho = 0.479$, $p = 0.115$) or the average number of rainy days ($\rho = 0.423$, $p = 0.171$).

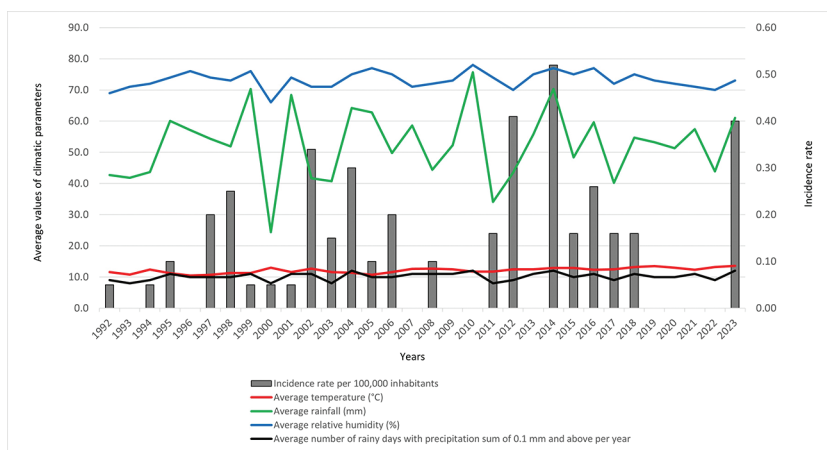


Figure 6. Annual incidence rates of Haemorrhagic Fever with Renal Syndrome per 100,000 population in relation to climatic parameters in AP of Vojvodina, 1992–2023.

DISCUSSION

This 32-year analysis provides the most comprehensive assessment to date of HFRS epidemiology in AP of Vojvodina. The annual incidence of HFRS across the European Union/European Economic Area (EU/EEA) for the period 2019–2023 ranged between 0.4 and 1.1 per 100,000 population (ECDC, 2025). Overall incidence of HFRS in AP of Vojvodina during 1992–2023 was low (0.13 per 100,000 population), with pronounced interannual variability and sporadic case occurrence, consistent with patterns reported in other parts of Serbia and neighboring Balkan countries (Papa et al., 2006; Stamenković et al., 2015; Štrbac et al., 2022).

Our results showed that district heterogeneity was substantial. South Bačka and Srem districts demonstrated the highest long-term average incidence, while some districts—for example North Banat—reported no cases. Similar fine-scale spatial clustering has been described in Slovenia, Croatia, and Belgium, where landscape structure, forest cover, agricultural practices, and rodent reservoir abundance shape local HFRS risk (Avsic-Zupanc et al., 1999; Tersago et al., 2009; Cekinović

Table 1. Monthly distribution of Haemorrhagic Fever with Renal Syndrome cases and correlation with climatic parameters in AP of Vojvodina,

Variable	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	ρ (rho)	p value
No of cases	4	4	3	11	7	20	11	6	1	7	6	2		
Aver. temp. (°C)	0.8	2.7	6.8	12.4	17.6	21.3	22.9	22.6	17.4	12.2	6.9	2.0	0.60565	0.03688
Aver. rainfall (mm)	39.1	37.0	35.0	45.1	66.6	79.0	65.9	54.5	59.1	49.9	49.1	49.5	0.47889	0.11523
Aver. rel. hum (%)	85.0	78.8	69.3	66.3	66.7	67.8	65.7	66.3	71.7	75.9	81.9	86.2	-0.62082	0.03122
Aver. no of rainy days (precipitation $\Sigma \geq 0.1$ mm / year)	8.7	7.9	9.4	11.7	13.4	12.0	9.5	8.4	10.1	9.8	10.6	10.3	0.42255	0.17118

Values that differ significantly ($p < 0.05$) are marked in bold

Grbeša et al., 2023). The identification of municipalities such as Beočin and Irig as micro-hotspots suggests the presence of favorable ecological niches supporting *Myodes glareolus* or *Apodemus* species, the primary reservoirs of PUUV and DOBV (Olsson et al., 2010; Avšič Županc et al., 2014).

The gender and age distribution observed in AP of Vojvodina is consistent with well-established epidemiological patterns. Higher incidence among males and adults aged 20–39 years has been consistently reported across EU/EEA countries and is commonly attributed to socio-behavioral factors and increased occupational or recreational exposure to rodent habitats (ECDC, 2025). A similar gender- and age-specific pattern has been described in a study of HFRS covering five Western Balkan countries (Bosnia and Herzegovina, Croatia, Montenegro, North Macedonia and Serbia) (Štrbac et al., 2022). Agricultural workers, including tractor drivers, mechanics, field and livestock farmers, as well as forestry workers, are particularly exposed to infection during rural outbreaks (Tkachenko et al., 2025). Low incidence among children is also typical and may reflect reduced exposure or underdiagnosis of mild or subclinical infections (Olsson et al., 2010; ECDC, 2025; Tkachenko et al., 2025).

Seasonality was clearly defined, with a dominant peak in late spring and early summer. During the 2019–2023 period in Europe, HFRS incidence peaked in July and August. A previously published study conducted in five Western Balkan countries similarly reported the highest average incidence during the summer months over a 10-year period (2006–2015) (Štrbac et al., 2022). This seasonal pattern is likely influenced by agricultural activities, rodent population dynamics, and climatic conditions (Papa et al., 2006; Štrbac et al., 2022). Notably, HFRS incidence increased markedly in 2014, coinciding with severe floods that affected several countries in southeastern Europe, including Serbia (Štrbac et al., 2022).

The analysis of climatic variables revealed no significant associations at the annual level; however, monthly data demonstrated a positive correlation between temperature and HFRS incidence and a negative correlation with relative humidity. We are aware that correlations between the number of HFRS cases and climatic variables may be compromised by underreporting; however, our findings are consistent with previous studies suggesting that warmer conditions may enhance rodent activity and human–rodent interactions, while higher humidity may reduce viral aerosol stability (Klempa, 2009). A systematic review screening 434 studies, with the majority conducted in central Europe (Belgium, France and Germany), reported a strong positive association between temperature and HFRS incidence in central Europe, while evidence for northern Europe remains insufficient. Findings related to precipitation were inconsistent across studies (Diaz, 2015). Our analysis did not reveal a statistically significant relationship between the number of cases and average monthly rainfall in AP of Vojvodina during the study period. Interestingly, the highest number of cases was recorded in 2014, a year marked by major floods caused by heavy rainfall. This apparent discrepancy suggests that, although monthly

rainfall alone does not consistently predict HFRS risk, extreme weather events such as floods can lead to rodent overpopulation and alter environmental conditions in a way that increases human exposure to hantavirus. Similar observations have been reported in other countries (Diaz, 2015), where rare and extreme climatic events have been associated with local increases in HFRS cases. Therefore, while our correlation analysis captures long-term trends, it may not fully reflect the impact of episodic climate extremes on disease incidence. Overall, climatic influences on HFRS transmission are complex, often mediated by delayed effects on rodent population dynamics, and may not be fully captured by simple correlation models (Klempa, 2009; Roda Gracia et al., 2015; Tian and Stenseth, 2019).

Mortality from HFRS in AP of Vojvodina remained very low, with only two deaths recorded over the 32-year study period. This finding is consistent with the predominance of PUUV in AP of Vojvodina (Štrbac et al., 2022), which is associated with mild to moderate clinical disease and low case fatality rates across much of the Balkan region (Papa et al., 2006; Stamenković et al, 2015). Nevertheless, occasional importation of severe cases from DOBV-endemic areas, along with sporadic circulation of more virulent DOBV lineages, has been documented in Serbia (Stanojevic et al., 2019), underscoring the need for continued epidemiological surveillance and laboratory characterization.

This study has several strengths, including its long duration, population-based design, and integration of demographic, spatial, and climatic data. However, it also has several limitations that should be considered when interpreting the findings. First, the analysis relied on routine surveillance data collected over more than three decades, during which diagnostic practices and reporting procedures evolved. Second, the surveillance system captures only clinically recognized and laboratory-confirmed cases. Mild, subclinical, or atypical infections are therefore likely underreported, resulting in underestimation of the true population incidence of HFRS. Third, information on key epidemiological variables—such as occupation, rodent exposure, recreational activities, or precise locations of exposure—is not systematically collected. This limits the interpretation of interannual variability and the identification of specific risk factors underlying the higher incidence observed in certain districts (e.g., South Bačka and Srem). In addition, data on circulating hantavirus species (e.g., PUUV versus DOBV) were unavailable, precluding assessment of genotype-specific differences in disease severity or geographic distribution. Integrating molecular typing into routine surveillance systems could substantially enhance the understanding of transmission dynamics and enable more detailed epidemiological analyses. Fourth, environmental and ecological drivers of HFRS transmission could not be comprehensively assessed. Longitudinal data on rodent population dynamics, forest cover, land use change, or agricultural activity were not available, limiting the ability to link incidence trends to ecological fluctuations. Fifth, despite the long observation period, the absolute number of HFRS cases was relatively small in several years, reducing statistical

power for analyses of mortality patterns and long-term trends. Underdiagnosis or misclassification — particularly in earlier years or in rural areas with limited access to laboratory diagnostics — cannot be excluded. Finally, the absence of seroprevalence data in the general population prevents estimation of cumulative exposure and the proportion of inapparent infections. Consequently, interpretation of temporal trends relies solely on clinically detected cases, which may not fully reflect underlying transmission dynamics. Finally, a potential limitation of spatial analyses is that cases are presented according to place of residence, while the actual location of exposure may differ, as illustrated by the imported case observed in this study.

Despite these limitations, the study provides the most comprehensive overview to date of long-term HFRS epidemiology in AP of Vojvodina and identifies important temporal and spatial patterns that are relevant for public health planning and risk mitigation. Overall, the findings indicate endemic but low-intensity circulation of hantaviruses in AP of Vojvodina, with marked spatial clustering and clear demographic and seasonal patterns. Future studies incorporating time-lag models may provide a more accurate assessment of delayed climatic effects on human infection.

CONCLUSION

HFRS remains a rare but endemic zoonotic infection in AP of Vojvodina, characterized by pronounced spatial clustering, distinct demographic differences, and consistent seasonal trends. Although the overall incidence remained low over the three decades studied, distinct hotspots in certain districts, together with the predominance of cases among younger and middle-aged males, suggest ongoing localized transmission linked to specific environmental and behavioral exposures. The consistent seasonal peak in late spring and early summer highlights the role of rodent-related activities and environmental conditions in shaping transmission dynamics.

Despite substantial interannual variability, mortality remained very low. The absence of significant long-term associations between incidence and major climatic parameters at the annual level suggests that ecological and behavioral factors beyond broad meteorological trends more strongly modulate transmission in this setting. However, the significant monthly correlation with temperature and inverse correlation with humidity highlight the value of finer-resolution environmental data for understanding short-term transmission patterns.

These findings emphasize the need to strengthen targeted risk communication in high-incidence districts, enhance early clinical recognition in primary care, and improve diagnostic coverage to reduce under-ascertainment. Integrating epidemiological surveillance with rodent monitoring and environmental data would substantially improve predictive capacity and allow more timely public health

responses. Given the observed geographic heterogeneity, sustained surveillance and periodic risk reassessments remain essential for preventing future outbreaks and mitigating the burden of HFRS in the AP of Vojvodina.

Author's contribution:

MK and MR contributed to the conception and design of the article; MK and TP were involved in data collection; MK and TP drafted the manuscript; MR coordinated the work and revised the manuscript; MK and SM prepared the final draft; MR and TP performed statistical analysis, SM and MŠ contributed to results analysis and the way of results presentation. All authors have read and approved the final manuscript.

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Ethical statement

This study was conducted in accordance with the principles of the Declaration of Helsinki and applicable national regulations. The study was based on routinely collected surveillance data obtained from the Institute of Public Health of Vojvodina. As the study involved secondary analysis of anonymized and aggregated data with no possibility of identifying individual participants, formal ethical approval and informed consent were not required according to national legislation. Data confidentiality and privacy were strictly maintained throughout the study.

Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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EPIDEMIOLOGIJA HEMORAGIJSKE GROZNICE SA BUBREŽNIM SINDROMOM U AUTONOMNOJ POKRAJINI VOJVODINI, SRBIJA, 1992–2023

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Kratak sadržaj

Iako je hemoragijska groznica sa bubrežnim sindromom u Srbiji prisutna endemski, višedecenijski epidemiološki podaci na subnacionalnom nivou su ograničeni. Ova studija je imala za cilj da opiše hronološke, topografske, sezonske i demografske obrasce ove bolesti u Autonomnoj Pokrajini Vojvodini, severna Srbija, tokom perioda od 32 godine (1992–2023), kao i da utvrdi potencijalnu povezanost između pojave ove bolesti i odabranih klimatskih parametara. Sprovedena je retrospektivna deskriptivna studija zasnovana na podacima rutinskog nadzora. Izračunate su godišnje opšte, kao i uzrasno- i polno-specifične stope incidencije. Prostorno grupisanje slučajeva procenjeno je na nivou okruga i opština. Korelacija između incidencije ove bolesti i temperature, količine padavina, relativne vlažnosti vazduha i broja kišnih dana analizirana je korišćenjem *Spearman*-ovog koeficijenta rang-korelacije. Tokom posmatranog perioda prijavljena su ukupno 82 slučaja (prosečna godišnja stopa incidencije: 0,13/100.000; 95% CI: 0,10-0,16) i dva smrtna ishoda. Bolest je registrovana sporadično, uz izraženu godišnju varijabilnost. Najviše stope incidencije zabeležene su 2012. (0,41/100.000) i 2014. godine (0,52/100.000). Prisutna je izrazita topografska heterogenost oboljenja, sa najvišim vrednostima u Južnobačkom i Sremskom okrugu i u nekoliko opština (Beočin, Irig, Sremski Karlovci). Muškarci uzrasta 30–39 godina imali su najvišu stopu incidencije (0,56/100.000), dok su slučajevi bili retki među decom. Izražen sezonski vrhunac obolevanja zabeležen je u junu, kada je registrovano 24,4% svih slučajeva. Nisu utvrđene statistički značajne povezanosti između godišnje incidencije i klimatskih parametara. Međutim, dokazana je pozitivna korelacija između broja slučajeva po mesecima i temperature ($\rho = 0,606$; $p = 0,037$) i negativna korelacija sa relativnom vlažnošću vazduha ($\rho = -0,621$; $p = 0,031$). Naši rezultati ukazuju da je hemoragijska groznica sa bubrežnim sindromom endemski prisutna zoonoza sa niskom incidencijom u AP Vojvodini. Karakterišu je prostorna žarišta, jasno definisani demografski obrasci i stabilna sezonska pojava u ranom letnjem periodu. Pojačan integrisani nadzor, dostupnost dijagnostike i uključivanje ekoloških i klimatskih podataka neophodni su za bolje razumevanje rizika na lokalnom nivou i podršku pravovremenim javnozdravstvenim intervencijama.

Ključne reči: Hantavirus, zoonoze, epidemiologija, morbiditet, geografska distribucija, glodari, klimatski faktori, godišnja doba

Original research article

ANTIBACTERIAL AND ANTIBIOFILM ACTIVITIES OF ECO-FRIENDLY HYDROGEL *MORINGA OLEIFERA*– CHITOSAN FORMULATION AGAINST *ESCHERICHIA COLI* AND *STAPHYLOCOCCUS AUREUS*

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Abstract

Escherichia coli and *Staphylococcus aureus* represent the dominant etiological agents of pyometra in cattle. Their strong biofilm-forming ability critically contributes to therapeutic failure and escalating antibacterial resistance. This study aimed to develop and evaluate an eco-friendly chitosan hydrogel formulation containing *Moringa oleifera* leaf extract as an alternative antibacterial and antibiofilm agent. The ethanolic extract of *M. oleifera* was characterized using Gas Chromatography–Mass Spectrometry (GC–MS). Hydrogel formulations (10,

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20, 30, and 40%) were prepared and assessed for bacterial growth inhibition and biofilm degradation over 24, 48, and 72 h using microplate spectrophotometry. The formulations exhibited clear concentration- and time-dependent activities. The 10% formulation produced the strongest inhibition against *E. coli*, whereas *S. aureus* responded most effectively to the 30% formulation, which maintained stable inhibitory effects across all incubation periods. Biofilm assays revealed transient suppression in *E. coli*, with maximal degradation at 48 h, while *S. aureus* showed more sustained inhibition, particularly with the 30% formulation. Correlation analysis demonstrated a stronger alignment between growth inhibition and biofilm reduction in *E. coli*, indicating more synchronized antibacterial and antibiofilm effects ($p < 0.05$). The *M. oleifera*–chitosan hydrogel formulation exhibited promising dual activity against planktonic and biofilm-associated bacteria, highlighting its potential as an eco-friendly therapeutic candidate against bovine uterine infections and reducing reliance on conventional antibiotics.

Keywords: *Moringa oleifera*, chitosan, *Escherichia coli*, *Staphylococcus aureus*, biofilms

INTRODUCTION

Pyometra is a serious uterine disorder in cattle, characterized by purulent accumulation resulting from secondary bacterial infection, along with hormonal imbalance and suboptimal reproductive performance (Amin et al., 2021). This condition contributes to delayed estrus, prolonged calving intervals, reduced fertility, and increased systemic risks, ultimately causing substantial economic losses (Kasimanickam et al., 2025).

In Aceh cattle, various opportunistic bacteria, including *E. coli*, *Corynebacterium* sp., *Enterobacter* sp., and *Pseudomonas* sp. are frequently isolated from the uterus (Thasmi et al., 2021). Among these, *E. coli* and *S. aureus* are commonly reported as associated with uterine infections, along with other pathogens (Talib et al., 2023). The ability of these bacteria to adhere to host tissues and form biofilms contributes to their persistence. Biofilm development enhances bacterial resistance, rendering infections more difficult to treat.

Conventional therapies, which include antibiotics, prostaglandins, and uterine irrigation, are increasingly limited due to rising antibacterial resistance driven by alterations in bacterial targets, enzymatic degradation, and biofilm-mediated protection (Iancu et al., 2025; Mekibib et al., 2024). These challenges underscore the need for alternative treatments that can effectively target both planktonic and biofilm-associated bacteria. Herbal-based approaches have gained attention, and *M. oleifera*, rich in antibacterial and antioxidant compounds, shows promising potential for managing uterine infections (Armansyah et al., 2023; Rosmaidar et al., 2021).

Several medicinal plants have been reported to exhibit antibacterial, anti-inflammatory, and immunomodulatory properties relevant to infection control

(Japheth et al., 2021). Among these, *M. oleifera* has attracted considerable attention due to its rich content of bioactive compounds, including flavonoids, alkaloids, and saponins, which contribute to its antibacterial and antioxidant activities (Armansyah et al., 2023). These compounds are known to inhibit bacterial growth through mechanisms such as disruption of cell membranes and interference with bacterial metabolic processes, highlighting the potential of *Moringa oleifera* extract as a natural antibacterial agent (Wen et al., 2022).

In this study, an eco-friendly hydrogel combining *M. oleifera* extract and chitosan was developed. Chitosan, a biodegradable and biocompatible biopolymer, exhibits intrinsic antibacterial activity due to its polycationic nature, which enables the interaction with bacterial cell membranes and inhibits biofilm formation (Ardean et al., 2021). The incorporated *M. oleifera* is expected to enhance the responsiveness and functionality of the chitosan matrix, improve its physicochemical and biological properties, including strength, biocompatibility, and biodegradability (Gani et al., 2023). This combination may also facilitate controlled release of active compounds, leading to enhanced antibacterial and antibiofilm effects (Jadhav et al., 2022; Jaferník et al., 2023).

Sustainable nanotechnology through green synthesis offers a novel strategy to enhance the stability, delivery, and bioactivity of plant-derived compounds (Jadhav et al., 2022). Chitosan nanoparticles possess inherent antibacterial properties, are biocompatible, effectively penetrate cell membranes, and have controlled release behavior (Herdiana et al., 2021; Jayathilaka et al., 2022). The antibiofilm activity of the *M. oleifera*–chitosan hydrogel is supposed to involve multiple complementary mechanisms, including disruption of bacterial cell membranes due to chitosan's polycationic interactions, inhibition of initial bacterial adhesion, and interference with biofilm matrix integrity. In addition, bioactive compounds from *M. oleifera* may penetrate the extracellular polymeric substances (EPS) of biofilms, interfere with quorum sensing, and enhance bacterial susceptibility through sustained release from the hydrogel system (Menon et al., 2026; Suhartono et al., 2019). However, an eco-friendly *M. oleifera* extract combined with a chitosan-based hydrogel formulation targeting *E. coli* and *S. aureus* has not yet been thoroughly investigated. Therefore, this study aimed to develop and evaluate an eco-friendly *M. oleifera*–chitosan hydrogel as an antibacterial and antibiofilm agent utilizing an *in vitro* experimental approach.

MATERIAL AND METHODS

Experimental design

This study was a randomized laboratory *in vitro* investigation. Ethanolic extracts of *M. oleifera* leaves were first subjected to phytochemical profiling using gas chromatography–mass spectrometry (GC–MS). The extracts were subsequently

formulated into chitosan-based hydrogels at different concentrations (10%, 20%, 30%, and 40%). Next, each was evaluated for its antibacterial and antibiofilm activities against the tested bacterial strains.

Preparation, extraction, and GC-MS analysis of M. oleifera

One kilogram of *M. oleifera* leaves was washed, air-dried for two days, and oven-dried at 50°C for 48 h before being powdered and stored airtight. The powder was macerated in 96% ethanol (100 mL), with solvent replaced every three days, and the filtrate was concentrated in a rotary evaporator at 50°C and 75 mmHg (BUCHI R-300) (Permadi et al., 2018). GC–MS analysis was conducted using a Shimadzu QP2010PLUS equipped with a 30 m × 0.25 mm polymethyl silicone column, employing an oven ramp of 80–200°C in 5 min, held for 20 min. FID and injection temperatures were 300°C and 220°C; nitrogen served as the carrier gas at 1 mL/min (116.9 kPa) with 50 mL/min column flow (Thasmi et al., 2024).

Preparation of chitosan–Moringa oleifera hydrogel formulation

The chitosan–*M. oleifera* hydrogel formulation was prepared by dissolving 1% chitosan in distilled water according to the designated volume for each treatment group (10–40%). Under continuous magnetic stirring, 70% ethanol and 2% glycerol were added sequentially, with each component mixed for 30 minutes to ensure uniform dispersion. The *M. oleifera* extract was then incorporated into the chitosan solution and stirred until a homogeneous hydrogel matrix was formed. All formulations were allowed to stand at room temperature to ensure complete gel formation and stabilization, as summarized in Table 1.

Table 1. Composition of eco-friendly *Moringa oleifera*–Chitosan hydrogel formulations

Ingredients	Hydrogel concentration			
	10%	20%	30%	40%
Chitosan 1% (ml)	25	25	25	23
Ethanol 70% (ml)	2	2	2	2
Glycerol 2% (ml)	5	5	5	5
<i>M. oleifera</i> extract (ml)	5	10	15	20
Distilled water (q.s.) (ml)	13	8	3	0

Preparation of Staphylococcus aureus and Escherichia coli

Staphylococcus aureus ATCC 25923 and *Escherichia coli* ATCC 25922 (Culti-Loops™, Thermo Scientific, Lenexa, USA) were used in this study. A single colony

of *S. aureus* and *E. coli* was inoculated separately into 5 mL of nutrient broth and incubated at 37°C for 24 h. Following incubation, the bacterial suspensions were adjusted to the 0.5 McFarland standard, equivalent to approximately 1.5×10^8 CFU/mL (Pitjamit et al., 2024).

Inhibition assessment of E. coli and S. aureus

The antibacterial activity of chitosan-*M. oleifera* hydrogel against *E. coli* and *S. aureus* was evaluated using a modified broth microdilution method according to El-Sherbiny et al. (2024). Briefly, 50 µL of tryptic soy broth (TSB) was dispensed into each well of a sterile 96-well microplate, followed by the addition of test samples at concentrations of 10%, 20%, 30%, and 40%, with penicillin-streptomycin as positive controls and distilled water as the negative one. A standardized bacterial suspension (0.5 McFarland) was diluted to obtain a final inoculum of $\sim 5 \times 10^5$ CFU/mL, and 100 µL was added to each well (final volume 200 µL). The plates were incubated at 37°C for 24, 48, and 72 h. Bacterial growth was quantified spectrophotometrically at 620 nm using a microplate reader (Bio-Rad, USA), where optical density (OD) values of 0.08–0.10 correspond to the 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/mL). The inhibitory activity was defined as the lowest concentration that did not lead to a significant increase in turbidity compared to the negative control.

Biofilm formation assay

Inhibition of biofilm formation was evaluated using a modified crystal violet assay as described by Adeyemo et al. (2022). Bacterial suspensions of *E. coli* and *S. aureus* were adjusted to the 0.5 McFarland standard and further diluted to approximately 1×10^6 CFU/mL in TSB. A hundred µL of the bacterial inoculum was co-incubated with 100 µL of chitosan-*M. oleifera* hydrogel at concentrations of 10%, 20%, 30%, and 40% in 96-well microplates at 37°C for 24, 48, and 72 h, with penicillin-streptomycin and distilled water as the positive and negative controls, respectively. Following incubation, the wells were gently washed three times with distilled water to remove non-adherent cells, air-dried, and stained with 0.1% crystal violet for 15 min. Excess stain was removed, and the wells were rinsed and dried. The bound dye was subsequently solubilized with 96% ethanol, and absorbance was measured at 595 nm with a microplate reader (Bio-Rad, USA). All experiments were performed in duplicate with two independent replications.

Statistical analysis

Data were analyzed using IBM SPSS Statistics version 24.0 (IBM Corp., Armonk, NY, USA). Data on growth inhibition and biofilm formation were analyzed using

the Kruskal–Wallis test. The relationship between growth inhibition and biofilm formation was evaluated based on the frequency distribution at each *M. oleifera* concentration. The correlation analysis was performed using Pearson's correlation coefficient. Statistical significance was set at $p < 0.05$.

RESULTS

Figure 1 illustrates the GC–MS chromatogram of the *M. oleifera* leaf extract, showing the overall distribution of its volatile and semi-volatile constituents. GC–MS analysis identified 45 compounds in the extract. The chromatographic profile is characterized by a series of small early peaks followed by a cluster of more prominent peaks between 22 and 28 minutes, indicating that the extract is largely composed of moderately non-polar compounds such as fatty acids, fatty alcohols, and ester derivatives. Consistent with this pattern, Table 2 summarizes the major compounds identified within this retention window, most of which are lipophilic metabolites known for their antibacterial, antifungal, or anti-inflammatory activities. The GC–MS profile of *M. oleifera* leaf extract is dominated by *cis*-9-tetradecen-1-ol (RT 23.53 min, 23.74% of TIC), with several other medium-abundance fatty acids and esters (including octadecanoic acid, glyceryl oleate, and a hexadecanoic acid ester) and minor amounts of phytol.

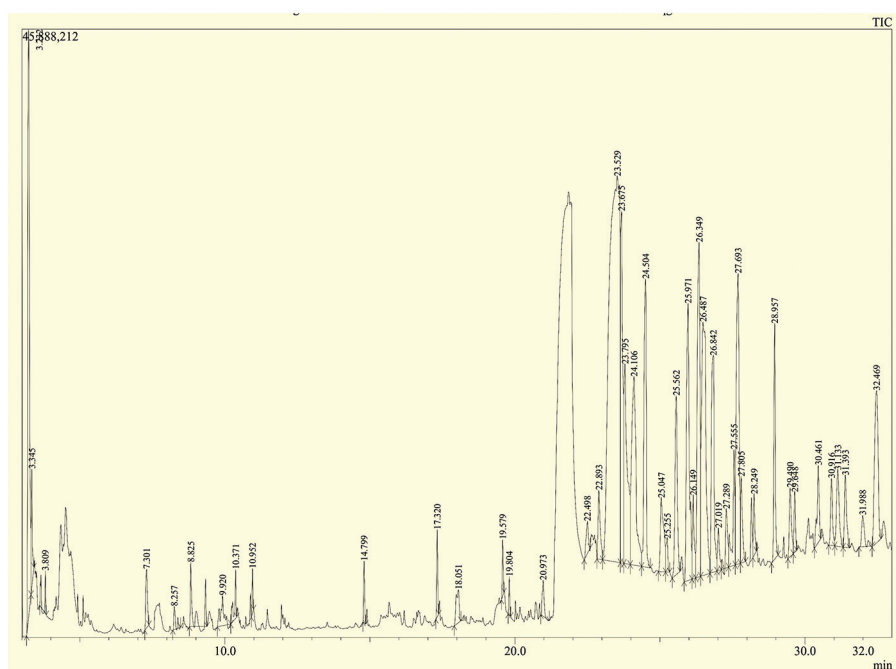


Figure 1. GC–MS chromatograms of *M. oleifera* leaf extract.

Table 2. Chemical composition (major compounds) of *M. oleifera* extract

R. Time	Compound	Chemical formula	Area%	Bioactivity	Reference
22.893	Phytol	C ₂₀ H ₄₀ O	00.87	Antibacterial	(Almeida-Bezerra et al., 2024)
23.529	cis-9-Tetradecen-1-ol	C ₁₄ H ₂₈ O	23.74	Antifungal, and antibacterial	(Mugayi & Mukanganyama, 2024)
23.675	Octadecanoic acid	C ₁₈ H ₃₆ O ₂	40.65	Antibacterial	(Casillas-Vargas et al., 2021) (Ghareeb et al., 2022); (Thasmi et al., 2024)
25.971	9-Octadecenoic acid, 1,2,3-propanetriyl ester	C ₅₇ H ₁₀₄ O ₆	40.65	Antibacterial, antiviral, anticancer, anti-spasmodic, and immune modulators	(Al-Marzoqi et al., 2016); (Ojo et al., 2021); (Najah & Alshawish, 2023)
26.349	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	C ₁₉ H ₃₈ O ₄	50.31	Antibacterials, antifungal, anti-Alzheimer's	(Subash & Srinivasa Rao, 2021); (Idris et al., 2022)
26.487	Bicyclo[4.2.0]oct-1-ene, exo-7-(1-cyclohexen-1-yl)-	C ₁₄ H ₂₀	60.58	Antibacterial	(Sundarajan & Abdurahman, 2019)
27.693	Propylene glycol monooleate	C ₂₁ H ₄₀ O ₃	40.97	Anti-inflammatory, antiviral, and antioxidant	(Qadir et al., 2018)

Table 2 presents the GC–MS analysis of the *M. oleifera* extract, which identifies several bioactive compounds with potential antibacterial properties. The GC–MS profile shows that cis-9-tetradecen-1-ol is the predominant component, contributing substantially to the extract’s antibacterial and antifungal effects, while other compounds—such as hexadecanoic acid derivatives, octadecanoic acid, bicyclo[4.2.0]oct-1-ene, propylene glycol monooleate, and 9-octadecenoic acid propanetriyl ester—provide additional antibacterial, antioxidant, anti-inflammatory, and immunomodulatory activities. Although present in smaller amounts, phytol may further enhance the extract’s antibacterial activity, suggesting that its overall bioactivity likely results from the synergistic action of these metabolites.

Chitosan–*M. oleifera* hydrogel produced significant ($p < 0.05$) antibacterial effects against both *E. coli* and *S. aureus* at 24, 48, and 72 h (Table 3). In *E. coli*,

the 10% concentration consistently yielded the lowest OD values, indicating the strongest and most stable inhibition compared with higher concentrations. By contrast, *S. aureus* was most sensitive to 30%, which was reflected in the lowest OD readings after all incubation periods, while the 10%, 20%, and 40% groups were less suppressed. These findings demonstrate that the optimal inhibitory concentration of the hydrogel depends on the bacterial species: 10% was most effective against *E. coli*, and 30% produced the highest inhibition of *S. aureus*.

Figure 2 demonstrates that chitosan-*M. oleifera* hydrogel produced concentration-dependent antibacterial effects against *E. coli* (Figure 2A) and *S. aureus* (Figure 2B) across all incubation periods. The 10% formulation showed the strongest and most sustained inhibition of *E. coli*, consistently outperforming the positive control at 24 and 48 h and remaining comparable at 72 h. By contrast, *S. aureus* displayed a different response pattern: the 30% and 40% concentrations yielded the highest inhibition at 24 h, though only the 30% treatment maintained relatively stable activity at later time points. The 10% and 40% groups showed marked reductions over time, and the 20% concentration exhibited minimal inhibition throughout. In summary, the findings indicate that the 10% formulation is optimal for *E. coli*, whereas the 30% concentration provides the most durable inhibition of *S. aureus*.

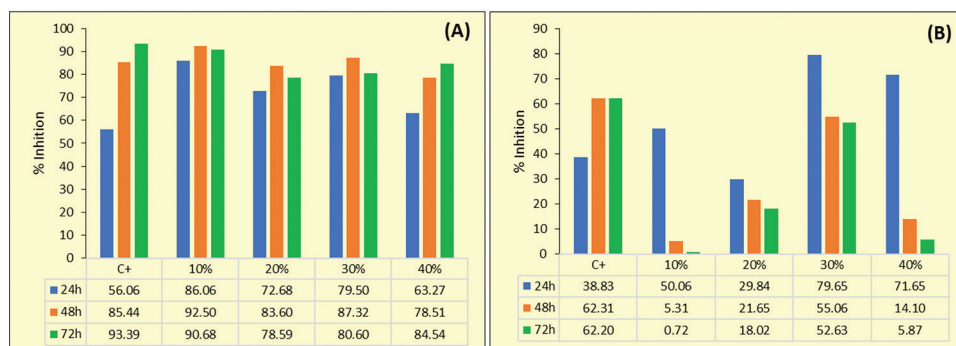


Figure 2. Growth inhibition (%) of *E. coli* (A) and *S. aureus* (B) after treatment by chitosan-*M. oleifera* hydrogel at 24, 48, and 72h

The antibiofilm activity of chitosan-*M. oleifera* hydrogel in *E. coli* and *S. aureus* after treatment is shown in Table 4. In *E. coli*, all concentrations showed limited inhibition at 24 h, a clearer reduction at 48 h, and renewed biofilm growth by 72 h, with the 40% formulation performing best. *S. aureus*, however, exhibited a clearer dose-dependent pattern: the 30% formulation consistently produced the lowest OD values and the most stable inhibition. The 40% treatment weakened over time, while the 10% and 20% concentrations remained ineffective. Significant differences appeared at 24 and 48 h only. Overall, antibiofilm effects were short-lived in *E. coli* but more sustained in *S. aureus*, especially with the 30% formulation.

Table 3. Optical density (OD) of *E. coli* and *S. aureus* inhibition after treatment by chitosan-*M. oleifera* hydrogel at 24, 48, and 72 h

Treatment Groups	n	OD (Mean ± SD)							
		<i>E. coli</i>			<i>p-value</i>	<i>S. aureus</i>			<i>p-value</i>
		24h	48h	72h		24h	48h	72h	
C+	3	1.00±0.03	0.31±0.32	0.13±0.01		0.48±0.05	0.12±0.00	0.12±0.00	
10%	3	0.32±0.03	0.16±0.01	0.18±0.02		0.39±0.07	0.31±0.11	0.32±0.11	
20%	3	0.62±0.02	0.35±0.07	0.41±0.07	0.038	0.55±0.06	0.26±0.02	0.27±0.02	0.009
30%	3	0.47±0.13	0.27±0.04	0.37±0.03		0.16±0.05	0.15±0.02	0.15±0.02	
40%	3	0.84±0.21	0.45±0.07	0.29±0.16		0.22±0.03	0.28±0.04	0.30±0.04	
p		0.007	0.038	0.015		0.009	0.033	0.03	

Statistical test: Kruskal–Wallis ($p < 0.05$).

Table 4. Optical density (OD) values of *E. coli* and *S. aureus* biofilm after treatment by chitosan-*M. oleifera* hydrogel at 24, 48, and 72 h

Treatment	n	OD (Mean±SD)							
		<i>E. coli</i>			<i>p</i> -value	<i>S. aureus</i>			<i>p</i> -value
		24 h	48 h	72 h		24 h	48 h	72 h	
C+	3	0.10±0.00	0.01±0.00	0.10±0.03		0.48±0.05	0.12±0.00	0.12±0.00	
10%	3	0.59±0.04	0.03±0.01	0.20±0.17		0.39±0.07	0.31±0.11	0.32±0.11	
20%	3	0.59±0.06	0.04±0.05	0.64±0.11	0.064	0.55±0.06	0.26±0.02	0.27±0.02	0.019
30%	3	0.50±0.32	0.02±0.01	0.41±0.47		0.16±0.05	0.15±0.02	0.15±0.02	
40%	3	0.36±0.11	0.01±0.01	0.39±0.19		0.22±0.03	0.28±0.04	0.30±0.04	
p		0.064	0.087	0.074		0.019	0.015	0.085	

Statistical test: Kruskal–Wallis ($p < 0.05$)

Figure 3 illustrates the inhibition of biofilm formation of chitosan-*M. oleifera* hydrogel against *E. coli* and *S. aureus*. In *E. coli*, degradation was minimal at 24 h but peaked at 48 h, especially in the 30% and 40% groups, before declining at 72 h, with moderate residual activity in the 10%, 30%, and 40% treatments. *S. aureus* showed a similar trend: early degradation appeared in the 10% and 20% groups, maximal removal occurred at 48 h with the 30% and 40% formulations, and activity decreased by 72 h, although the 40% and 10% groups showed higher remaining effects. Overall, biofilm degradation was strongest at 48 h, with the 30% and 40% formulations demonstrating the most consistent performance against both species.

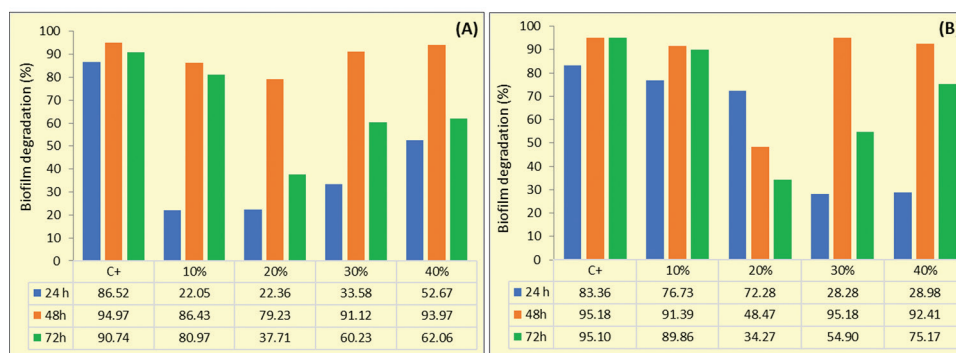


Figure 3. Biofilm degradation (%) of *E. coli* (A) and *S. aureus* (B) after treatment by chitosan-*M. oleifera* hydrogel at 24, 48, and 72 h

Figures A, B, and C represent *E. coli* at 24 h, 48 h, and 72 h, respectively, and D, E, and F represent *S. aureus* at 24 h, 48 h, and 72 h, respectively. At 24 h, both bacteria showed varying patterns between inhibition activity and biofilm percentage across concentrations. At 48 h, a closer relationship between inhibition and biofilm values was detected at specific concentrations (indicated by boxed areas). At 72 h, certain concentrations demonstrated a stronger inverse relationship, suggesting improved antibiofilm performance at these points. The boxed areas highlight concentrations with the closest inhibition-biofilm relationship.

The relationship between inhibition and biofilm formation in *E. coli* and *S. aureus* becomes more coordinated over time (Figure 4). At 24 h, correlations were weak across concentrations, but by 48 h, a clearer pattern emerged—especially at 30%, where both species showed more aligned inhibition and biofilm responses. At 72 h, *E. coli* displayed a stronger inverse relationship, particularly at 10%, while *S. aureus* remained less consistent. However, the 40% concentration produced the most stable and closely aligned patterns against both bacteria species. These trends indicate that the formulation achieved its most synchronized antibacterial and antibiofilm activity between 48 h and 72 h.

Table 5 shows that *E. coli* exhibits more consistent relationships between inhibition and biofilm responses than *S. aureus*. In *E. coli*, strong correlations across incubation periods—particularly at 48–72 h—indicate stable antibacterial activity that increasingly aligns with biofilm reduction. By contrast, *S. aureus* displays weaker and irregular correlations, with significant links appearing only at certain time points, although a notable biofilm correlation at 24–48 h suggests a more coordinated early response. Overall, the nanoencapsulation achieves more synchronized antibacterial and antibiofilm effects in *E. coli* than in *S. aureus*.



Figure 4. Relationship between growth inhibition and biofilm formation of *E. coli* and *S. aureus* treated with chitosan-*M. oleifera* hydrogel at different incubation times.

Table 5. Correlation between inhibition and biofilm formation of *E. coli* and *S. aureus* treated by chitosan-*M. oleifera* hydrogel

		<i>E. coli</i>						<i>S. aureus</i>					
		Inhibition			Biofilm			Inhibition			Biofilm		
		24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h
Inhibition	24 h	<i>r</i>											
			0.940**	0.898**	0.226	0.516*	0.555*			0.165	-0.101	0.443	0.497*
	48 h	<i>p</i>	0.000	0.000	0.367	0.028	0.017		0.193	0.514	0.691	0.066	0.036
		<i>r</i>											
			0.940**	0.962**	0.436	0.600**	0.587*	0.322		0.483*	0.190	0.211	0.207
	72 h	<i>p</i>	0.000	0.000	0.071	0.008	0.010	0.193		0.042	0.449	0.401	0.411
Biofilm	24 h	<i>r</i>											
			0.898**		0.497*	0.565*	0.674**	0.165	0.483*		0.365	0.550*	0.043
		<i>p</i>	0.000	0.000	0.036	0.015	0.002	0.514	0.042		0.137	0.018	0.867
		<i>r</i>											
			0.226	0.436		0.166	0.265	-0.101	0.190	0.365		0.634**	0.148
	72 h	<i>p</i>	0.367	0.071		0.511	0.288	0.691	0.449	0.137		0.005	0.557
Biofilm	48 h	<i>r</i>											
			0.516*	0.565*	0.166		0.005	0.443	0.211	0.550*	0.634**		0.104
		<i>p</i>	0.028	0.008	0.511		0.985	0.066	0.401	0.018	0.005		0.682
Biofilm	72 h	<i>r</i>											
			0.555*	0.587*	0.265	0.005		0.497*	0.207	0.043	0.148	0.104	
		<i>p</i>	0.017	0.010	0.002	0.288		0.036	0.411	0.867	0.557	0.682	

Significant correlations are indicated by * ($p < 0.05$) and ** ($p < 0.01$)

DISCUSSION

Figure 1 and Table 2 show that *M. oleifera* extract contains predominantly lipophilic compounds, particularly cis-9-tetradecen-1-ol, with additional fatty acids, esters, and their derivatives, which are widely reported to exhibit antibacterial and antioxidant properties (Casillas-Vargas et al., 2021; Mugayi & Mukanganyama, 2024). Minor metabolites such as phytol may further enhance antibacterial action through terpenoid-mediated stress modulation (Almeida-Bezerra et al., 2024). Together, these compounds support a synergistic, multi-target mechanism consistent with lipid-based agents that disrupt membranes, metabolism, and biofilm formation processes (Pérez-Flores et al., 2025). These bioactive components may contribute to membrane perturbation and metabolic interference in bacterial cells. Previous studies have also highlighted that *M. oleifera* contains diverse functional compounds, including polysaccharides and fatty acid derivatives, which are associated with antibacterial and biofunctional activities (Dzuovor et al., 2022; Yang et al., 2022).

Nanoencapsulation enhanced antibacterial activity, potentially by improving the stability and dispersion of bioactive compounds. In *E. coli*, the 10% formulation showed the most consistent inhibitory effect (Table 3 and Figure 2A), whereas in *S. aureus*, the 30% formulation demonstrated more sustained activity (Figure 2B). These differences may be related to variations in bacterial cell wall structure and permeability between Gram-negative and Gram-positive bacteria (Mulat et al., 2025). A reduction in activity at higher concentrations may be associated with aggregation effects that limit effective interaction with bacterial cells.

The antibiofilm assay in this study specifically evaluated inhibition of biofilm formation rather than disruption of preformed biofilms. The results demonstrated time-dependent and species-specific responses. In *E. coli*, inhibition peaked at 48 h before declining at 72 h (Table 4 and Figure 3A), suggesting an effect on early-stage biofilm development. This observation is consistent with previous findings indicating that *Moringa*-based materials may inhibit the initial attachment of *E. coli* to abiotic surfaces (Bessalah et al., 2024). Similarly, plant-derived compounds have been reported to interfere with bacterial adhesion and early biofilm formation processes (Liu et al., 2019; Neto et al., 2017).

In *S. aureus*, the 30% formulation showed relatively stable inhibition at 48 h (Figure 3B), which may reflect interference with early biofilm matrix formation. Previous studies have reported that bioactive compounds from *M. oleifera*, including fatty acids such as behenic acid, can inhibit biofilm formation in *S. aureus* (Kim & Kim, 2025). However, these effects should be interpreted within the context of early-stage biofilm inhibition, as mature biofilm disruption was not assessed in the present study.

The current results are also consistent with previous findings that chitosan effectively inhibits biofilm formation by *Streptococcus mutans*, *Streptococcus*

sanguinis, and *Candida albicans* (Djunaidy & Putri, 2020; Garcia et al., 2023; Haniastuti et al., 2023). In this study, the combination of chitosan and *M. oleifera* extract suggested a potential synergistic effect: chitosan offered physical antibacterial properties, while *Moringa* bioactive interfered with quorum sensing (Haris & Ahmad, 2024) and oxidative stress pathways (González-Romero et al., 2022).

The relationship between antibacterial activity and biofilm formation showed a clear pattern in *E. coli*, where stronger growth inhibition was associated with reduced biofilm production (Figure 4), suggesting that antibacterial effects may contribute to limiting early-stage biofilm development. By contrast, this relationship was less consistent in *S. aureus*, possibly due to differences in cell wall structure and biofilm architecture. These findings are consistent with previous reports on variability in biofilm formation and susceptibility among bacterial species (Jayathilaka et al., 2022). The observed effects may be associated with bacterial membrane perturbation and subsequent interference with early biofilm formation processes (Ghareeb et al., 2022).

Table 5 shows that *E. coli* exhibited a clearer and more consistent relationship between growth inhibition and biofilm reduction, particularly at 48–72 h, suggesting sustained antibacterial activity and its association with reduced biofilm formation. By contrast, *S. aureus* showed a weaker and less consistent relationship, which may be related to its thicker peptidoglycan layer and more complex biofilm structure (Jayathilaka et al., 2022; Mulat et al., 2025). The correlation observed at 24–48 h may reflect an effect on early-stage biofilm development before maturation.

Taken together, the findings suggest that chitosan–*M. oleifera* hydrogel exhibits antibacterial activity and inhibits early-stage biofilm formation under *in vitro* conditions, particularly at concentrations of 10–30% and at 48 h. These effects may be associated with interactions between chitosan and the bioactive compounds present in *M. oleifera*, which could influence bacterial viability and the initial adhesion process. However, the present findings are limited to *in vitro* observations and do not directly reflect clinical or therapeutic efficacy. Therefore, further studies, including mechanistic investigations and *in vivo* evaluations, are required to elucidate the potential applications and limitations of this formulation.

CONCLUSION

Chitosan–*M. oleifera* hydrogel demonstrated concentration- and time-dependent antibacterial activity: the 10% formulation showed greater inhibition against *E. coli* and the 30% formulation against *S. aureus*. The strongest bacterial inhibition and reduction in biofilm formation were observed at 48 h. These results demonstrate that the hydrogel formulation possesses antibacterial characteristics and can impede early-stage biofilm development under *in vitro* conditions. However, as this study was conducted in *in vitro* conditions, further studies are required to confirm its broader applicability.

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Authors' contribution

CNT and HH substantially contributed to the conception and design of the study, methodology development, and drafting of the initial manuscript. SR, NNN, AF, and SLA made substantial contributions to data acquisition and laboratory investigation. HZ, AS, and TNS contributed to data curation, interpretation of the results, and critical revision of the manuscript for important intellectual content. HA, FJ, and DD facilitated project administration, oversight, and fundraising efforts. All authors have read and approved the final manuscript.

Ethical statement

This study did not involve experimental research on live animals. All procedures were conducted in accordance with applicable institutional and ethical standards. ARRIVE guidelines are not applicable to this study.

Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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ANTIBAKTERIJSKA I ANTIBIOFILM AKTIVNOST EKOLOŠKI PRIHVATLJIVE HIDROGELNE FORMULACIJE MORINGA OLEIFERA–HITAZANA PROTIV *ESCHERICHIA COLI* I *STAPHYLOCOCCUS AUREUS*

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Kratak sadržaj

Escherichia coli i *Staphylococcus aureus* predstavljaju dominantne uzročnike piometre kod goveda, a njihova izražena sposobnost formiranja biofilma značajno doprinosi neuspehu terapije i eskalaciji antibakterijske rezistencije. Cilj ovog ispitivanja bio je razvoj i procena ekološki prihvatljive hidrogel formulacije hitozana koja sadrži ekstrakt lista biljke *Moringa oleifera* kao alternativnog antibakterijskog i antibiofilmskog agensa. Etanolni ekstrakt *M. oleifera* je analiziran korišćenjem GC-MS. Formulacije hidrogela (10, 20, 30 i 40%) su pripremljene i njihova sposobnost inhibicije rasta bakterija i razgradnje biofilma tokom 24, 48 i 72 h procenjena korišćenjem spektrofotometrije mikroploče. Formulacije su pokazale jasne aktivnosti zavisne od koncentracije i vremena. Koncentracija od 10% dovela je do najjače inhibicije *E. coli*, dok je *S. aureus* najbolje reagovao na formulu od 30%, koja je održala stabilne inhibitorne efekte tokom svih perioda inkubacije. Testovi biofilma su otkrili prolaznu supresiju kod *E. coli*, sa maksimalnom razgradnjom nakon 48 h, dok je inhibicija *S. aureus* bila dugotrajnija, naročito u prisustvu 30% formulacije. Korelaciona analiza pokazala je jaču usklađenost između inhibicije rasta i smanjenja biofilma kod *E. coli*, što ukazuje na sinhronizovanje antibakterijske i antibiofilmske efekte ($p < 0,05$). Hidrogel *M. oleifera*-hitozan pokazao je obećavajuću dvojaku aktivnost protiv planktonskih i bakterija koje stvaraju biofilm, čime se ističe njegov potencijal kao ekološki prihvatljivog terapijskog kandidata protiv infekcija materice kod goveda i smanjuje zavisnost od konvencionalnih antibiotika.

Ključne reči: *Moringa oleifera*, hitozan, *Escherichia coli*, *Staphylococcus aureus*, biofilmovi

Original research article

DECLINE OF MATERNAL ANTIBODIES AGAINST SWINE INFLUENZA A AND PRRS IN PIGLETS FROM DIFFERENT SOW VACCINATION PROGRAMS

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Abstract

The primary objective of this study was to evaluate the effect of sow influenza vaccination timing on the persistence and decay of maternally derived antibodies (MDAs) against swine influenza A virus (IAV-S) in piglets. PRRSV antibody dynamics were additionally assessed to provide contextual information on maternally derived antibody decline and post-vaccination seroconversion under the same herd conditions. A total of 200 piglets (20 per group at each of five time points: days 5, 28, 42, 56, and 70) were sampled in a cross-sectional design. Serum antibodies against IAV-S and PRRSV were measured using commercial ELISA assays. Piglets born to sows vaccinated against IAV-S three weeks before farrowing (Group A) exhibited higher and more consistent MDA titers compared to those from sows vaccinated four months earlier (Group B). At day 56, 75% of piglets in Group A and 60% in Group B remained seropositive for IAV-S. By day 70, seropositivity declined to approximately 40–45% in both groups. A marked decline in antibody levels was observed over time, with a significant proportion of piglets becoming seronegative by eight weeks of age. For PRRSV, antibody levels declined until day 42 and increased thereafter following piglet vaccination,

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indicating successful induction of active immunity. These results highlight the critical role of sow vaccination timing in shaping MDA kinetics and the need to align piglet vaccination strategies accordingly. Furthermore, they emphasize the necessity of integrated control approaches, beyond vaccination alone, to effectively manage PRRSV and IAV-S transmission in endemically infected herds.

Key words: swine, porcine reproductive and respiratory syndrome virus, swine influenza, maternal antibodies, vaccination

INTRODUCTION

Swine Influenza and Porcine Reproductive and Respiratory Syndrome (PRRS) are among the most significant infectious diseases in modern swine production (Saade et al., 2020). Both are viral diseases caused by RNA viruses, which are characterized by high mutation rates. As such, continuous monitoring and adaptation of control programs are essential. Swine Influenza is most commonly caused by three subtypes of Influenza A virus (IAV): H1N1, H1N2, and H3N2, originating from human, avian, or swine sources (Ma, 2020). Pigs are considered mixing vessels for influenza viruses, facilitating the emergence of new strains with pandemic potential (Ma et al., 2009). PRRS virus (PRRSV) is highly diverse but generally classified into two species: *Betaarterivirus suid 1* (PRRS-1) and *Betaarterivirus suid 2* (PRRSV-2) (Taira et al., 2025). Ongoing viral evolution and genetic diversification continue to result in the emergence of novel variants, some of which are associated with increased pathogenicity. (Li et al., 2024). PRRSV infections are especially significant in neonatal and early post-weaning pigs because their adaptive immune systems are not fully developed and they depend on maternally derived antibodies during a critical window of immunological development (Butler et al., 2014). In contrast, endemic swine influenza A virus (IAV-S) circulation within herds is maintained by continuous introduction of susceptible hosts, and as maternal antibodies decline in the nursery and early grower stages, stress and increased exposure contribute to disease susceptibility (Schmies et al., 2024). Additionally, PRRS causes reproductive disorders in sows and impairs semen quality in boars (Schulze et al., 2013). Following IAV-S infection, the pigs mount a humoral immune response with the production of specific antibodies (Salvesen and Whitelaw, 2021). However, this immunity is short-lived, often necessitating repeated vaccination (Petro-Turnquist et al., 2025). In contrast, PRRSV rather modulates the cellular immune response, although antibody levels are still used as indicators of infection and immune status (Toman et al., 2019). IAV-S infection typically leads to efficient viral clearance, whereas PRRS infection is characterized by prolonged persistence and incomplete viral elimination (Toman et al., 2019). Vaccination against both diseases is widely used, with vaccines tailored to match circulating strains for optimal efficacy. Although vaccination is a valuable tool for disease control, it does not completely prevent viral circulation or the emergence of new strains (Ma,

2020; Li et al., 2024). Therefore, vaccination protocols must be carefully designed and regularly updated. In early life, piglets are protected by maternally derived antibodies (MDA), whose high levels can neutralize vaccine antigens and block immune response, a phenomenon known as maternal antibody interference. Thus, the optimal time for vaccination is when MDA levels decline below the threshold of detection, allowing the piglet's immune system to respond effectively. The primary aim of this study was to compare maternally derived antibody kinetics against IAV-S in piglets born to sows vaccinated either three weeks or four months prior to farrowing. Additionally, PRRSV antibody dynamics were evaluated under a uniform sow vaccination schedule. Although IAV-S and PRRSV differ substantially in pathogenesis and immune response, both infections are endemic respiratory pathogens commonly controlled through sow and piglet vaccination programs in commercial swine production systems. The inclusion of PRRSV in this study was not intended as a direct comparison with IAV-S pathogenesis, but rather to provide contextual insight into maternally derived antibody decline and post-vaccination serological responses within the same herd environment under standardized management conditions.

MATERIALS AND METHODS

Animals and experimental design

All procedures were approved by the Ethics Committee of the Institute of Veterinary Medicine of Serbia under approval No. EK-2025/M06 and were conducted in accordance with national animal welfare regulations. The study was conducted on a commercial farrow-to-nursery farm with approximately 2000 sows operating a batch-farrowing system of 300 sows per batch. The farm operated under endemic vaccination protocols for IAV-S and PRRSV and no clinical outbreaks were observed during the study period. Two groups of piglets born to sows with different influenza vaccination timings were included: Group A sows were vaccinated with RespiPorc Flu3 (Ceva Santé Animale), an inactivated trivalent swine influenza vaccine containing H1N1, H1N2, and H3N2 strains, administered three weeks prior to farrowing. Group B sows received the same vaccine four months before farrowing. All sows were vaccinated against PRRSV using ReproCyc PRRS EU (Boehringer Ingelheim), a modified live PRRSV-1 vaccine, three weeks prior to farrowing. Piglets were vaccinated at weaning (day 21) with PRRS FLEX EU (Boehringer Ingelheim), a modified live PRRSV-1 vaccine. The study involved sows of parities 1–5, representing a typical mid-parity distribution for commercial herds, and all were selected from the same production batch to minimise batch-related variability. This study followed a cross-sectional design. A cross-sectional approach was chosen to reflect herd-level immunity dynamics under commercial production conditions. Individual piglets were not followed longitudinally

throughout the study period. Repeated sampling of the same piglets was avoided to minimize handling stress and potential immune modulation. Limiting inclusion to a maximum of two piglets per litter reduced litter-related bias while enabling population-level estimation of MDA distribution. At each sampling point (days 5, 28, 42, 56, and 70), twenty different piglets per group were randomly selected, with no re-sampling of individuals across time points. A maximum of two piglets per litter was included at each sampling to avoid overrepresentation of individual sows. In total, 200 piglets were sampled throughout the study (20 piglets $\times$ 2 groups $\times$ 5 sampling points). The analytical approach differed between IAV-S and PRRSV due to differences in sow vaccination schedules. For IAV-S, two distinct vaccination intervals were compared, whereas for PRRSV all sows followed a standardized schedule, allowing evaluation of MDA decline and post-vaccination seroconversion without between-group comparison.

Sample collection

Blood samples were collected from piglets in both groups on days 5, 28, 42, 56, and 70 post-farrowing. Serum was separated by centrifugation and stored at -20°C until analysis. Nasal swabs were collected from a subset of 20 piglets per time point, primarily those exhibiting clinical signs, to monitor potential field IAV and PRRSV circulation at the herd level. Nasal samples were obtained using sterile cotton swabs inserted into both nostrils and rotated to ensure adequate mucus collection. Samples were immediately chilled, transported to the laboratory within the same day, and stored at -80°C until nucleic acid extraction and PCR analysis.

Serological testing

Serum samples were tested for the presence of antibodies against PRRSV and IAV-S using commercial enzyme-linked immunosorbent assay (ELISA) kits, following the manufacturers' instructions. Detection of PRRSV antibodies was performed using the PrioCHECK® PRRSV Antibody ELISA Kit (Thermo Fisher Scientific). For Influenza A virus antibodies, the INGEZIM Influenza A ELISA kit (Ingenasa) was used. Samples with competition percentages (%C) ≤ 30 were considered seropositive, in accordance with the manufacturer's recommended cut-off value. The PrioCHECK® PRRSV Antibody ELISA detects antibodies directed against PRRSV nucleocapsid (N) protein antigens. The INgezim Influenza A ELISA detects antibodies against conserved Influenza A nucleoprotein (NP) antigens, enabling broad detection of antibodies against multiple IAV-S subtypes.

Molecular testing

RNA was extracted from nasal swabs immersed in 1 mL phosphate-buffered saline (PBS), vortexed, and centrifuged at 2000 g for 10 min using the IndiSpin Pathogen Kit (Indical Bioscience, Germany). For molecular detection, real-time reverse transcription quantitative PCR (RT-qPCR) assays targeted the the ORF7 region of PRRSV and matrix (M) gene of Influenza A virus using the Luna® Universal Probe One-Step Kit (NEB, USA) according to previously published protocols (Opriessnig et al., 2006, Shu et al., 2011). IAV testing was done at all sampling points, while PRRSV testing was limited to pre-vaccination samples due to possible detection of the live vaccine strain.

Statistical analysis

Data were analyzed using a two-way ANOVA to assess the effects of sampling time and vaccination group on ELISA antibody reactivity, including their interaction (Time $\times$ Vaccination). As different piglets were sampled at each time point, a between-subjects design was applied. Homogeneity of variance was tested with Levene's test. For PRRSV, only sampling time was analyzed using one-way ANOVA, since all piglets came from vaccinated sows. Analyses were performed in Numiqo online application (<https://numiqo.com/>) with significance set at $P < .05$.

RESULTS

Most animals in both groups remained seropositive up to the fourth sampling point (day 56), with 75% of piglets in Group A (Figure 1) and 60% in Group B (Figure 2) testing positive.

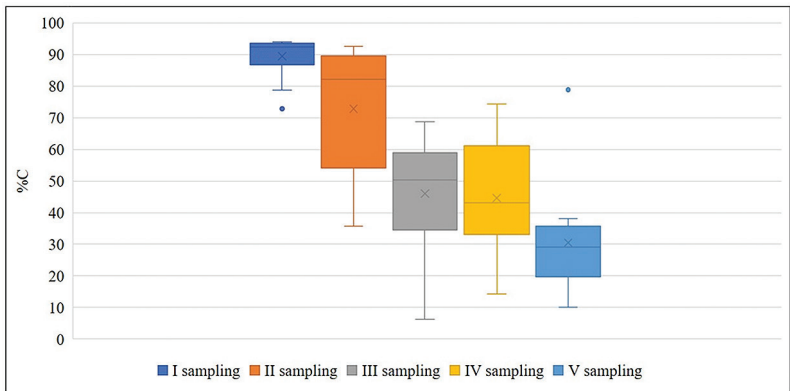


Figure 1. Distribution of %C values for IAV-S antibodies in Group A piglets across five sampling points. The data represent all tested piglets, including both seropositive and seronegative animals

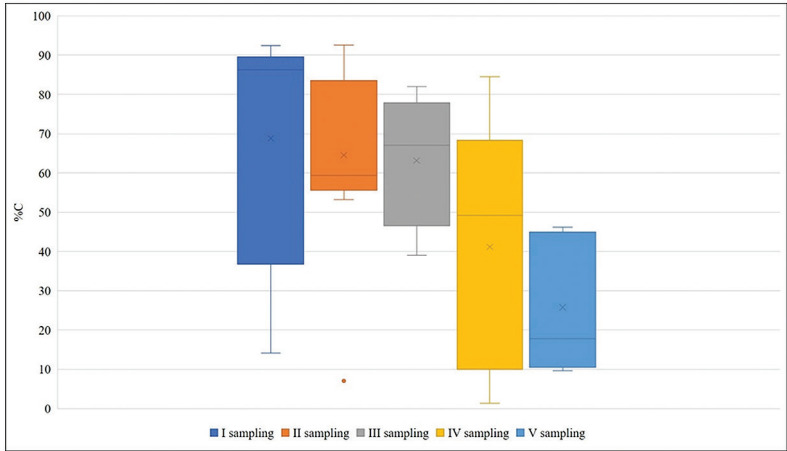


Figure 2. Distribution of %C values for IAV-S antibodies in Group B piglets across five sampling points. The data include all tested piglets, incorporating both seropositive and seronegative animals

A significant variance difference was observed at the first sampling ($P = .018$), with Group A showing more consistent values and Group B greater variability. No variance differences were detected thereafter. By day 70, most piglets in both groups were seronegative; Group B's mean % competition fell below 30%, while Group A remained slightly higher (Table 1). Two-way ANOVA showed a significant effect of sampling time on antibody levels ($F(4, 64) = 19.98, P < .001$), but no significant effects of vaccination group ($F(1, 16) = 0.12, P = 0.739$) or interaction ($F(4, 64) = 0.12, P = 0.054$), indicating similar antibody decline patterns between groups.

Table 1: Percentage of seropositive piglets and mean competition percentage (%C) for IAV-S antibodies in Group A and Group B across five sampling points

	Group A days post-farrowing					Group B days post-farrowing				
	5	28	42	56	70	5	28	42	56	70
% of seropositive piglets	100	100	80	75	45	80	95	100	60	40
% C	89.5	72.8	46.1	44.6	30.46	68.8	64.5	63.2	40.6	25.7

Regarding PRRSV, MDA levels, reflected by seropositivity rates and percentage positivity (PP) values, declined until day 42. Following vaccination of piglets at day 21, PP values subsequently increased, indicating seroconversion and the development of active humoral immunity. By day 70, nearly all piglets were seropositive with high antibody levels. The results showed no significant effect of sampling time ($F(4, 40) = 2.37, P = 0.069$), though trends indicated waning maternal antibodies followed by a post-vaccination rise. All nasal swabs tested negative for IAV-S and PRRSV, confirming absence of viral circulation or shedding.

DISCUSSION

Vaccination against PRRSV and IAV-S is routinely implemented in modern swine production systems. The standard PRRSV vaccination protocol involves immunizing piglets at one day of age if born to seronegative sows, or around the time of weaning if born to seropositive sows (Balasch et al., 2018). Gilts and sows are typically vaccinated every 3–4 months and again approximately three weeks prior to breeding. The study was conducted in a commercially managed herd with endemic vaccination against IAV-S and PRRSV and no evidence of active viral circulation during the sampling period, as confirmed by negative PCR results. Therefore, maternal antibody dynamics were evaluated under conditions of stable vaccination pressure rather than acute outbreak exposure.

Vaccination against IAV-S is performed multiple times per year. The primary objective of vaccinating breeding females is to enhance the level of maternal antibodies transferred to piglets, thereby providing early protection against IAV-S. The results of this study indicate that piglets born to sows vaccinated three weeks prior to farrowing exhibited more persistent maternally derived antibody responses against IAV-S, as reflected by ELISA %C values and seropositivity rates. This underscores the critical importance of optimal sow vaccination timing in enhancing passive immunity during the early life stages of piglets. Although MDA levels can vary, protective titers can be detected in piglets up to 14 weeks of age (Pyo et al., 2015), which is consistent with our findings, where 40–45% of piglets remained seropositive at 70 days of age. Despite the early protection conferred by MDAs, fewer than 80% of piglets remained protected (presumed protective threshold based on manufacturer cut-off values) at 8 weeks of age in our study, resulting in a critical period of susceptibility to IAV-S infection. Therefore, in endemic farms, it is essential to implement additional biosecurity measures, as intra-subtype variations of IAV-S have been shown to compromise cross-protection and may result in acute outbreaks. Moreover, infection with homologous strains in piglets possessing MDAs can lead to prolonged viral shedding and potentially facilitate the emergence of new viral variants (Ryt-Hansen et al., 2019). The presence of MDA at vaccination may also promote wild-type virus transmission, contributing to on-farm persistence (Andraud et al., 2023). While manufacturers recommend piglet vaccination at 56 days, this should be considered a minimum, as 60–75% of piglets in our study were still seropositive at that age. Maternal immunity has been shown to reduce viral titers in piglets challenged with homologous IAV-S strains, but not with heterologous ones (Keay et al., 2023). Once infection occurs, no significant difference in the duration of viral shedding was observed between MDA-positive and MDA-negative pigs (Keay et al., 2023). In our study, all nasal swabs tested negative for both IAV and PRRSV throughout the observation period, confirming the absence of viral shedding or field virus circulation. These results suggest that MDAs, in combination with vaccination and implemented biosecurity measures,

were associated with the absence of detectable viral circulation under the study conditions. However, as the level of infection pressure on the farm was not directly assessed, definitive conclusions regarding prevention of viral replication cannot be drawn (Schulze et al., 2013). While our study focused on herd-level protection, other studies emphasize that piglets with low MDA levels respond more similarly to MDA-negative individuals, underscoring the importance of analyzing individual-level data rather than relying solely on group means (Keay et al., 2023).

Similarly to IAV-S, vaccination of sows with modified live PRRSV vaccines results in the passive transfer of MDAs to piglets, providing transient protection that typically lasts from 2 to 11 weeks post-farrowing (Augustyniak and Pomorska-Mól, 2023). However, high MDA titers suppress early vaccine virus replication, resulting in no neutralizing antibodies detected until 14 weeks of age. In contrast piglets with low MDA titers exhibited earlier viral replication and a more robust cellular immune response, including increased numbers of interferon gamma IFN- γ -secreting cells (Fablet et al., 2016). Similarly, it was reported (Renon et al., 2019) that at five weeks of age, only 44% of piglets with high MDA levels seroconverted following vaccination, compared to 94% in the low-MDA group. These findings are consistent with our observations, which show an initial decline in antibody levels, followed by a secondary increase, suggesting delayed seroconversion in the presence of MDAs. In our study, MDA levels peaked during the first four weeks of life and declined rapidly thereafter, reaching minimal titers between six and eight weeks. This decline may be further influenced by vaccination, as antibodies are consumed during antigen opsonization, rendering them undetectable by the ELISA method employed. However, given that effective herd immunity usually develops four to five weeks after vaccination, immunizing piglets at three weeks may serve as a practical compromise between early intervention and MDA interference. By ten weeks of age, all piglets in our study population had seroconverted, indicating successful induction of active immunity despite the initial presence of maternal antibodies. This study suggests that the timing of maternal vaccination plays a crucial role in determining both the duration and uniformity of antibody protection in piglets against IAV-S and PRRSV. The greater variability in initial IAV-S antibody levels observed in Group B piglets (born to sows vaccinated four months before farrowing) suggests that longer intervals between sow immunization and parturition may result in less consistent passive immunity transfer. In contrast, piglets from sows boosted three weeks pre-farrowing (Group A) exhibited more uniform antibody levels, underscoring the practical value of a pre-farrowing booster to achieve a more homogeneous maternal antibody profile and enhance early-life protection. These findings highlight the importance of optimizing sow vaccination schedules, together with appropriately timed piglet vaccination and strict biosecurity measures, for effective disease control. Although antibody measurements from sows at farrowing would have further strengthened the interpretation of maternal antibody transfer, such data were not available within this

study design. Antibody levels in piglets may vary substantially within litters due to differences in colostrum intake and individual immune response. Colostrum intake was not individually quantified in this field-based study. Although all piglets were managed under standardized commercial conditions with no evidence of impaired lactation, variation in colostrum consumption may contribute to individual differences in early MDA levels. The cross-sectional design and inclusion of piglets from multiple litters were intended to reduce litter-specific bias. However, residual variability related to passive transfer efficiency cannot be excluded. The observed heterogeneity in MDA levels further underlines the importance of herd-level monitoring and tailored management strategies to minimize protection gaps and reduce the risk of viral persistence and evolution in swine populations. While ELISA testing offered valuable insight into the temporal dynamics of humoral immunity, the obtained results represent relative measures of antibody reactivity rather than absolute concentrations. The %C and PP values provided by commercial ELISA kits reflect the strength of antigen-antibody interactions, allowing differentiation between seropositive and seronegative animals and comparison of antibody trends over time. However, these optical density-based indices are not linearly correlated with serum antibody concentration and should be interpreted as semi-quantitative indicators of immune status. Although the present findings describe relative changes in antibody reactivity associated with the decline of maternally derived antibodies and the onset of vaccine-induced responses, they do not quantify antibody concentration or neutralizing capacity. To better assess protective immunity, future studies should include quantitative or functional assays such as virus neutralization (VNT) or hemagglutination inhibition tests (HIT), along with cellular immune parameters like cytokine profiling to more accurately define the dynamics of maternal immunity. Unlike ELISA, which measures antibody reactivity, VNT and HIT quantify protective antibody titers and therefore provide a more direct assessment of the piglets' ability to neutralize circulating virus. Including such functional measures would help define meaningful correlates of protection, reduce uncertainty associated with interpreting %C values alone, and offer clearer insight into the true dynamics of maternally derived immunity. The present study was designed as a cross-sectional field investigation; therefore, individual piglets were not followed longitudinally throughout the sampling period. Consequently, the findings reflect herd-level trends in antibody dynamics rather than precise individual decay trajectories. While a longitudinal design with individually tagged piglets sampled from birth through the nursery phase would provide more accurate characterization of individual MDA kinetics, the cross-sectional approach employed here allowed robust estimation of population-level immunity patterns under commercial production conditions. Future studies incorporating longitudinal monitoring alongside functional antibody assays would further refine understanding of individual MDA decay and protective immunity development. Despite these limitations, this study provides robust field-based

evidence by comparing two practical sow vaccination intervals under standardized production conditions, offering directly applicable insights for refining vaccination schedules and strengthening herd-level immunity.

CONCLUSION

This field-based study demonstrated that piglets born to sows vaccinated against IAV-S three weeks prior to farrowing exhibited more uniform and prolonged maternally derived antibody responses compared with piglets from sows vaccinated four months earlier. PRRSV antibody dynamics additionally illustrated the decline of maternally derived antibodies followed by post-vaccination seroconversion under commercial herd conditions. The findings suggest that sow vaccination timing may influence the duration and uniformity of passive immunity in piglets. However, the study was conducted in a single herd using a cross-sectional design and relatively limited sample size. Therefore, broader conclusions regarding optimal vaccination protocols and herd-level protection should be interpreted cautiously. Further longitudinal studies incorporating functional immunity assays and larger multi-herd populations are needed to better define protective antibody thresholds and optimal vaccination timing.

Author's contribution

VM was responsible for the conceptualization and writing of the original draft. SŠ conducted the formal analysis. SS carried out the investigation. GR contributed to the development of the methodology. DR and SR provided resources. VM supervised the work. DG prepared the visualizations. DR contributed to the review and editing of the manuscript.

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Ethical statement

All procedures were approved by the Ethics Committee of the Institute of Veterinary Medicine of Serbia under approval No. EK-2025/M06 and were conducted in accordance with national animal welfare regulations.

Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

One of the co-authors is an employee of Boehringer Ingelheim (BI). Boehringer Ingelheim had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. All other authors declare no conflicts of interest.

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OPADANJE MATERNALNIH ANTITELA PROTIV INFLUENCE A SVINJA I PRRS-a KOD PRASADI KOD RAZLIČITIH VAKCINALNIH PROGRAMA KRMAČA

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Kratak sadržaj

Primarni cilj ove studije bio je procena uticaja vremena vakcinacije krmača protiv virusa influenza svinja (IAV-S) na održavanje i opadanje titra maternalnih antitela (MDA) kod prasadi. Dinamika antitela protiv virusa respiratornog i reproduktivnog sindroma svinja (PRRSV) dodatno je ispitivana radi dobijanja kontekstualnih informacija o opadanju maternalnih antitela i serokonverziji nakon vakcinacije u istim uslovima zapata.

Ukupno 200 prasadi (20 po grupi u svakoj od pet vremenskih tačaka: 5., 28., 42., 56. i 70. dana života) uzorkovano je tokom istraživanja sprovedenog po principu presečnog ispitivanja. Antitela protiv IAV-S i PRRSV u serumima određivana su primenom komercijalnih ELISA testova. Prasad poreklom od krmača vakcinisanih protiv IAV-S tri nedelje pre prašenja (Grupa A) ispoljila su više i ujednačenije titre maternalnih antitela u poređenju sa prasadi poreklom od krmača vakcinisanih četiri meseca ranije (Grupa B). Pedeset šestog dana života, 75% prasadi u Grupi A i 60% prasadi u Grupi B ostalo je seropozitivno na IAV-S. Do 70. dana života seropozitivnost je opala na približno 40–45% u obe grupe. Tokom vremena zabeleženo je izraženo opadanje nivoa antitela, pri čemu je značajan procenat prasadi postao seronegativan do osme nedelje života. Kod PRRSV, nivo antitela opadao je do 42. dana života, nakon čega je usled vakcinacije prasadi došlo do njegovog porasta, što ukazuje na uspešnu indukciju aktivnog imuniteta. Ovi rezultati ukazuju na značajnu ulogu vremena vakcinacije krmača u oblikovanju kinetike maternalnih antitela, kao i na potrebu usklađivanja strategije vakcinacije prasadi sa tim promenama. Pored toga, rezultati naglašavaju neophodnost primene integrisanih mera kontrole, koje prevazilaze samu vakcinaciju, u cilju efikasnog suzbijanja transmisije PRRSV i IAV-S u endemski inficiranim zapatima.

Ključne reči: svinje, reproduktivni i respiratorni sindrom svinja, influenza svinja, maternalna antitela, vakcinacija

Original research article

CLINICAL AND EPIDEMIOLOGICAL INVESTIGATION OF FELINE CHRONIC GINGIVOSTOMATITIS AND PERIODONTAL DISEASE IN BLIDA, NORTH OF ALGERIA

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Abstract

Oral diseases are frequent issues in cats. The objectives of this study were to determine the prevalence, clinical signs, and risk factors associated with these conditions. Out of the 470 cats presented to a private veterinary clinic in the Blida region of northern Algeria, 76 (16.17%) were diagnosed with oral pathologies and included in the study. For each animal, potential risk data were obtained from medical record and cat owner interviews. The most frequent oral condition was gingivitis (60.52%), followed by feline chronic gingivostomatitis (44.73%), periodontitis (31.57%), eosinophilic granuloma complex (6.57%), jaw fracture (3.94%), mouth obstruction by chicken bone with, hypodontia (2.63%), prognathism and tooth caries with 1.31% for each clinical finding. The most common clinical signs of oral conditions were halitosis (85.52%), followed by pain (73.68%), anorexia (72.36%), tartar buildup (68.42%), dehydration (53.49%), ulceration (47.36%), palatoglossitis (40.78%), fever (34.21%), bleeding and dental mobility with 32.89% observed in each group. The breed, vaccination status, diet, home dental care, living environment and owner's awareness of oral diseases were found to be significantly associated with periodontal disease and feline chronic gingivostomatitis. Indeed, European breeds, unvaccinated cats, those fed a mixed diet, cats that did not receive home dental care, cats with outdoor access, and those

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whose owners lacked knowledge about oral diseases were the most susceptible to these two conditions. The present study revealed a non-negligible prevalence of oral conditions, particularly periodontal disease and stomatitis. Appropriate recommendations need to be introduced to control these diseases.

Key words: mouth diseases, cats, symptoms, clinical trials, risk factors, Algeria

INTRODUCTION

Oral health is a fundamental yet often overlooked aspect of overall feline health. The oral cavity of cats can be affected by a number of diseases, ranging from tartar and plaque buildup to more serious problems such as gingivitis, periodontal disease, tooth resorption, tumors, and chronic gingivitis and periodontitis (Chaubey et al., 2021). These conditions are not only painful but can also lead to systemic complications affecting the heart, kidneys, and liver through the entry of oral bacteria into the bloodstream (Niemic, 2012). These oral diseases are among the most common health problems diagnosed in domestic cats, yet they are often not recognized by owners or even veterinarians. A survey in Finland reported that dental and oral diseases were the most prevalent disease category in cats (prevalence 28%), and dental calculus and gingivitis were the most prevalent clinical signs noted (Vapalahti et al., 2016). Additionally, periodontal disease was the most prevalent diagnosis-level disorder in cats based on UK clinic data, accounting for 13.9% of all feline diagnoses (O'Neill et al., 2014). These inflammatory oral diseases in cats are complex and multifactorial conditions consisting primarily of local or widespread inflammation in the oral cavity (Kornya et al., 2014). Viral diseases such as feline leukemia virus (FeLV), feline immunodeficiency virus (FIV), calicivirus can show the signs of oral inflammation. Nutritional disorders also contribute to oral diseases. There is evidence that breed and therefore genetics play some role in periodontal disease development in cats (O'Neill et al. 2023). In Algeria, cats are often brought to the clinic only when they exhibit advanced symptoms. Routine oral exams are rare, veterinary dental care is often not prioritized, and information on the prevalence and nature of feline oral diseases is still limited. Insufficient knowledge about these conditions in Algeria cats motivated this study. Therefore, a survey was conducted in order to determine the prevalence, clinical signs, and risk factors of oral conditions in cats in a veterinary office in Blida.

MATERIAL AND METHODS

A total of 470 cats of different breeds, ages, and sex from a private veterinary clinic located in the region of Blida were examined from July 1st to August 30th, 2024. Only patients displaying clinical signs of oral diseases (oral pain, halitosis, anorexia, tartar...) and cats whose owners consented to their participation in the

study and completed the questionnaire face-to-face were included. Demographic data and risk factors were collected through face-to-face questionnaires with the cats' owners. The following data were acquired: the animal's age, breed, sex, lifestyle, dental hygiene, vaccinations, hunting behavior, type of diet and the owner's knowledge of oral diseases. The types of food included dry, wet and mixed food. The lifestyles included indoor housing or outdoor housing. Additionally, the owners provided information on the duration and persistence of symptoms, as well as potential stressors.

The cat vaccination protocol includes the feline leukemia virus vaccine, and a combined vaccine against feline panleukopenia and feline viral rhinotracheitis (herpesvirus and calicivirus): The first vaccination, around 8 weeks of age, 2 to 3 injections spaced approximately 3 weeks apart are required to ensure adequate immunization. Rabies vaccination is administered from 3 months of age. Annual boosters are required.

Detailed clinical examination of each patient was carried out. Visual inspection was applied while respiration, pulse rates and rectal temperature were carefully recorded. Oral and dental clinical examination was performed on cats admitted to the clinic. Evaluation of the animal's oral health in a conscious state was performed as a visual intraoral examination in sternal recumbency using a headlight. An examination of the teeth, gingiva, and oral cavity was conducted as thoroughly as the animal permitted. Attention was paid to whether any gingivitis, stomatitis, plaque, calculus, persisting deciduous teeth, missing or mobile permanent teeth, dental defects, or malocclusions were found. For aggressive animals, sedation was performed using 2% Xylazine (Vetopharm pro, Algeria) of 0.1 to 0.2 mL/kg (2 to 4 mg/kg) intramuscular/subcutaneous. If a problem was suspected, a detailed dental examination was performed using specialized dental instruments, including William's periodontal probe to evaluate gingival and periodontal health, and a dental explorer to assess the hard surfaces of the teeth. Mouth mirrors were used to check the posterior teeth and surfaces.

Data analysis was performed using Microsoft Excel 2010 and Statistical Data for Social Science (SPSS) 26 © IBM Copyright, IBM Corporation and its licensors 1989.2019 IBM, USA. Univariate analysis was performed to assess each independent variable for its unadjusted association with disease. The chi-square test of homogeneity and in dependence as well as the test of Mac-Nemar were performed. The statistical significance level used for this study was $p < 0.05$.

RESULTS

From 470 cats inspected in veterinary office, 76 patients were diagnosed with oral affections with a prevalence of 16.17%.

Of the 76 diagnosed cases, the results (Table 1) showed that most of the cats in this study were suffering from gingivitis (Figure 1A, Figure 1D), followed by

FCGS (Figure 1C), periodontitis (Figure 1D, Figure 2A), eosinophilic granuloma complex, while very low frequencies were observed for jaw fractures (Figure 1B) and mouth obstruction by chicken bones, as well as for hypodontia, prognathism, and tooth caries (Figure 2B). A highly significant difference (<0.0001) was recorded between the different conditions.

Table 1: Number and frequencies of oral conditions recorded in examined cats

Clinical finding	No. of cats affected	% of cats affected
Gingivitis	46	60.52
FCGS	34	44.73
Periodontitis	24	31.57
Eosinophilic granuloma complex	5	6.57
Jaw fracture	3	3.94
Mouth obstruction by chicken bone	2	2.63
Hypodontia	1	1.31
Prognathism	1	1.31
Tooth caries	1	1.31



Figure 1. A: Gingivitis (black arrow), B: Fracture of the left lower jaw (black arrow), C: Ulcero-proliferative lesions of FCGS with maxillary gingivitis (black arrow) and alveolar mucositis (white arrow), D: Gingivitis (black arrow) and periodontitis in the upper right premolar (white arrow).



Figure 2. A : Severe periodontitis with oral abscess (black arrow) : severe attachment loss, exposure of furcation and tooth root, B : Tooth caries in the upper right premolar (black arrow).

For each cat, we documented either a single pathology or a combination of two to three pathologies. Highly significant difference was recorded between the three cases ($p < 0.0001$). Periodontal disease was the most common pathology and was found alone in 36 cats (47.36%) or associated with other pathologies especially FCGS in 32 cases (42.10%). With the exception of two cases, FCGS was always associated with periodontal disease in 32 cases (42.10%) (Table 2).

In all cats diagnosed with oral conditions, the most common clinical signs are showed in Table 3. The chi-square test was highly significant (< 0.0001) between the different clinical signs recorded.

Among the 70 cats diagnosed with periodontal disease, 50 (71.42%) had tartar, whereas 20 (28.57%) did not. Conversely, of the 52 cats that showed the presence of tartar, 50 (96.15%) were diagnosed with periodontal disease, while only 2 (3.84%) were not. The Mac-Nemar test was highly significant ($p = 0.00012$) (Table 4).

Table 2: Distribution of clinical cases among the 76 cats examined

	Clinical findings	N° of cats	%
Presence of one pathology	Periodontal disease	36	47.36
	Jaw fracture	1	1.31
	Mouth obstruction by chicken bone	1	1.31
	Prognathism	1	1.31
	Hypodontia	1	1.31
Total		40	52.63
Presence of two concurrent pathologies	Periodontal disease + FCGS	27	35.52
	FCGS + Eosinophilic granuloma complex	2	2.63
	Periodontal disease + Jaw fracture	1	1.31
	Periodontal disease + Mouth obstruction by chicken bone	1	1.31
Total		31	40.78
Presence of three concurrent pathologies	Periodontal disease + FCGS + Eosinophilic granuloma complex	3	3.94
	Periodontal disease + FCGS + Jaw fracture	1	1.31
	Periodontal disease + FCGS + Tooth caries	1	1.31
Total		5	6.57
Total number of cats with oral conditions		76	100

Periodontal disease (gingivitis alone or periodontitis alone or gingivitis + periodontitis)

Table 3: Proportion of clinical symptoms recorded in cats

Clinical signs	N	%
Halitosis	65	85.52
Pain	56	73.68
Anorexia	55	72.36
Tartar (calculus)	52	68.42
Dehydration	41	53.49
Ulcerations	36	47.36
Palatoglossitis	31	40.78
Fever	26	34.21
Dental mobility	25	32.89
Bleeding	25	32.89
Glossite	15	19.37
Ptyalism	5	6.57

Table 4: Association between tartar and periodontal disease

		Periodontal disease		Total
		Presence	Absence	
Tartar	Presence	50	2	52
	Absence	20	4	4
Total		70	6	76

Associations of risk factors with the prevalence of periodontal diseases and FCGS

The association of demographic data and risk factors with periodontal disease and FCGS are shown in Table 5. Chi-square analysis revealed significant associations between these conditions and breed, vaccination status, diet, home dental care, living environment, and owners' awareness of oral pathologies. Cats aged 1 year and older were the most affected by periodontal disease. Regarding FCGS, the highest prevalence was observed in cats under 1 year of age. No significant difference ($p > 0.05$) was found between age groups for the two pathologies. For both pathologies, females were slightly more affected than males. No significant difference ($p > 0.05$) was recorded for sex of cats for each pathology. Regarding breed, the European breed had a higher risk of periodontal disease and FCGS. The chi-square test was highly significant ($p < 0.0001$). Unvaccinated cats were more susceptible to both conditions than cats that received vaccination. The difference was significant for periodontal disease ($p < 0.0168$) and highly significant for FCGS ($p < 0.00001$). Cats fed a mixed diet had higher prevalence than those fed only a dry or wet diet. The chi-square test was highly significant ($p < 0.0001$) for each pathology. The prevalence of both pathologies was too high for cats that did not receive home dental care with a highly significant difference ($p < 0.0001$) for each condition. Cats allowed outdoors had a higher likelihood of developing these diseases compared to strictly indoor cats. The statistical difference was very significant for the two diseases. Cats that hunt seem to have a higher risk of developing periodontal disease and FCGS than cats that do not hunt, but with no significant statistical difference for both pathologies. Owners generally had limited knowledge about oral diseases and were not well-informed about oral pathologies. The statistical difference was highly significant for periodontal disease ($p < 0.00001$) and very significant for FCGS ($p = 0.006$).

Table 5: Analyses of cats affected with periodontal disease and stomatitis according to risk factors.

Group	Periodontal disease			FCGS		
	N	%	P-value	N	%	P-value
Age						
< 1 year	27	38.57	0.558	21	61.76	0.17
≥ 1 year	43	61.42		13	38.23	
Sex						
Male	32	45.71	0.473	14	41.17	0.303
Female	38	54.28		20	58.82	
Breed						
Angora	1	1.42	<0.0001	1	2.94	<0.0001
Persian	6	8.57		0	0	
Siamese	8	11.42		1	2.94	
European	55	78.57		32	94.11	
Vaccination						
Yes	25	35.71	<0.0168	2	5.88	<0.00001
No	45	64.28		32	94.11	
Diet						
Dry	10	14.28	<0.00001	6	17.64	<0.00001
Wet	6	8.57		6	17.64	
Mixed	54	77.14		22	64.70	
Home dental care						
Yes	2	2.58	<0.00001	0	0	<0.00001
No	68	97.14		34	100	
Living environment						
Indoor	23	32.85	0.0041	7	20.58	0.0006
Outdoor	47	67.14		27	79.41	
Hunting						
Yes	42	60	0.094	22	64.7	0.086
No	28	40		12	35.29	
Owner's knowledge about oral pathologies						
Yes	18	25.71	<0.00001	9	26.47	0.006
No	52	74.28		25	73.52	

DISCUSSION

The prevalence of oral disorders in cats in this study was almost similar to 15.1% reported by O'Neill et al. (2014), but lower than the 21.2% and 84% reported by O'Neill et al. (2023) and Aula (2018), respectively. The variation in results could be due to differences in the populations studied (feral versus domestic cats) and the diagnostic methods employed (clinical versus radiographic) (Verhaert and Van Wetter, 2004), diet, life style, popular breeds of cats in each country and duration of sample collection.

In the present study, the periodontal disease (92.10%) was the most frequent diagnosis (60.52 % for gingivitis, 31.57% for periodontitis). Similarly, studies from different countries reported that the main oral disease observed in felines is periodontal disease (Perry and Tutt, 2015) with a reported prevalence ranging from 13.4 % to 92.75%. Indeed, our results are similar to 91.7% (gingivitis 73.2% periodontitis 18.5%) found by Verhaert and Wetter (2004) and 92.7% found by Elseddawy et al. (2023). Da Silva et al. (2019) reported lower prevalences (65.11%: gingivitis 13.95%, periodontitis 51.16%), followed by Kornya et al. (2014) at 31.8% (gingivitis 20.7%, periodontitis 11.1%), Whyte et al. (2022) at 30.9%, O'Neill et al. (2023) at 15.2%, and O'Neill et al. (2014) at 13.4%. This difference in prevalence between studies may be due to the criteria and method applied for diagnosis and the cohort demographics (O'Neill et al., 2023). According to Long (2020), an important proportion of cats with periodontal disease still goes undiagnosed. The underdiagnosis of oral diseases may be due to factors such as problematic patient temperament, which can prevent a conscious clinical examination, and the fact that clinical signs of oral disease are often nonspecific to the oral cavity (Gorrel, 2013). The second most common oral disease recorded in our study was FCGS (44.73 %) which was close to the result of Da Silva et al. (2019) who noted a prevalence of 34.88% and Öztürk Gürgen et al. (2022) who recorded 45.76%. However, it was higher than the 12% reported by Verhaert and Van Wetter (2004), 5.5% recorded by Girard et al (2009) and 0.7% noted by Healey et al. (2007). These studies were conducted on cats with no contact with the external environment or on cats whose interactions with other cats could be controlled. However, as the cats in the present study were exposed to the external environment, contact with other cats could not be controlled. This represents a significant distinction compared to other studies. Eosinophilic granuloma complex was identified as the pathology of oral cavity in 6.57 % of the cats included in the present study. Our results were lower than 11.1% found by Falcão et al. (2020), but higher than 1.63% noted by Scott et al. (2012). Bone fracture, congenital and developmental problems (hypodontia and prognathism with 1.31% for each disease), tooth caries were not seen frequently in our study. The same findings were recorded by Verhaert and Van Wetter (2004) who reported low rates of malocclusion (4.9 %) and supernumerary tooth (1.2%), by Aula (2018) who noted a rate of 1% of retained deciduous teeth and Elseddawy

et al. (2023) who found a prevalence of 0.40% of missing teeth, 1.61% of persistent deciduous teeth and 0.8% of tooth caries. No cases of bone fracture were reported in the aforementioned studies.

Different studies observed nearly identical clinical symptoms. Wiggs and Lobprise, (1997) noted that the most common clinical signs of periodontitis are halitosis, ptyalism, dysphagia, pain on chewing and bleeding gingiva. For FCGS, clinical signs include severe oral pain with reduced food prehension ability, ptyalism, mandibular lymphadenopathy, dysphagia, anorexia, « unkempt appearance » (Wiggs and Lobprise, 1997; Niemiec, 2012), halitosis, loss of appetite, depression, weight loss (Dai et al., 2024) and (in severe cases) even dehydration.

According to Chaubey et al. (2021), the calculus was the main cause for the development of gingivitis. However, many other factors also predispose the disease progress: tooth crowding and morphology, low saliva flow and systemic illnesses such as renal dysfunction and diabetes mellitus (Albuguergue, 2012). Additionally, we found that among cats with tartar, 96.15% had periodontal disease, while the remaining cats without periodontal disease may have had a stronger immune system. Periodontal disease results from an imbalance between the host and the microbes.

For periodontal disease, we found that cats aged 1 or older were the most affected. Age was the most significant predictor of periodontal disease (O'Neill et al., 2023) and seems to be the main factor because older cats are more susceptible to the developing oral pathologies (Villegas-Ferre et al., 2024). In addition, Williams et al., (2024) noted that significant proportion of young cats can develop gingival inflammation. This inflammation at a young age may be due to a syndrome called juvenile periodontal disease, which is the inflammation of the gingiva occurring shortly after the permanent dental eruption (Villegas-Ferre et al., 2024). Regarding FCGS, the youngest cats—those under 1 year of age—were the most affected. This could be explained by the fact that these young animals were not vaccinated and exposed to the outside environment. FCGS is most frequently observed in adult cats (Healey et al., 2007; Öztürk Gürgen et al., 2022), whereas Nakanishi et al. (2019) reported that cats can also be affected at an early age. On the other hand, some studies showed that there was no significant difference in the age distribution of cats with and without FCGS (Healey et al., 2007; Dai et al., 2024). For both pathologies, females were slightly more affected than males, although the difference was not statistically significant, which aligns with the findings of Verhaert and Van Wetter (2004) and Girard et al. (2009) who reported that the sex does not appear to be associated with the risk of periodontal disease in cats. Also, our results are in agreement with many studies that showed no significant correlation between FCGS and sex (Healey et al., 2007; Öztürk Gürgen et al., 2022; Dai et al., 2024). However, Villegas-Ferre et al. (2024) reported that sex appears to be associated with a predisposition to gingivitis, with males being more susceptible, although the underlying reason remains unclear. Martijn (2009) noted that the male sex was

significantly associated with stomatitis. A higher prevalence of stomatitis was also identified in males than in females in a study conducted by Kim et al. (2023), but the difference was not statistically significant.

The European breed had a significantly higher risk of periodontal disease and FCGS than other breeds. O'Neill et al. (2023) noted that breed and therefore genetics play some role in periodontal disease development in cats. It was also shown that some breeds (Himalayan, Siamese, Persian, Balinese, Oriental, Seychellois, Abyssinians, Ocicats and Somalis) are more likely to have dental diseases than other breeds or mixed breed cats (Verhaert and Van Wetter 2004; Nakanishi et al., 2019). On the other hand, Healey et al. (2007) and Dai et al. (2024) found that there is no significant correlation between the breed of cats and stomatitis. The variability in the results is probably due to the popularity of particular cat breeds at different times. This variability probably shows that in the areas where pedigree cats are more common, they become affected. We recorded that cats that did not receive vaccination were more susceptible to periodontal disease and stomatitis. Several viruses of worldwide distribution have been associated with FCGS: Calicivirus (FCV) (Healey et al., 2007), Feline Herpesvirus (FHV) (Lommer and Verstraete, 2003), and Feline Leukemia Virus (FeLV), and with periodontal disease (FeLV, Feline Immunodeficiency Virus (FIV), FCV) (Wiggs and Lobprise 1997; Kornya et al. 2014). Da Silva et al. (2019) concluded that even when associations are found, other factors must also be considered in the development of oral cavity lesions in cats. Most cats affected in our study were unvaccinated against these viruses, leading us to conclude that there is consistent evidence linking FCV and/or some of these viruses to the disease, suggesting a potential etiologic role. Additionally, the infected cats in our study exhibited free-roaming behavior, which is a known risk factor for FeLV, FIV, FHV and FCV infection (Whyte et al., 2017). This condition causes permanent exposure of cats to viruses shed by chronic carriers, by promoting high rates of viral evolution and by facilitating cyclic reinfection of susceptible animals. Numerous studies showed an influence of diet on periodontal health. Williams et al. (2024) have reported that the odds of gingivitis in cats were higher in cats fed a wet only or mixed wet/dry diet compared to dry only. It is suggested that dry foods offer protection from gingivitis compared to wet diets, potentially due to the mechanical cleaning of dental surfaces and the reduced rate of dental plaque and calculus accumulation. This helps to prevent plaque accumulation and gingival inflammation (Williams et al., 2024). Dry diets have, however, been associated with an increased risk of obesity (Rowe et al., 2015). Therefore, gingivitis should not be the only factor guiding dietary choices made by cat owners (Williams et al., 2024). Tooth brushing is usually considered to be the most effective way to remove dental plaque and prevent or reduce calculus formation. Lertpimonchai et al. (2017) found that average and poor oral hygiene (OH) significantly increased the risk of periodontitis by two- and five-fold, respectively, compared to good OH. In contrast, regular tooth brushing and visits to the dentist could reduce periodontitis

by 34% and 32% respectively. The accumulation of dental plaque and tartar, associated with poor oral hygiene, is one of the risk factors in the development of periodontal disease (Lertpimonchai et al., 2017). According to Verstraete et al. (1996), a natural lifestyle, including feral cats or house cats with outdoor access and hunting behavior, does not prevent periodontal disease. Additionally, Verhaert and Van Wetter (2004) found that hunting behavior does not appear to influence periodontal status. Recent studies showed that the prevalence of FCGS was higher in multicat than single-cat households, and that each additional cat in the household increased the odds of FCGS by more than 70% (Peralta and Carney, 2019). Kim et al. (2023) noted that the high prevalence of FCGS in the feral cats may have an infectious mechanism. Free-roaming behavior is a known risk factor for FeLV, FIV, FHV and FCV infection, but non-infectious mechanisms related to multi-cat environments like stress could also play a role in the etiopathogenesis (Peralta and Carney, 2019). On the other hand, Verhaert and Van Wetter (2004) found that different lifestyles (living inside, allowed to go outside) do not seem to affect periodontal status and the outdoor access was not significantly associated with periodontal disease (Soltero-Rivera et al., 2023). Additionally, contrary to our findings, some authors reported that cats whose owners indicated they hunt were associated with a lower prevalence of periodontal disease (Verstraete et al., 1996 ; Williams et al., 2024). It is plausible that some owners may be unaware of hunting activity in their cats, or that the level of hunting activity is not directly linked to the actual consumption of captured prey (Williams et al., 2024). Further studies are required to investigate the association between hunting behaviour and oral health in cats. This finding is consistent with previous literature, which indicates that many pet owners underestimate the importance of routine dental checkups, resulting in inadequate home care and missed early signs of disease (Oskarsson et al. 2021). Owners who receive proper education from veterinarians about oral care are more likely to adhere to preventive measures, including tooth brushing, dental diets, and regular clinical check-ups (Girard et al., 2009).

CONCLUSION

These findings suggest that oral illness in cats, such as gingivitis, periodontal disease, and FCGS are not only common but also have severe implications if not treated. These illnesses progress subtly, making continuous monitoring and early detection essential. The survey also highlighted that most cat owners have limited knowledge about the importance of dental care. This offers practical recommendations for improving the management of oral diseases and highlights the urgent need for enhanced education and advocacy by veterinarians, animal welfare organizations, and society at large. Some limitations, particularly the small number of animals studied, may affect the generalizability of our findings. Further

studies are needed to determine the true prevalence of oral diseases in cats across different regions of Algeria.

Author's contribution

A.D. was responsible for the study's concept and design, data collection, manuscript drafting, data analysis, result interpretation, and final manuscript preparation. S.Z. carried out the statistical analysis, critically revised the manuscript, and approved the final version. All authors reviewed and approved the final manuscript for publication.

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Ethical statement

This study was based exclusively on observations recorded during routine oral examinations of cats conducted in a private veterinary clinic. All procedures were performed solely for diagnostic and therapeutic purposes and did not involve any additional interventions beyond standard veterinary care. Animal welfare was fully respected throughout the study. Written informed consent for participation and publication of the results was obtained from all owners. In line with applicable regulations, ethical committee approval was not required for this type of non-experimental clinical study.

Competing interests

The authors declare that there is no conflict of interest.

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KLINIČKO I EPIDEMIOLOŠKO ISTRAŽIVANJE HRONIČNOG GINGIVOSTOMATITISA I PARODONTALNE BOLESTI KOD MAČAKA U BLIDI, SEVERNI ALŽIR

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Kratak sadržaj

Oralne bolesti su široko rasprostranjene kod mačaka. Cilj ovog rada je da se utvrde učestalost, klinički znaci kao i faktori rizika za ta oboljenja. Od ukupno 470 mačaka koje su bile pregledane u privatnoj veterinarskoj klinici u oblasti Blida, na severu Alžira, kod 76 jedinki (16,17%) dijagnostikovane su oralne bolesti i one su uključene u studiju. Za svaku životinju podaci o potencijalnim faktorima rizika pribavljeni su iz medicinske dokumentacije i razgovora sa vlasnicima mačaka. Najčešće oralno oboljenje bilo je gingivitis (60,52%), zatim hronični gingivostomatitis (44,73%), parodontitis (31,57%), kompleks eozinofilnog granuloma (6,57%), fraktura vilice (3,94%), opstrukcija usne duplje pilećom kosti sa hipodencijom (2,63%), kao i progenija i karijes zuba, svaki sa po 1,31% kliničkih nalaza. Najučestaliji simptomi oralnih oboljenja bili su halitoza (85,52%), zatim bol (73,68%), anoreksija (72,36%), nakupljanje zubnog kamenca (68,42%), dehidracija (53,49%), ulceracije (47,36%), palatoglositis (40,78%), febrilnost (34,21%), kao i krvarenje i mobilnost zuba, sa učestalošću od 32,89% u svakoj grupi. Utvrđeno je da su rasa, vakcinalni status, ishrana, nega zuba kod kuće, životno okruženje i stepen informisanosti vlasnika o oralnim bolestima značajno povezani sa parodontalnom bolešću i felinim hroničnim gingivostomatitisom. Evropske rase, nevakcinisane mačke, mačke koje su hranjene mešovitom ishranom, mačke bez adekvatne kućne dentalne nege, jedinke koje imaju kontakta sa spoljašnjom sredinom, kao i mačke čiji vlasnici nisu upoznati sa oralnim bolestima, bile su najpodložnije ovim oboljenjima. Ova studija pokazala je značajnu prevalenciju oralnih oboljenja, naročito parodontalne bolesti i stomatitisa, kao i to da je neophodno uvesti odgovarajuće preporuke za kontrolu ovih bolesti.

Ključne reči: bolesti usne duplje, mačke, klinička ispitivanja, faktori rizika, Alžir

Original research article

PREVALENCE, RISK FACTORS AND MOLECULAR CONFIRMATION OF *EIMERIA* SPP. IN ONE-HUMPED CAMELS (ALGERIA)

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Abstract

This study had for objective to investigate by molecular tools the intestinal *Eimeria* infection in slaughtered dromedaries (*Camelus dromedarius*) from two abattoirs in Southern Algeria. In all 89 paraffin-embedded intestinal samples were analysed, and the DNA was extracted with a commercial kit. Polymerase chain reaction (PCR) was done after optimising the method, and correlations between the DNA concentrations and PCR results were assessed. Of the 85 DNA extracts tested, 12 (14.1%) were PCR-positive for *Eimeria*. Higher infection rates were observed in Sahraoui camels compared with the Targui breed, and in older animals compared with adults. A strong positive correlation was observed between DNA concentration and the success of PCR amplification. This work provides one of the first molecular confirmations of *Eimeria* infection in Algerian

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dromedaries. Further research is needed to identify *Eimeria* species and construct a phylogenetic tree to support epidemiological control strategies.

Keywords: Algeria, camels, *Eimeria*, molecular characterization, parasites

INTRODUCTION

Often referred to as the “ship of the desert”, the camel is not only used as a means of transport, but is also an important source of meat, milk and wool for desert nomads in Asia and Africa (Toaleb and Raafat, 2024). The world’s camel population continues to grow in most countries, particularly in Africa. It is now estimated to be less than 40 million head (Faye et al., 2023). In Algeria, this species plays an important social and economic role for Saharan families. Its breeding does not require substantial resources or advanced breeding techniques (Boukert et al., 2023). Previous studies claimed that camelids are susceptible to many parasitic diseases (Ahmed et al., 2018; Dubey and Schuster, 2018). For example, Maxamhud et al. (2023) reported that the dromedary camels in Algeria can be infected by many parasitic protozoan, helminth, and arthropod species. Most widely studied gastrointestinal protozoan parasites of camels include *Cryptosporidium spp.*, *Eimeria spp.*, *Giardia duodenalis*, *Balantidium coli* and *Enterocytozoon bieneusi* (Dubey and Schuster, 2018), which all have intracellular life stages that inhabit the intestinal epithelium (Craig et al., 2007). Tafti et al. (2000) reported that the most important and frequent pathological lesions in the gastrointestinal tract of camels were caused by *Eimeria* infections (detected in 63 out of 100 slaughtered camels). *Eimeria* genus is an Apicomplexan group, with plenty of species that can infect various animals (e.g., mammals, birds, cattle, poultry, dogs, cats, sheep and goats), although many remain asymptomatic (Dubey and Schuster, 2018). According to Chartier and Paraud (2012) *Eimeria* are monoxenous. Their life cycle includes an exogenous phase (sporogony) - a free-living phase outside the host and an endogenous parasitic phase inside the host. In the host, an asexual (merogony) and sexual reproduction (gamogony) take place. Unsporulated oocysts are eliminated with faeces, and sporulation occurs in 2 to 7 days, depending on the species and environmental conditions (temperature, oxygen and humidity). *Eimeria* infections are transmitted between animals by the faecal-oral route (Al-Afaleq et al., 2018; Abbas et al., 2019). Eimeriosis, AKA coccidiosis, is an important intestinal parasitic disease in pre-weaned and recently weaned camels (Yakhchalim and Cheraghi, 2007). It is also considered an important parasitic disease of camels (Dubey and Schuster, 2018). Several studies have found three species of *Eimeria* in camels in the Turkestan region: *E. bactriani*, *E. cameli* and *E. dromedarii* (Abbas et al., 2019; Hussein et al., 1987). Research on the prevalence of *Eimeria spp.* in camels reported different infection rates (Metwally et al., 2020). For instance, Djerbouh et al. (2018) reported *E. dromedarii* and *E. cameli*, in 9.6% of the samples in Algeria. In Egypt,

Abbas et al. (2019) found *E. cameli*-like parasites in 31%, *E. rajasthani* in 18%, and *E. dromedarii* in 14% of examined animals. In Iran, the reported range of *Eimeria* infections in dromedary camels was 9.51%–63%. According to Metwally et al. (2020), the different infection rates may reflect risk factors that are summarised by geographical distribution and the influence of varying environmental conditions (humidity, temperature, oxygen levels and soil type) affecting oocyst sporulation, and even age and gender, which play an important role in the infection.

In recent years, research has focused on more sensitive methods to improve the diagnostics of parasitic diseases, such as molecular approaches (Hassan et al., 2016). PCR is a simple, fast, specific, and sensitive assay for the confirmation of protozoa (Verweij et al., 2004). Currently, diagnostic laboratories are increasingly turning to DNA-based technology for the identification of particular *Eimeria* spp. (Ekawasti et al., 2022). It should be noted that PCR diagnosis of *Eimeria* is a more reliable, sensitive and rapid method (Malek and Kuraa, 2018): it is easy to amplify DNA from very small amounts and there is a high degree of variability between closely related species (Ekawasti et al., 2019; Hastutiek et al., 2022). However, few studies have investigated the presence of intestinal parasites in dromedary camels in Algeria, most based on microscopic faecal analysis, as reported by Saidi et al. (2022) and Bouragba et al. (2020). On the other hand, a recent study on the histopathology of intestinal coccidiosis in dromedaries was carried out by Boukert et al. (2024) in Algeria, but molecular diagnostics of coccidiosis in dromedaries has never been done in Algeria. Thus, this study aimed to determine, for the first time, the prevalence of coccidia in the small intestines of dromedaries (*C. dromedarius*) in two regions of the southern Algerian Sahara using molecular diagnostics.

MATERIAL AND METHODS

Study area

The camels in this study were slaughtered in two abattoirs: (1) the Royal Slaughterhouse located in the north-east of the Algerian Sahara, in the province of El-Oued (33° 22' 06" N, 6° 52' 03" E), approximately 512 km from the capital, Algiers, and (2) the municipal slaughterhouse of the province of Tamanrasset (22° 47' 13" N, 5° 31' 38" E), in the far south of Algeria, roughly 1,900 km from Algiers.

Sample collection

The study evaluated the use of the PCR to detect the presence of intestinal *Eimeria* spp. in dromedary camels. The samples analyzed in the present work were derived from the same cohort of animals as reported in our previous publication (Boukert et al., 2024). Specifically, 31 samples were collected from clinically healthy

dromedaries intended for meat consumption, these samples were obtained during post-mortem examinations and included different segments of the intestinal tract, namely the duodenum, jejunum, ileum, and cecum. From these samples, a total of 89 paraffin blocks were prepared for molecular diagnosis. This higher number reflects the fact that multiple tissue segments were collected and processed from each animal. In the present study, all samples from the previous study, regardless of their histopathological status (positive or negative), were subjected to PCR-based molecular analysis.

DNA extraction and purification

The parasite DNA was extracted from paraffin blocks following the protocol described by Pikor et al. (2011). Genomic DNA was isolated using the DNA extraction kit (Molecular Genomique Tools, GM Biolab Co., Ltd). The concentrations and purity of the DNA extracted from the 89 samples were measured using a NanoDrop Micro-Spectrophotometer. The required DNA dilutions were prepared with molecular-grade water. All extraction procedures were done at the Microbiology Research Laboratory, Acibadem University in Istanbul (Turkey). However, PCR was performed under conditions that targeted the partial 18S rRNA and the Internal Transcribed Spacer (ITS) region is commonly used as the target gene. Species-level identification and sequencing were not done in this study. Therefore, the detection is reported at the genus level (*Eimeria spp.*) only, and the lack of sequence confirmation limits the epidemiological and diagnostic specificity of the results.

PCR thermocycler conditions

According to Kawahara et al. (2010), the PCR amplification was used to determine the presence of genomic DNA of *Eimeria spp.* Subsequently, the cycle parameters of the PCR reaction were as follows by the thermocycling procedure as shown in Table 1, using the protocol by Salama et al. (2023). A Bio-Rad thermal cycler was used to do PCR. The reaction protocol consisted of 12.5 µL of 2X PCR Master Mix (#RP02-II-400, GeneMark), 1 µL of 10 µM Forward Primers (Table 2), 1 µL of 10 µM Reverse Primer (ITS-F 5'-GCAAAAGTCGTAACACGGTTTCC-3'); (ITS-R 5'-CTGC AATT CACAATGCGTATCG-3'). These ingredients were provided by Eurofins Genomics. So, 25 ng/µL of DNA sample and dH₂O up to 25 µL (Table 3).

Table 1. Thermocycler protocol

Temperature	Time	Number of cycles
94° C	5 min	X 35
94° C	30 sec	
57° C	40 sec	
72° C	45 sec	
72° C	10 min	

Table 2: Primers Used for PCR Amplification of *Eimeria* spp.

Primer Name	Sequence (5' → 3')
ITS-F	GCAAAAGTCGTAACACGGTTTCC
ITS-R	CTGCAATTCACAATGCGTATCG

Table 3: PCR Reaction Components

Component	Volume / Concentration
2X PCR Master Mix	12.5 µL
Forward Primer	1 µL of 10 µM
Reverse Primer	1 µL of 10 µM
DNA Template	25 ng/µL
dH ₂ O	Up to 25 µL total reaction volume

Standard gel electrophoresis procedures

To detect positivity, 2% agarose gel electrophoresis was run at 120 V for 50 min. Samples were prepared by mixing 5 µL of PCR product with a 1:2 ratio of TIBO Gold: Orange G dyes to observe the DNA. To estimate the size of the amplicons, a 300-1000 bp DNA ladder was used. Finally, the gels were visualised using the ChemiDoc™ MP Imaging System. The first group of samples included a positive sample, one of the samples, which served as a positive control for the remaining samples. On the contrary, negative control without DNA was prepared for each run. However, a non-specific amplification band was observed on the 100 bp line. To address this issue, three different optimization studies were performed.

Optimization experiments

Firstly, gradient PCR was performed between 55 °C and 60 °C for annealing, and although there was still a weak non-specific band, 57 °C was chosen as the most suitable annealing temperature. Secondly, instead of using a commercial master mix, a mix was prepared with different MgCl₂ concentrations in the range of 1 mM and 3.5 mM, but without a significant difference. Finally, as the band was suspected to be due to Taq polymerase contamination by *E. coli*, the Taq polymerase enzyme was treated with DNase. However, the non-specific band did not become weaker. It was therefore concluded that this band did not affect positivity/negativity.

Statistical analysis

Statistical analysis was done with SAS software (SAS, 2005). Descriptive statistics was used to analyze the prevalence of *Eimeria* in dromedaries in the study area and

certain risk factors (sex, age and breed). The geographical area was not taken into consideration due to the inequality in the number of samples. Differences were considered significant at $p < 0.05$ with a 95% confidence interval and the X-Squared statistical test was calculated. In the form of a heat map, a correlation matrix provides a measure of the linear relationship between pairs of numerical variables (amount of DNA, H2O and the positive (1), negative (-1) amplification).

RESULTS

Detection of *Eimeria* spp. by PCR

As suggested by Salama et al. (2023), amplification (positivity) was expected in the 500 bp band. Figure 1 (a, b, c and d) summarises the gel electrophoresis snapshots for positive samples. Eleven samples were identified as positive, namely nos. 14, 22, 27, 30, 39, 58, 64, 65, 66, 80 and 85 (Figure 1).

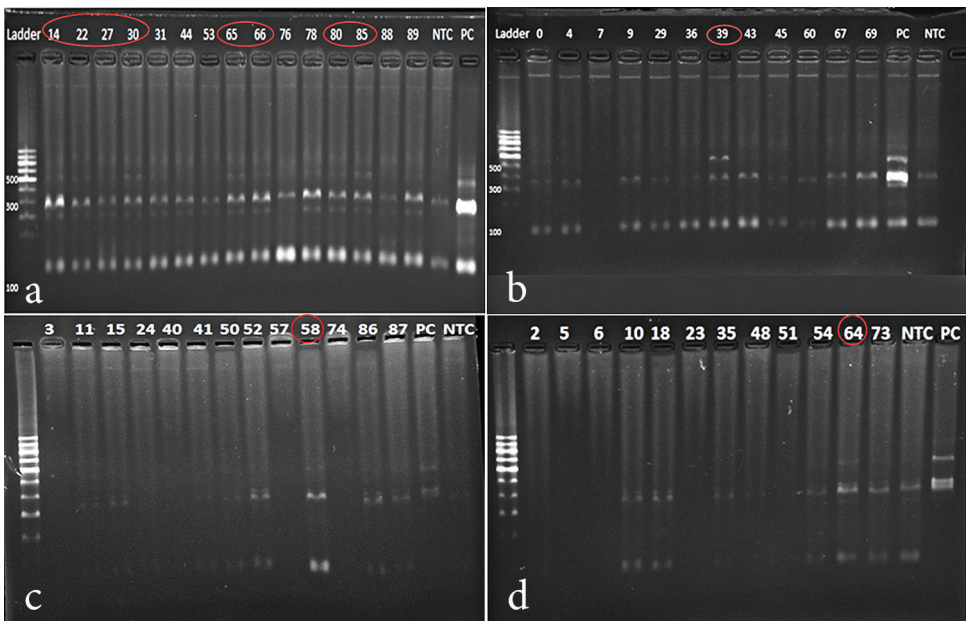


Figure 1. Representative positive PCR amplifications for (a): positive samples: 14, 22, 27, 30, 65, 66, 80, 85 (NTC: Negative Control, PC: Positive Control); (b): 39 was positive; (c): 58 was positive; (d): 64 was positive

Prevalence of Eimeria spp. in camels detected by PCR

Several samples (4 out of 89) showed abnormally high PCR signals and were therefore considered false positives and excluded from the study to prevent their

impact on the statistical analysis. Consequently, 85 DNA samples were retained, out of which 11 tested positive for *Eimeria* spp. (Table 4).

Table 4. Prevalence of *Eimeria* spp. in camels

Technical	No. of DNA samples tested	Positive (+)	Prevalence (%)	IC
PCR	85	11	12.94	5.8 – 20.0

Risk factors for intestinal Eimeria infection in camels

The study identified certain risk factors associated with *Eimeria* spp. infection in dromedaries, including breed, sex and age (Table 5). Statistical analysis showed that there were no significant differences between the three risk factors.

Table 5. Risk factors for Eimeriosis

Variables	Category	EI (%)	NEI (%)	χ^2	<i>p</i> -value
Sex	Female	8.18	47.81	3.87	> 0.05
	Male	4.13	23.18	-	-
Age	< 10 years	4.23	25.76	2.10	> 0.05
	> 10 years	7.66	47.23	-	-
Breed	Sahraoui	9.45	57.53	1.24	> 0.05
	Targui	2.54	15.43	-	-

*EI: *Eimeria* infection; NEI: Non *Eimeria* infection; χ^2 : Chi-square

Correlation matrix for numerical variables used in PCR

A correlation was calculated between the amount of DNA concentration, H₂O and PCR amplification of positive and negative samples. The results are illustrated by a heat map (Figure 2). The results were as follows:

- DNA (25 ng): This variable had a strong negative correlation with DNA (ng/μL) and dH₂O, and a strong positive correlation with PCR amplification (Figure 2).
- dH₂O had a strong negative correlation with ng/μL and DNA (25 ng), and a strong positive correlation with PCR amplification (Figure 2).
- PCR amplification had a weak positive correlation with DNA (ng/μL), DNA (25 ng) and dH₂O, and a perfect positive correlation with itself (autocorrelation). This suggests that amplification increases as DNA concentration and dH₂O increase (Figure 2).

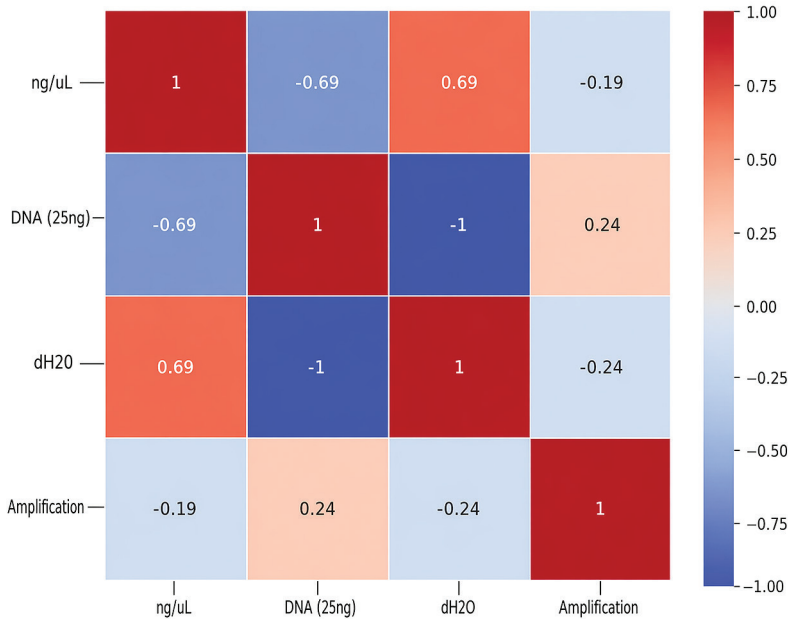


Figure 2. A heat map for three variables

DISCUSSION

Eimeriosis in camelids worldwide has recently been summarised (Dubey and Schuster, 2018). The present study highlighted intestinal infection with *Eimeria spp.* using molecular diagnostics with PCR in camels slaughtered in two abattoirs in southern Algeria. However, there is a paucity of data on intestinal infections in camels, particularly based on molecular methods, which makes it difficult to discuss our results. The studies carried out in Algeria were based exclusively on the detection of intestinal parasites in faecal samples. For example, data reported by Djerbouh et al. (2018) proved *Eimeria dromedarii* (4.8%) and *Eimeria cameli* (9.6%) in five localities in the South of Algeria. According to Bouragba et al. (2020) *Eimeria spp.* (3.21%) were found on 28 farms at the Steppe and Northern Sahara regions and Saidi et al. (2022) detected *Eimeria* species in 20% of camels in the Laghouat region (Southern Algeria). The molecular study, using the PCR assay, confirmed the positive amplification of *Eimeria spp.* DNA in 12.94% (11/85) animals. This result is important due to the high sensitivity and specificity of PCR technique in comparison with other diagnostic methods (Mahmmoud et al., 2010; Qablan et al., 2013). However, this is in sharp contrast to what Salama et al. (2023) found in Egypt, who failed to detect *Eimeria spp.* in dromedaries by PCR, despite the presence of oocysts in faeces. This negative identification of *Eimeria spp.* by PCR is possibly related to fecal PCR inhibition and/or a failure to break oocyst walls

during the DNA extraction (Raj et al. 2013). However, the DNA in our study was extracted directly from the paraffin blocks. Moreover, the investigation method, the time of sampling, and the number of screened animals are considered as important factors (Bouragba et al., 2020). In China (Inner Mongolia), Wei and Wang (1990) detected *Eimeria* spp. in up to 50% Bactrian camels. The study by Tafti et al. (2000) indicated that the most important and frequent pathological lesions in the digestive tract of camels are those resulting from *Eimeria* infections (63% of 100 slaughtered animals). However, the prevalence of *Eimeria* spp. depend on variable factors such as the animal species, age, immune status, stress factors, environment, and management practices (Sazmand et al., 2012). An important fact that contributes to the epidemiology of coccidiosis is the high resistance of oocysts to environmental conditions (Utebaeva et al., 2021). In this study, we considered some risk factors. The seasonal influence was not one of them because we conducted our research in late autumn and winter (November and December 2022). The study by Utebaeva et al. (2021) showed that spring and autumn are the two seasons when *Eimeria* infection is significant. Female dromedaries (8.18%) were more infested by this parasite than the males (4.13%). Therefore, this finding was approved by many studies, such as in histopathological research by Boukert et al. (2024) and by a coprological examination by Metwally et al. (2020). Regarding the age, animals over 10 years old were the most infected by *Eimeria* spp. Our results differ from those published by Sazmand et al. (2012), who found that young males are the most infected with eimerians by coprology examination, and that the Sahraoui breed is more prone to infection compared to the Targui. A statistical analysis was also carried out to assess the association between age, race and the presence of parasites. Therefore, the intestinal damage caused by *Eimeria* spp. leads to both clinical disease and production losses (reduced weight gain, poor feed conversion, lower meat and milk yield). The economic impacts arise from reduced performance, increased management costs, and lower reproductive efficiency, which are significant for producers who depend heavily on camel products (Ahmed et al., 2025). After calculating the X², we found no relationship. The heat map revealed a weak positive correlation between DNA concentration and PCR amplification, indicating that more DNA leads to greater amplification. Conversely, a strong negative correlation was observed between water (dH₂O) and PCR amplification, suggesting that excess water can dilute the reaction and reduce amplification efficiency. Although there was a weak negative correlation between DNA and water, this was not considered a significant factor that influenced the reaction. The heat map confirmed the general understanding of PCR and highlighted the importance of maintaining optimal levels of DNA and water for efficient amplification. According to Kramer and Coen (2001), the efficiency of target DNA amplification in the PCR assay is highly dependent on its initial concentration in a given sample. However, further experiments are needed to establish a direct cause-and-effect relationship and to take into account other factors that may influence the amplification results. It is

important to note that the present study did not include histopathological evaluation or clinical data correlation, despite the availability of paraffin-embedded tissue samples. The absence of microscopic confirmation of *Eimeria* oocysts or associated intestinal lesions limits the ability to link PCR detection to pathological changes or clinical disease. Future investigations should integrate molecular and clinical approaches to provide a more comprehensive understanding of the epidemiology and pathogenicity of *Eimeria spp.* in camels.

It would be plausible to complete this study by sequencing. However, to date, there are no 18S rRNA sequences of *Eimeria cameli* available in GenBank (Narnaware et al., 2024). Consequently, further research is needed to develop new molecular methods, as well as means of prevention and/or treatment of *Eimeria* infection, as indicated by Bussière et al. (2018). Although there are well-established methods for reproducing life cycles in *in vitro* cultures, such as those used for *Toxoplasma gondii* and *Babesia spp.*, they are not yet available for *Eimeria spp.* (Bussière et al., 2018).

CONCLUSION

The results of this study provide, for the first time, a molecular diagnosis of the prevalence of *Eimeria spp.* in the host intestinal epithelium in dromedaries from two slaughterhouses in southern Algeria. In order to reduce this infection, adequate control of coccidiosis must be established, especially in dromedary calves. First, animals should be separated according to age group (avoid mixing young and adult animals), high-quality colostrum should be provided, and stress should be minimised (for example, weaning, transport, dietary changes). However, few studies have been conducted on the treatment of coccidiosis in camels. Some of them have proven the efficacy of aqueous suspension of sulfadimidine administered orally to dromedary calves for 10 days at a dose of 30 mg/kg body weight. Therefore, our previous findings were based solely on molecular investigation by PCR. Thus, a prevalence of *Eimeria spp.* in dromedaries was determined, proving the efficacy of the PCR used. Further molecular studies are needed to determine the exact taxonomic status of various *Eimeria* species found in camels in different areas of southern Algeria. Larger sample sizes are needed to gain a better understanding of the epidemiology of the infection, as eimeriosis can lead to significant economic losses.

Author's contribution

RB and DS designed, wrote the manuscript and reviewed the study. SB, BB and MC analyzed the data. AAB, BŞ, EEIC, KBK, GS and TK contributed to the investigation and methodology. NS reviewed the manuscript. All authors read and approved the final manuscript.

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Ethical statement

Animal experiments in the present study were performed under the approval of the guidelines of the Institutional Committee for the Protection of Animals in Algerian Higher Education and Scientific Research (agreement no. 45/DGLPAG/DVA.SDA.14).

Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no conflict of interest.

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PRELAVENCIJA, FAKTORI RIZIKA I MOLEKULARNA POTVRDA PRISUSTVA VRSTA RODA *EIMERIA* KOD JEDNOGRBIH KAMILA (ALŽIR)

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Kratak sadržaj

Cilj rada bio je primenom molekularnih metoda ustanoviti infekciju creva vrstama *Eimeria* kod kamila (*Camelus dromedarius*) zaklanih u dve klanice na jugu Alžira. Pregledano je ukupno 89 uzoraka creva kalupljenih u parafinu, a DNK je izolovana primenom komercijalnog kita. Lančana reakcija polimerizacije (PCR) je urađena posle metodološke optimizacije, i izračunata korelacija između koncentracije DNK i rezultata PCR. Od 85 izolata DNA koji su testirani, 12 (14,1%) je bilo pozitivno na prisustvo *Eimeria*. Infekcija je bila raširenija kod Sahraoui kamila nego kod kamila rase Targui, kao i kod mlađih životinja u odnosu na odrasle. Ustanovljena je visoka pozitivna korelacija između koncentracije DNK i uspešne amplifikacije u PCR. Ovaj rad je jedan od prvih koji pruža molekularno-biološku potvrdu infekciju vrstama roda *Eimeria* kod kamila u Alžiru. Potrebna su dalja ispitivanja da bi se ustanovile vrste *Eimeria* i konstruisalo filogenetsko stablo kako bi se unapredile strategije epidemiološke kontrole.

Ključne reči: Alžir, kamile, *Eimeria*, molekularna karakterizacija, paraziti

Original research article

CLINICOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL EVALUATION OF MAMMARY TUMOURS IN INTACT FEMALE DOGS

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Abstract

Canine mammary tumors (CMTs) are among those most frequently diagnosed in intact female dogs. They share biological and hormonal similarities with human breast cancer, which renders them valuable models in comparative oncology. This study aimed to evaluate the clinical, histopathological, and immunohistochemical characteristics of CMTs in intact female dogs. A total of 22 archived CMT cases submitted between 2013 and 2023 was conducted at the Federal University of Agriculture, Abeokuta. Samples were obtained through mastectomy or nodulectomy from female dogs presented at private veterinary clinics and a teaching hospital. Clinical data, including age, tumour size, location, lymph node involvement, and spay status, were recorded. Most dogs were German Shepherds (45.5%) with a mean age of 8.15 years; none were spayed. The tumours were predominantly malignant (77.3%) and commonly located in the inguinal mammary glands (40.9%). Stage T₂ (45.5%) and histological grade II (72.7%) were the most prevalent. E-cadherin was immunopositive in 89% of tumours, with stronger expression in malignant cases. N-cadherin expression was significantly associated with malignancy ($p = 0.001$), indicating epithelial-mesenchymal transition (EMT). Oestrogen receptors α (OR α) was strongly expressed in all tumours, with significantly higher levels in cases of malignancy ($p = 0.002$). Progesterone receptor (PR) and vimentin were observed only in

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malignant tumours, while Ki-67 showed mild, non-significant expression. These findings suggest that N-cadherin, OR α , and PR may serve as potential biomarkers of malignancy and hormonal responsiveness in CMTs. They also support the relevance of molecular profiling in the diagnosis and management of canine mammary tumours.

Keywords: Canine mammary tumors, comparative oncology, immunohistochemistry, N-cadherin, oestrogen receptors

INTRODUCTION

Mammary gland tumors have become a significant concern in both human and veterinary medicine due to their increasing incidence (Vascellari et al., 2016). In dogs, canine mammary tumors (CMTs) are the second most frequently diagnosed neoplasms after skin tumors (Terzaghi et al., 2020). Studies estimate that CMTs account for 50–70% of all tumors diagnosed in sexually intact female dogs (Sorenmo et al., 2013; Terzaghi et al., 2020).

Three major risk factors have been identified in the development of CMTs: prolonged hormonal exposure, advancing age (particularly in middle-aged and older dogs), and breed predisposition (especially among purebred dogs). Other contributing factors include nutritional influences and genetic susceptibility (Sorenmo et al., 2013).

CMTs are histologically classified as simple (comprising a single cell type—either epithelial or myoepithelial), complex (containing both epithelial and myoepithelial components), or mixed (featuring epithelial and/or myoepithelial cells with areas of cartilage or bone formation) (Pena et al., 2014).

Due to shared features in spontaneous development, clinical behavior, histopathology, and prognosis, dogs with CMTs are considered excellent comparative models for human breast cancer research (Hussain et al., 2018; Kumar et al., 2019). The canine mammary gland exhibits a complex branching architecture and diverse immunohistochemical profiles (Sorenmo et al., 2013). The heterogeneous histological and immunohistochemical nature of CMTs often complicates diagnosis (Kumar et al., 2019).

Several immunohistochemical markers, including cytokeratin, calponin, smooth muscle actin (SMA), p63, protein 14-3-3 σ , and CD-10 antigen, have been investigated to assist in identifying myoepithelial cells and improving diagnostic accuracy (Sánchez-Céspedes et al., 2013; Lopuszyński et al., 2019).

Ovarian hormones play a critical role in the etiology of mammary tumours in both species. Oestrogen receptors (ORs) and progesterone receptors (PRs) have been detected in benign and malignant CMTs (Chang et al., 2009). Notably, intact, young female dogs in estrus are more likely to develop receptor-positive tumors than older, spayed, or anestrus dogs (Sorenmo et al., 2013).

The pathogenesis of CMTs involves complex interactions among genetic mutations, epigenetic changes, hormonal dynamics, and alterations in the tumour microenvironment (Carvalho et al., 2013). Epithelial-mesenchymal transition (EMT), a key process wherein epithelial cells lose their adhesion and gain invasive mesenchymal characteristics, is central to tumour progression and metastasis (Dongre and Weinberg 2019).

This study correlated the expression patterns of E-cadherin (E-CAD), N-cadherin (N-CAD), Ki-67, vimentin and hormonal receptors in CMTs to enhance diagnostic precision and support informed treatment decisions, including surgery and adjuvant therapy. While previous research has assessed individual biomarkers in CMTs, few have taken an integrated approach. This study aimed to fill that gap by carrying out a comprehensive molecular profile to better predict tumor progression and therapeutic response in dogs.

MATERIALS AND METHODS

Tissue samples collection

Twenty-two canine mammary tumour (CMT) samples were obtained via excision biopsy (mastectomy or nodulectomy) from female dogs (n = 22) submitted to the Department of Veterinary Pathology, Federal University of Agriculture, Abeokuta. These cases originated from various private veterinary clinics and the University's Veterinary Teaching Hospital between 2013 and 2023.

Histopathological examination

Tissues were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin wax. Sections measuring 4-5 µm were cut from each block for haematoxylin and eosin (H&E) staining. Histological evaluations were independently performed by four pathologists. Additional sections were prepared for immunohistochemistry (IHC) analysis. Clinical data—including breed, age, body size, tumour location, ovariohysterectomy (OHE) status, and lymph node involvement or metastasis (based on cytological evaluation) were extracted from accompanying case history forms. Tumours were histologically classified and graded according to the modified World Health Organization system (Goldschmidt et al., 2011) into grades I, II, and III. Staging followed the Tumour–Node–Metastasis (TNM) system, depending on the tumour size: T₁ (<3 cm), T₂ (3–5 cm), and T₃ (>5 cm) (Sorenmo et al., 2003).

Immunohistochemistry (IHC)

Immunohistochemical analysis was done using a panel of antibodies targeting E-cadherin, N-cadherin, Ki-67, vimentin, progesterone receptor (PR), and oestrogen receptors (OR α and OR β). The 2-Step plus Poly-HRP anti-rabbit IgG detection system was used to detect antigen expression in neoplastic mammary tissues. Sections were dewaxed, rehydrated, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. Antigens were retrieved by heating the sections in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven (650 W) for 10 minutes, which was followed by cooling for 20 minutes. Non-specific binding was blocked using normal goat serum for 30 minutes. Sections were incubated overnight at 4 °C with primary monoclonal antibodies against E-cadherin (Bioss, USA), N-cadherin (Elabscience, USA), Ki-67 (Novus, USA), vimentin (Novus, USA), and anti-progesterone receptor (Abcam, UK), as well as polyclonal antibodies against OR α and OR β (Abcam, UK). Secondary antibody incubation (polyperoxidase–anti-rabbit IgG) was done at room temperature for 30 minutes. Immunoreactivity was visualized using 3, 3'-diaminobenzidine (DAB), counterstained with haematoxylin, dehydrated, and mounted. Normal canine mammary gland and uterine tissues served as positive controls (Table 1).

Table 1: Primary antibodies used in immunohistochemistry

Antibody	Type/Clone	Dilution	Source	Positive control tissue
E-Cadherin	Rabbit/Polyclonal	1: 400	Bioss, USA	Mammary gland
N-Cadherin	Rabbit/Polyclonal E-AB-70061	1: 400	Elabscience, USA	Mammary gland
Ki-67	Rabbit/Polyclonal NB500-170	1: 150	Novus, USA	Mammary gland
Anti-Progesterone	Mouse/Monoclonal PR-AT4.14	1: 100	Abcam, U.K	Mammary gland
Anti-Oestrogen α	Rabbit/Polyclonal Ab 37438	1: 100	Abcam, U.K	Uterus, Mammary
Anti-Oestrogen β	Rabbit/Polyclonal Ab 3577	1: 100	Abcam, U.K	Uterus, Mammary

Source: Research's compilation (2025)

Immunohistochemical analysis

Out of the 22 canine mammary tumor samples collected, only 9 consisted of sufficient, well-preserved tissue available for the IHC analyses of E-cadherin, N-cadherin, and Ki-67. The remaining samples were depleted during diagnostic processing. In contrast, all 22 samples were suitable for the molecular analysis

of OR α . Following the immunohistochemical (IHC) procedure, each slide was examined under a light microscope for general assessment. Quantitative image analysis was subsequently performed to evaluate the expression of E-cadherin, N-cadherin, vimentin, Ki-67, OR α , OR β , and PR in tumour epithelial cells using AmScope pathology imaging software (AmScope Technologies, Chester Hill, NSW, Australia). Oestrogen receptors (OR α and OR β) were scored as described by Benavente et al. (2019). At 400 $\times$ magnification, 10 representative high-power fields per slide were selected randomly from representative tumour areas, avoiding necrotic regions and tissue edges. The proportion score (PS) was calculated as the percentage of positively stained cytoplasm or nuclei (brown signal) relative to the total area (positive + haematoxylin-stained). PS was rated on a scale of 0–5: 0 (no staining), 1 (<1%), 2 (1–10%), 3 (10–33%), 4 (33–66%), and 5 (66–100%). Intensity score (IS) evaluated staining intensity on a 0–3 scale: 0 (none), 1 (faint), 2 (moderate), 3 (strong). The total score (TS = PS + IS) categorized expression as negative (0–2) or positive (3–8) (Allred et al., 1998).

For PR and vimentin, the immunoreactive score (IRS) method was applied (Gartner et al., 1999; Arihiro et al., 2010). The percentage of positive cells was graded as: 0 (none), 1 (<10%), 2 (10–50%), 3 (51–80%), and 4 (>80%), with intensity scored as 0 (none), 1+ (weak), 2+ (moderate), and 3+ (strong). Final IRS ranged from 0 to 12: 0–1 (negative), 2–3 (mild), 4–8 (moderate), and 9–12 (strong). E-cadherin and N-cadherin expression was assessed semi-quantitatively based on the percentage of positive cells: <10%, 10–50%, or >50%. Ki-67 expression was determined by counting 50–100 tumour cells and calculating the labeling index (LI) as: Ki-67 LI (%) = (Number of positive nuclei / Total nuclei counted) $\times$ 100. Proliferative activity was categorized as low (<15–20%), moderate (20–40%), or high (>40%) (Markov et al., 2024). These cut-offs were adopted provisionally due to the lack of standardized thresholds for canine mammary tumours.

Statistical analysis

The results were analysed using descriptive statistics for each of the variables: tumour classification, histological subtypes, tumour size, location, staging, histological grade and lymph node metastasis. Means were compared in ANOVA; the Chi-square test was used to analyse the total score of the Allred TS and IRS for the associations between the expression of different antigens between benign and malignant CMTs. All statistical analyses were done with SPSS version 27 software. The p-values <0.05 were considered significant.

RESULTS

A total of 10 dog breeds were represented in the study, with the German shepherd (45.5%), and Lhasa apso (13.6%) being the most frequent. The mean

age of the affected was 8.15 years (range 4-13 years). No dog had undergone ovariohysterectomy. Most tumours were stage T₁ (n=8, 36.4%), and T₂ and T₃ were equally represented (31.8% each). T₂ and T₃ stages were more frequent in malignant tumours (77.3%) and T₁ in benign growths (22.7%). CMTs were mostly located on the left or right inguinal mammary gland, 40.9% in each; 18.2% (n=4) cases (malignant tumours) had lymph node involvement (Table 2).

Table 2: Prevalence, clinical, and pathological characteristics of CMTs

Variables	N (%)
Breed	
Pure breed	9 (90)
German shepherd	10 (45.5)
Lhasa apso	3 (13.6)
Cane Corso	1 (4.5)
Terriers	1 (4.5)
Samoyed	1 (4.5)
Dobermann	1 (4.5)
Rhodesian ridgeback	1 (4.5)
Rottweiler	1 (4.5)
Local	1 (4.5)
Mixed breed	1 (10)
Mongrel	2 (9.1)
Ovariohysterectomy	
Intact	22 (100)
Spayed	0
Tumour size	
T ₁ (<3cm)	7 (31.8)
T ₂ (3-5cm)	8 (36.4)
T ₃ (>5cm)	7 (31.8)
Tumour location	
Cranial thoracic mammary glands	2 (9.1)
Caudal abdominal mammary glands	2 (9.1)
Inguinal mammary glands	18 (81.8)
Lymph node involvement	
Yes	4 (18.2)
No	18 (81.8)

Histopathology

In the study, a total of 9 histological subtypes of CMT were diagnosed, out of which 22.7% (n=5) were benign and 77.3% (n=17) malignant. CMTs were diagnosed at stages T₁ (36.4%, n=8), T₂ (45.5%, n=10), and T₃ (18.2%, n=4). Stages T₁, T₂, and T₃ occurred more frequently in malignant tumours (77.3%) ($P = 0.03$) and all benign tumours were stage T₁ (22.7%). Regarding malignancy, grade I and grade II were observed: grade II was more frequent (72.7%, n=16) in comparison to grade I, which was 4.5% (n=1) (Table 3 and Figures 1A-F).

Table 3. Association between histological subtype with tumour staging and histological malignancy grading

Histological subtype	N (%)	Staging (%)	Histological grading (%)
Malignant	17 (77.3)		
Malignant myoepithelioma	2 (9.1)	V (9.1)	II (9.1)
Carcinoma (tubular)	4 (18.2)	I (4.5) II (9.1) V (4.5)	II (18.2)
Ductal carcinoma	1 (4.5)	II (4.5)	II (4.5)
Squamous cell carcinoma - Spindle cell variant	1 (4.5)	V (4.5)	II (4.5)
Inflammatory carcinoma	4 (18.2)	I (4.5) II (13.6)	II (18.2)
Carcinoma (solid type)	3 (13.6)	I (4.5) II (9.1)	II (9.1) I (4.5)
Carcinoma (mixed type)	2 (9.1)	II (9.1)	II (9.1)
Benign	5 (22.7)		
Simple adenoma	3 (13.6)	I (13.6)	0
Complex adenoma	2 (9.1)	I (9.1)	0
		<i>p</i> value	<i>p</i> value
		0.027	0.028

Chi-square ($P < 0.05$ was considered significant)

IHC expression of the receptor and cell differentiation

E-cadherin, N-cadherin, and vimentin were expressed in both the nuclei and cytoplasm of neoplastic epithelial cells, while ORa expression was observed in the cytoplasm of neoplastic epithelial cells; PR and Ki-67 expressions were confined to the nuclei of neoplastic cells. The negative controls were characterized by the absence of immunostaining (Table 4).

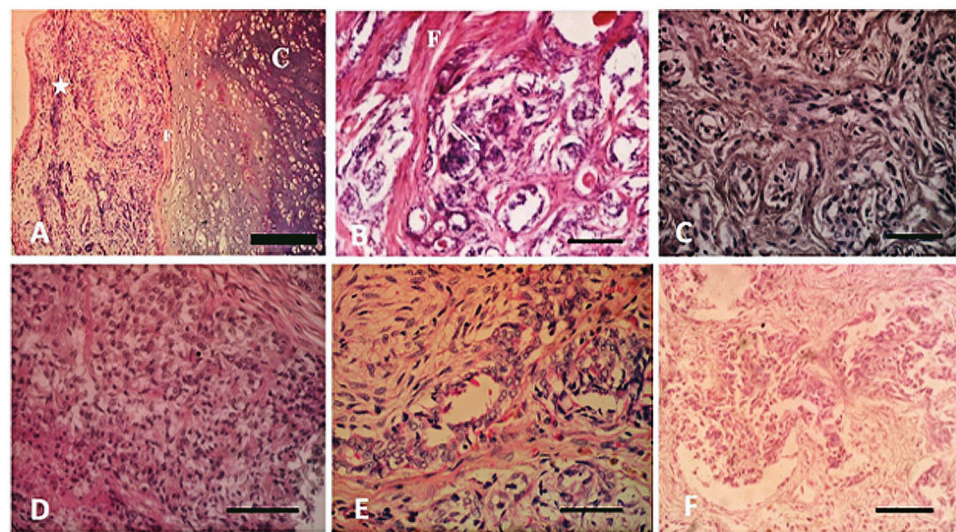


Figure 1. A. Mammary carcinoma (mixed type), neoplastic myoepithelial cells (star) and a large area of cartilage formation with an area of calcification (C) separated by fibrous connective tissue (F), H&E Stain. Scale bar=100µm; B. Mammary carcinoma, hyperchromatic myoepithelial cells (arrow) and separated by fibrous connective tissue (F); C. Carcinoma (tubular type), hyperchromatic myoepithelial cells and presence of mitotic figure; D. Simple carcinoma (solid type), neoplastic cells in solid sheets and cords; E. Complex adenoma, epithelial (tubular) and myoepithelial proliferation with spindle cells with poorly demarcated cell borders and a moderate amount of cytoplasm; F. Inflammatory carcinoma, mononuclear cells infiltration of the acini. H&E Stain. Scale bar=20µm.

Table 4. Immunohistochemical expression of E-cadherin, N-cadherin, Ki-67, Vimentin, OR α , OR β , and PR in canine mammary tumours

Immunohistochemical marker	Malignant CMT	Benign CMT	<i>p</i> value
	Positive Staining n (%)	Positive Staining n (%)	
E-cadherin	8(89)	1(11)	0.003
N-cadherin	9 (100)	0 (0)	0.001
Ki-67	5 (56)	4(44)	0.002
Vimentin	4 (18.2)	0 (0)	0.487
OR α	17 (77.3)	5 (22.7)	0.009
OR β	0 (0)	0 (0)	-
PR	9 (40.9)	0 (0)	0.214

Chi-square ($P < 0.05$ was considered significant)

E-cadherin expression

Out of the 9 samples analysed for E-CAD, 8 (89%) were immunopositive: both malignant and benign tumours expressed the antigen. The expression was predominantly observed in mesenchymal cells irrespective of the tumour malignancy (Figure 2A). However, the expression was mild in benign tumours, while malignant tumours showed severe to moderate expression. The mean E-CAD TS was 1.41 ± 0.150 for the malignant tumours and 1.00 ± 0.00 for benign tumours. The association between the E-CAD TS and the classes of tumours was not significant ($p=0.097$, Figure 3).

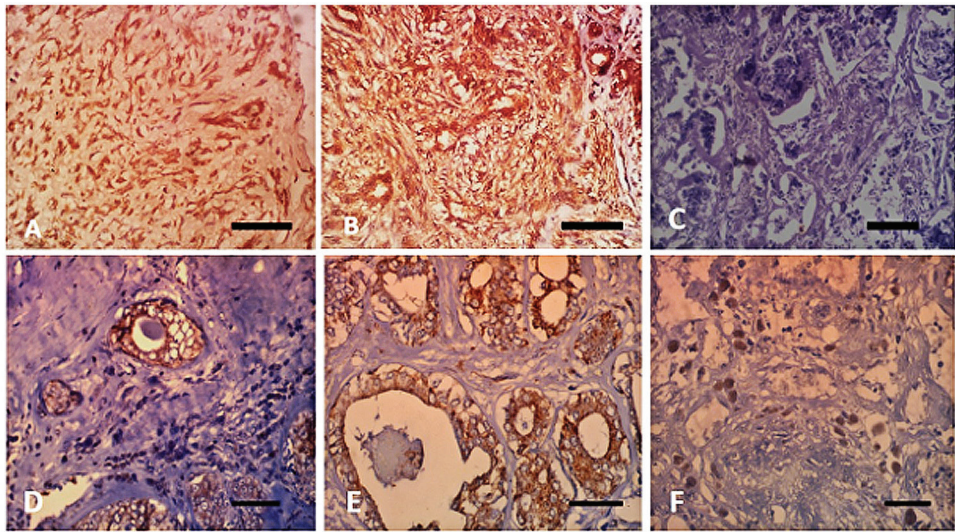


Figure 2. A. E-cadherin, IHC, diffuse severe cytoplasmic expression, mesenchymal cells (carcinoma, mixed type), scale bar=20μm; B. N-cadherin, IHC, diffuse severe cytoplasmic expression, epithelial cells, (mammary carcinoma, tubular), scale bar=20μm; C. Ki-67, IHC, mild nuclear expression, epithelial cells (inflammatory carcinoma), scale bar=20μm; D. Vimentin, IHC, moderate nuclear expression, mesenchymal cells (carcinoma, mixed type), scale bar=20μm; E. ORα, IHC, severe cytoplasmic expression by epithelial cells (mammary carcinoma, complex), scale bar=20μm; F. Progesterone, IHC, moderate nuclear expression, epithelial cells (carcinoma), scale bar=20μm.

N-cadherin expression

All of the 9 samples analysed for N-CAD were immunopositive, both the malignant and benign tumours expressed the antigen. The cells that mostly expressed the N-cadherin antigen were the epithelial cells of both the benign and malignant tumours (Figure 2B). The expression was severe in malignant tumours, and mild to moderate in the benign tumours. The mean N-CAD TS was 1.88 ± 0.81 for the malignant tumours and 1.00 ± 0.00 for benign tumours ($p = 0.001$). There

was a significant association between the N-CAD TS and the classes of tumours (Figure 3).

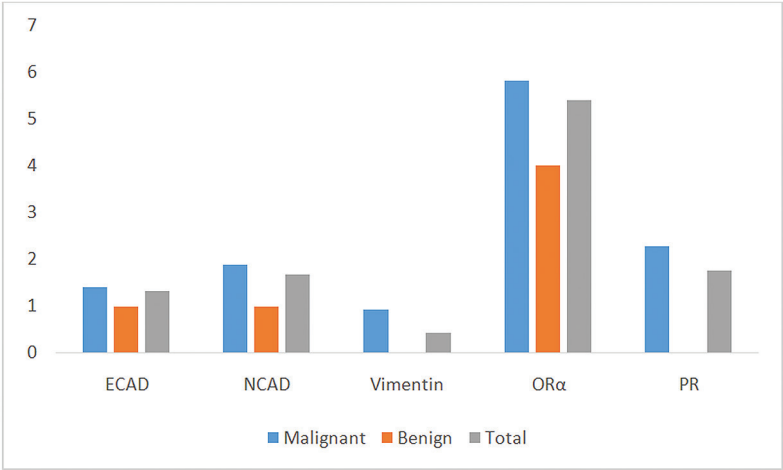


Figure 3. Mean immunohistochemical expression of E-cadherin, N-cadherin, vimentin, OR α , and PR in benign and malignant tumours. Malignant tumours generally showed higher expression levels compared with benign tumours, with statistically significant increases observed for N-cadherin, vimentin, OR α , and PR.

Ki-67 expression

Out of 9 samples analysed for Ki-67, very few were immunopositive; both the malignant and benign tumours expressed the antigen in a very mild degree. The epithelial cells of both the benign and malignant tumours mostly expressed Ki-67 antigen in this study. The expressions were mild in malignant tumours and benign tumours (Figure 2C).

Vimentin expression

About 18% ($n = 4$) of cases expressed immunopositive reactions for vimentin, in both the nuclei and cytoplasm of mesenchymal cells (Figure 2D). Half of them were moderately positive and the rest mildly positive. Vimentin expression was absent in benign tumours. The mean vimentin IRS for the malignant tumours was 0.94 ± 0.441 ($p = 0.269$, Figure 3).

Oestrogen receptor expression

Twenty-two samples were analysed for OR α , and all proved immunopositive (Figure 2E). The mean OR α TS in malignant tumours (simple and mixed) was 5.82

± 1.13 and 4.00 ± 0.00 in benign tumours (simple and complex) ($p = 0.002$, Figure 3). There was no expression for OR β .

Progesterone receptor expression

Out of 22 samples analysed for PR, 9 (40.9%) were immunopositive; all were of malignant tumour origin. Progesterone receptor expression was present mainly in the epithelial cells (Figure 2F): 31.8% were moderately positive and 9.1% mildly positive. The mean PR IRS was 2.29 ± 0.561 for the malignant tumours ($p = 0.041$, Figure 3). See also Supplementary table 1.

DISCUSSION

Mammary gland tumours have been shown over the years to be hormonally regulated in both dogs and humans, resulting from similar hormone dependency between human breast cancer and CMT (Sleeckx et al., 2011). Several studies have demonstrated the use of naturally occurring mammary gland tumours and other tumour types in dogs as sentinels for comparative studies between different types of mammary gland tumours and other tumour types in humans for diagnosis, prognosis, and development of novel therapies (Ranieri et al., 2013; Goebel and Merner, 2017). In this study, purebred dogs were overrepresented, which agrees with the findings of Vascellari et al., (2016): they reported a higher prevalence of CMT in purebred dogs, and a general increase in CMT, similar to that of human breast tumours. Only intact female dogs were affected in the current research, which affirmed the link between hormonal exposure (especially oestrogen and progesterone) and mammary tumour development. Moreover, unlike in some other countries, dog spaying at a young age is not a common practice in Nigeria, which may additionally influence the rise in the incidence of CMT. Similarly, the studies by Sorenmo et al. (2013) and Brønden et al. (2010) also reported that tumour incidence depends on the study region and the characteristics of the targeted population. The mean age of the affected dogs in this study aligns with the literature data (Sleeckx et al., 2011).

Stages T₂ and T₃ were more frequent in malignant tumours in this study, which is consistent with the results by Cassali et al. (2014), who reported that larger tumours are significantly associated with higher malignancy potential and poorer prognosis. However, it has been reported that the tumour size is irrelevant if the local lymph node is involved (Kurzman and Gilbertson, 1986). In this study, a few cases of malignant CMT had lymph node involvement, suggesting the risk of metastasis, a key prognostic indicator, which is comparable to previous studies noting metastasis rates between 10–50% depending on histological grade (Pérez Alenza et al., 1997). The inguinal mammary glands were the most common sites (40.9%), which aligns with previous findings that the caudal mammary glands are more frequently affected

due to their larger volume and higher hormonal responsiveness (Karayannopoulou et al., 2005).

Among malignant tumours, grade II was the most common, but benign tumours were only of grade I, confirming that higher grades are more indicative of malignant transformation. The high occurrence of histological grade II tumours also reflects the data provided by Pena et al. (2014), who reported it as the most prevalent in malignant CMTs, often associated with moderate differentiation and intermediate prognosis.

Simple carcinoma was significantly more frequent in this study, in which simple carcinoma (tubular) and inflammatory carcinoma occurred most. This contrasts with Vascellari et al. (2016), who reported a significantly higher incidence of complex carcinoma in their study. This could result from the difference in the study region, available samples, and the unwillingness of some dog owners to pay for diagnostics.

Both benign and malignant tumours expressed E-cadherin, although the latter showed stronger expression, contrary to many studies, which claimed that the loss of E-cadherin is associated with tumour invasiveness and metastasis (Gama et al. 2007). E-cadherin is typically considered an epithelial marker, and its expression in mesenchymal-like cells is unexpected. In this study, the mesenchymal cells were detected based on morphology in the tissue sections, but some of these cells may have retained epithelial characteristics or represented areas of tumour cells undergoing partial epithelial–mesenchymal transition (EMT). Therefore, the observed E-cadherin expression likely reflects heterogeneity within the tumour microenvironment rather than true mesenchymal cell expression. However, comparably to our findings, some studies have reported variable expression patterns of E-cadherin depending on tumour subtype and cell differentiation (Łopuszyński et al., 2015). The borderline association suggests a complex role of E-cadherin in tumour biology, beyond a simple on-off mechanism. This research found a statistically significant increase in N-cadherin expression in malignant tumours, supporting the role of epithelial–mesenchymal transition (EMT) in tumour progression. These results are consistent with Yoshida et al. (2014), who reported that increased N-cadherin expression correlates with aggressive phenotypes in canine mammary tumours (CMTs), and support the “cadherin switch” hypothesis as a hallmark of epithelial–mesenchymal transition (EMT), within the limitations of the sample size analysed.

Vimentin was expressed only in malignant tumours, further supporting its role as a mesenchymal marker in EMT processes. Although not statistically significant, its presence in a subset of malignant cases reflects the findings by Leonel et al. (2017), who showed that vimentin co-expression with epithelial markers may be indicative of invasiveness and stem-like tumour behaviour.

Only mild expression of Ki-67 was observed, with no significant difference between tumour classes. This contrasts with prior studies, where higher Ki-67 index

was significantly associated with malignant and poorly differentiated tumours (Salaritabar et al., 2019). The discrepancy in this study could be attributed to sample size limitations or variations in fixation and antigen retrieval protocols. Also, the observed low proliferative activity may suggest the predominance of low-grade tumours and the heterogeneity of canine mammary tumours, which can display lower Ki-67 indices compared to more aggressive tumours.

The strong expression of OR α in both tumour types supports the evidence that hormone receptor status is not always inversely correlated with malignancy (Nieto et al., 2000). This finding also aligns with Millanta et al. (2005), who emphasized the heterogeneity of OR α expression in CMTs and suggested that it could influence tumour behaviour and response to hormonal therapy.

The selective expression of PR supports the idea that PR may be a marker of hormonal responsiveness in malignant CMTs. Several studies have similarly found that PR expression may be retained even in more aggressive tumour forms (Chang et al., 2005), and that PR-negative tumours are often associated with poor prognosis.

CONCLUSION

This study supports that the known CMT patterns in age, intact status, tumour size, and location are critical factors. The observed associations of N-cadherin, OR α , and PR with tumor characteristics are preliminary and require validation in larger-scale studies before these markers can be considered reliable biomarkers of malignancy. The findings in this study have confirmed the similarities between CMT and human breast cancer, and suggested CMT as a spontaneous model for human breast cancer. The heterogeneous groups of different molecular-driven tumours were identified in this study; we recommend that comparative research on homogeneous groups of CMT should be carried out for more reliability and that oestrogen subtypes should be considered during therapy administration. To the best of the authors' knowledge, there has not been any documented study on the immunohistochemical study of CMT in this region.

Authors' contribution

TMO: Conceptualization, investigation, designing, data analysis, supervision, and writing of the original draft. OLA: Conceptualization, designing, supervision and writing – review. TMO & OLA: Writing – review. TMO, OLA, FMM & JTO: Sample collection and writing. The first draft of the manuscript was written by TMO and all authors commented on the previous versions of the manuscript. All authors read and approved the final manuscript.

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Ethical statement

No ethical approval was obtained because this study did not involve laboratory animals and consisted solely of non-invasive procedures (collection of tissue after surgery). All tissues were residual clinical specimens collected during routine surgery, and no additional procedures were performed for research purposes. This retrospective study was performed on archived diagnostic samples. All data were anonymized, and no owner consent was required according to institutional guidelines.

Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors state that there are no competing interests to declare.

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KLINIČKOPATOLOŠKA I IMUNOHISTOHEMIJSKA PROCENA TUMORA MLEČNE ŽLEZDE KOD INTAKTNIH KUJA

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Kratak sadržaj

Tumori mlečne žlezde kod pasa (TMŽP) spadaju u najčešće dijagnostikovane tumore kod intaktnih pasa ženskog pola. Po biološkim i hormonskih osobinama slični su tumorima mlečne žlezde kod žena, zbog čega predstavljaju dobar model u komparativnoj onkologiji. Ovaj rad ima za cilj da evaluiira kliničke, patohistološke i imunohistološke odlike TMŽP kod intaktnih ženki pasa. Ukupno su ispitivana 22 arhivirana slučaja TMŽP na Saveznom poljoprivrednom univerzitetu u Abeokuti, iz perioda od 2013. do 2023. godine. Uzorci tkiva dobijeni su od kuja mastektomijom ili nodulektomijom, koje su bile dovedene na privatne veterinarske klinike ili u nastavnu bolnicu. Beleženi su klinički podaci, uključujući starost životinja, veličinu tumora i njihovu lokaciju, prisustvo u limfnim čvorovima, i reproduktivni status kuja. Većina pasa bila je rase nemački ovčar (45,5%) prosečne starosti 8,15 godina; nije bilo sterilisanih.

Većina tumora (77,3%) bila je maligna i obično lokalizovana u ingvinalnim limfnim čvorovima (40,9%). Stadijum T₂ (45,5%) i histološki gradus II (72,7%) bili su najzastupljeniji. E-kaderin je bio imunopozitivan kod 89% tumora, a njegova ekspresija veća kod malignih. Ekspresija N-kaderina bila je značajno povezana sa malignitetom tumora ($p = 0,001$), što ukazuje na epitelno-mezenhimnu tranziciju (EMT). Estrogenski receptori α (OR α) su bili veoma izraženi kod svih tumora, i bili su na značajno višim nivoima kod malignih ($p = 0,002$). Progesteronski receptori (PR) i vimentin bili su detektovani samo kod malignih tumora, dok je Ki-67 ispoljio blagu, neznačajnu ekspresiju. Ovi nalazi sugerišu da N-kaderin, OR α i PR mogu biti potencijalni biomarkeri maligniteta i hormonske responzivnosti kod TMŽP. Oni takođe podržavaju relevantnost molekularnog profilisanja u dijagnostici i rešavanju pitanja tumora mlečne žlezde kod kuja.

Ključne reči: Tumori mlečne žlezde pasa, komparativna onkologija, imunohistohemija, N-kadherin, estrogenski receptori

Short communication

ASSOCIATION BETWEEN INTESTINAL ZONULIN EXPRESSION AND POLYCYSTIC OVARY SYNDROME IN RATS

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Abstract

Polycystic Ovary Syndrome (PCOS) is a complex endocrine disorder characterized by reproductive and metabolic disturbances, frequently associated with insulin resistance. Emerging evidence suggests that altered intestinal barrier function may be involved in the pathophysiology of PCOS, with zonulin recognized as a marker of increased intestinal permeability. This study aimed to investigate the association between PCOS and intestinal zonulin expression using an experimental animal model.

The research was done on female Wistar rats. Animals were randomly assigned to a control group or a PCOS-induced group. PCOS was induced through androgen

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administration, after which intestinal zonulin expression was measured and compared between groups. Statistical analyses were performed to evaluate intergroup differences and the association between PCOS induction and zonulin expression.

The results showed that PCOS-induced rats had significantly higher intestinal zonulin expression than controls (5.545 ± 1.163 vs. 3.775 ± 1.196 ; $p < 0.001$). Normality testing showed a non-normal distribution in the PCOS group (Shapiro–Wilk $p = 0.027$) and a normal distribution in controls ($p = 0.413$), supporting the use of non-parametric analysis. Mann–Whitney U test confirmed a significant difference between groups ($U = 58.0$, $Z = -3.849$), with a large effect size ($r = 0.61$). Structural modeling (SEM-PLS) demonstrated a significant direct effect of PCOS induction on zonulin expression ($T = 9.797$, $p < 0.001$), with substantial explanatory power (adjusted $R^2 = 0.511$) and predictive relevance ($Q^2 = 0.371$).

The results demonstrated that PCOS-induced rats exhibited significantly increased intestinal zonulin expression compared with healthy controls. Further analysis revealed a significant positive correlation between PCOS induction and elevated zonulin expression, suggesting altered intestinal barrier function, as inferred from the heightened zonulin levels in the PCOS model.

These findings suggest that PCOS is associated with increased intestinal permeability, supporting the involvement of gut barrier dysfunction in the metabolic features of the disorder. Intestinal zonulin expression may serve as a possible biomarker linked to metabolic disturbances, including insulin resistance, as indicated by previous studies and corroborative findings. This study provides experimental evidence highlighting the relevance of the gut–endocrine axis in PCOS pathophysiology and may contribute to the development of novel diagnostic or therapeutic strategies.

Keywords: Polycystic ovary syndrome, insulin resistance, rats, biomarkers, intestines

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting 4–18% of reproductive-age women, characterized by oligo/anovulation, hyperandrogenism, and polycystic ovarian morphology (Brosens et al., 2015). It is frequently associated with insulin resistance, obesity, and long-term metabolic complications including type 2 diabetes mellitus and cardiovascular disease. Infertility remains the major clinical consequence, affecting up to 10% of patients and significantly impairing the quality of life (Joham et al., 2015). Despite extensive research, the exact mechanisms underlying PCOS pathogenesis remain incompletely understood.

Emerging evidence suggests that gut dysbiosis and impaired intestinal barrier integrity contribute to the metabolic and endocrine disturbances observed in PCOS (Sun et al., 2023). Zonulin, a physiological regulator of intestinal tight

junctions, plays a central role in modulating gut permeability. Elevated zonulin levels promote the translocation of bacterial lipopolysaccharides (LPS), triggering low-grade inflammation, insulin resistance, and subsequent disruption of ovarian function (Mkumbuzi et al., 2020; Serek and Oleksy-Wawrzyniak, 2021). Recent studies have reported increased zonulin levels in women with PCOS and other metabolic disorders, supporting its role as a potential biomarker linking intestinal permeability with endocrine dysfunction (He and Li, 2020; Veres-Székely et al., 2023).

However, most existing evidence in humans is observational, with limited exploration of direct causal pathways, restricting the ability to infer causal relationships between intestinal barrier dysfunction and PCOS. Experimental animal models provide an opportunity to elucidate mechanistic links between PCOS and the pathophysiology of zonulin expression. Alterations in gut permeability may impact metabolic and reproductive functioning via the gut–brain–gonad axis, in which microbial-derived inflammatory signals and metabolic mediators regulate hypothalamic–pituitary control and ovarian steroidogenesis. Experimental animal models provide a controlled approach to elucidate these mechanistic interactions. Therefore, the objective of this study was to explicitly evaluate the association between PCOS induction and zonulin expression using a PCOS animal model. Furthermore, this study introduces a novel analytical approach, applying Structural Equation Modeling - Partial Least Squares (SEM-PLS) to assess the direct effect of PCOS induction on zonulin expression, providing new experimental insight into the role of intestinal permeability in PCOS pathogenesis and its potential relevance as a therapeutic target. We hypothesized that PCOS induction would be associated with increased intestinal zonulin expression, reflecting impaired gut barrier integrity.

MATERIAL AND METHODS

Study design and animals

This research was an experimental study using a post-test control-only group design to evaluate the effect of polycystic ovary syndrome (PCOS) induction on zonulin expression as a marker of intestinal permeability. Forty female Wistar rats (*Rattus norvegicus*), aged 8–10 weeks and weighing 180–220 g, were used. The animals were randomly allocated into two groups (n = 20 per group): a control group and a PCOS-induced group. The sample size was determined based on previous experimental studies employing similar PCOS animal models, where comparable group sizes were sufficient to detect biologically meaningful differences (Wang et al., 2020). Randomization was done using a simple random allocation method. All histological and immunohistochemical evaluations were conducted by an investigator blinded to group allocation to avoid observer bias.

PCOS induction

PCOS was induced by oral administration of testosterone propionate for 21 consecutive days. Control animals received standard feed and water without hormonal treatment. Successful induction of PCOS was confirmed by estrous cycle monitoring using daily vaginal smear cytology and by ovarian histopathological examination at the end of the experimental period (Figure 1).

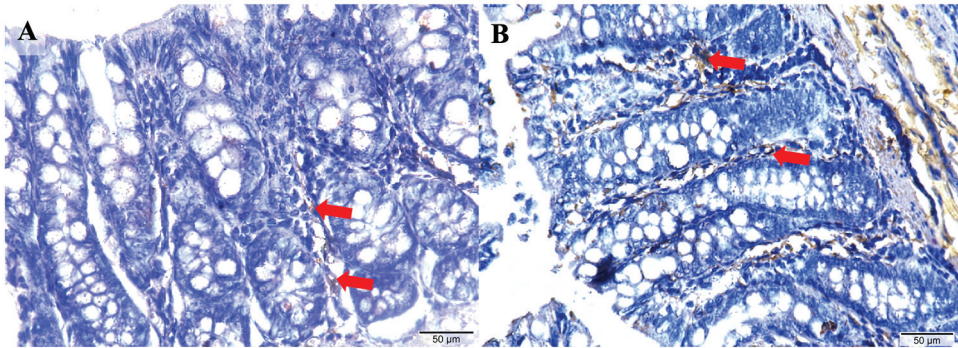


Figure 1. Zonulin expression in colonic epithelium (Immunohistochemistry, 400×). Representative micrographs show the colonic mucosal layer of Wistar rats evaluated via immunohistochemistry. Arrows indicate positive zonulin expression within the epithelial layer, highlighted by brown chromogen staining. (A) Control group showing baseline expression. (B) PCOS-induced group showing markedly increased, intense staining.

Tissue collection and histopathological evaluation

After the treatment period, the animals were sacrificed, and ovarian and colon tissues were collected. Ovarian tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. Microscopic examination demonstrated multiple cystic follicles, increased numbers of atretic follicles, and reduced corpora lutea in the PCOS group, consistent with polycystic ovarian morphology.

Immunohistochemical assessment of zonulin

Zonulin expression, an indirect indicator of intestinal permeability, was assessed using immunohistochemistry (IHC). Although serum-based assays were initially considered, only IHC-derived data were included in the final analysis to ensure methodological consistency.

Colon tissue samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 4 µm thickness. The sections were deparaffinized, rehydrated, and subjected to antigen retrieval using citrate buffer (pH 6.0).

Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The slides were incubated overnight at 4°C with a primary anti-zonulin antibody (rabbit polyclonal, Abcam, ab276405; dilution 1:200), followed by incubation with an HRP-conjugated secondary antibody. Visualization was achieved using 3,3'-diaminobenzidine, and the sections were counterstained with hematoxylin.

Zonulin expression was evaluated semi-quantitatively and expressed as the Immunoreactive Score (IRS). Staining intensity was graded as 0 (no staining), 1 (weak), 2 (moderate), or 3 (strong). The percentage of positively stained epithelial cells was scored as 0 (<10%), 1 (10–25%), 2 (26–50%), 3 (51–75%), or 4 (>75%). The IRS was calculated by multiplying intensity and percentage scores, yielding a total score ranging from 0 to 12. Ten randomly selected high-power fields were evaluated per sample.

Metabolic parameters, including fasting glucose, insulin levels, and HOMA-IR, were assessed as part of the same experimental protocol and animal population but will be reported separately.

Statistical analysis

Data were analyzed with SPSS version 26.0. Normality was assessed with the Shapiro–Wilk test. Normally distributed data were analyzed using independent t-tests, while non-normally distributed data were analyzed with the Mann–Whitney U test. To explore the association between PCOS induction and zonulin expression, SEM-PLS was done using SmartPLS version 4.0.8.6. Statistical significance was set at $p < 0.05$. SEM-PLS was applied to estimate the structural relationship between PCOS induction and zonulin expression, including path coefficients and predictive relevance indices (R^2 and Q^2). In this model, both PCOS induction and zonulin expression were treated as observed variables; therefore, outer model (measurement model) evaluation was not applicable. The use of SEM-PLS was intended to provide a scalable analytical framework for future studies incorporating multiple metabolic and inflammatory variables.

RESULTS AND DISCUSSION

Normality testing with the Shapiro–Wilk test revealed that zonulin expression scores in the control group were normally distributed ($p = 0.413$), but in the PCOS group were not ($p = 0.027$). Accordingly, between-group comparisons were done with the Mann–Whitney U test.

Immunohistochemical analysis revealed significantly higher zonulin expression in the PCOS-induced group compared with controls ($p < 0.05$) (Table 1, Figure 1). Descriptive analysis demonstrated an increased central tendency in the PCOS group, with a mean zonulin IRS of 5.545, when compared with 3.775 in the control group. The median IRS value was likewise higher in the PCOS group, and the

interquartile range was shifted upward, indicating a consistent increase in zonulin expression across samples. Although outliers were present in both groups, the overall distribution pattern clearly demonstrated higher zonulin expression in the PCOS model.

Table 1. Association between zonulin expression and PCOS

Variable	Group	Mean $\pm$ SD	Shapiro–Wilk Sig.	p-value ^a
Zonulin	Control (n=20)	3,775 $\pm$ 1,195	0.413	0.000
	Treatment (n=20)	5,545 $\pm$ 1,163	0.027	

^aMann-Whitney

The magnitude of the group difference was further supported by effect size analysis. The Mann–Whitney U test confirmed a significant difference ($U = 58.0$, $Z = -3.849$, $p < 0.001$), yielded a large effect size ($r = 0.61$), with a 95% confidence interval indicating a robust separation between control and PCOS groups, reinforcing the biological relevance of the observed difference.

All panels presented in Figure 2 (Panels A–I) depict zonulin staining across control and experimental groups, with Panels A–D showing normal folliculogenesis in healthy rats and Panels E–H showing disrupted folliculogenesis in PCOS-induced rats; Panel I shows an aberrant Graafian follicle showing follicular atresia. Representative images demonstrated stronger zonulin staining intensity and a higher proportion of positively stained epithelial cells in the colon tissue of PCOS-induced rats. Zonulin expression patterns were visualized using boxplots, which illustrated higher median values and a wider distribution in the PCOS group compared with controls (Figure 3).

To assess the strength of the association between PCOS induction and zonulin expression, SEM-PLS analysis was performed. The structural model demonstrated a strong explanatory capacity, with an adjusted $R^2 = 0.511$, indicating that 51.1% of the variance in zonulin expression was explained by PCOS induction. Predictive relevance testing yielded a positive Q^2 value of 0.371, confirming adequate predictive accuracy of the model.

Path analysis using SEM-PLS demonstrated a direct significant effect of PCOS induction on zonulin expression ($p < 0.05$; T -statistic > 1.96), reinforcing the strong association between PCOS and increased zonulin expression. The structural model revealed a significant direct effect of PCOS on zonulin expression (β = path coefficient, $T = 9.797$, $p < 0.001$), indicating that PCOS induction strongly and positively influences zonulin expression. The high t -statistic and low standard error further confirm the robustness of this relationship (Table 2).

This study demonstrated a significant increase in zonulin expression in the PCOS group, indicating enhanced intestinal permeability associated with PCOS. These experimental findings are consistent with clinical observations reporting elevated

zonulin in women with PCOS and its correlation with metabolic dysfunction (Zhang et al., 2015; Lindheim et al., 2017). Using an immunohistochemical approach, this study provides histological evidence of increased zonulin expression in the intestinal tissue.

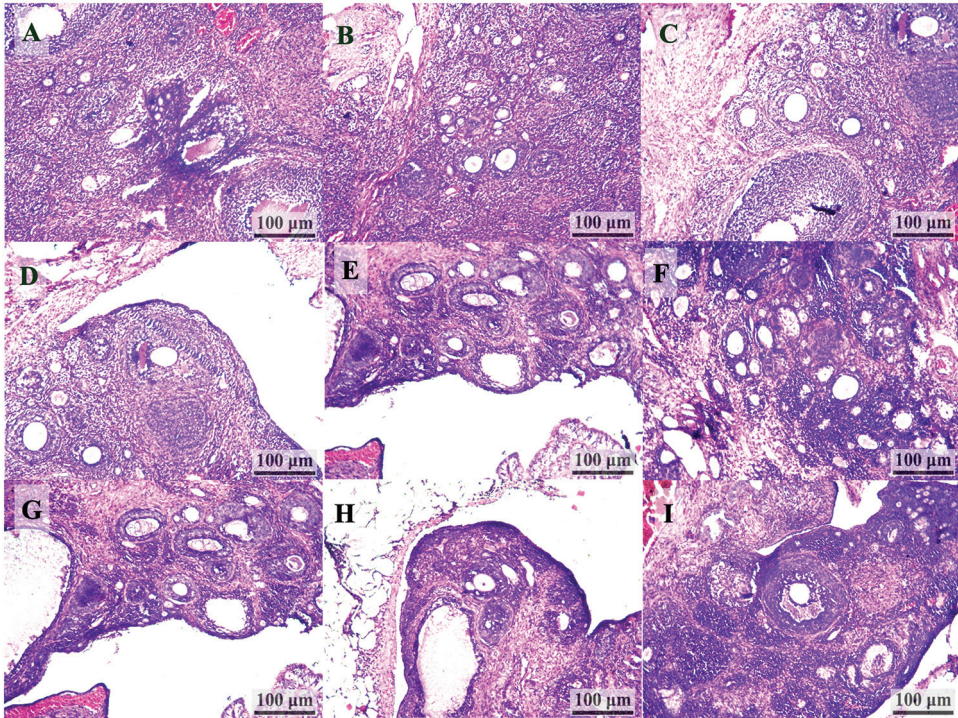


Figure 2. Comparative ovarian histopathology of healthy control and PCOS-induced Wistar rats (H&E, 100×). Panels A–D show normal folliculogenesis in healthy rats: (A) primordial follicles, (B) primary follicles, (C) secondary follicles, and (D) tertiary follicle with antral cavity formation. Panels E–H illustrate disrupted folliculogenesis in adrogen-induced PCOS rats: accumulation of primordial (E) and primary follicles (F), (G) numerous arrested secondary follicles in the cortical region, (H) tertiary follicles with cystic changes and thinning of granulosa cell layers, and (I) aberrant Graafian follicle showing follicular atresia. These findings highlight the contrast between balanced follicular development in healthy ovaries and follicular arrest with cystic degeneration in PCOS.

Zonulin is a physiological regulator of intestinal tight junctions; its upregulation increases epithelial permeability and facilitates the translocation of luminal antigens and endotoxins (e.g., LPS) into the circulation, which can trigger low-grade inflammation and impair insulin signalling—mechanisms that plausibly link zonulin elevation to insulin resistance observed in PCOS (Sturgeon and Fasano, 2016). Nevertheless, increased zonulin expression may also reflect alternative or complementary processes. Factors such as systemic inflammation, hormonal stress, dietary influences, or androgen-driven alterations in gut microbiota

composition may independently contribute to increased zonulin expression (He and Li, 2020). In this hyperandrogenic animal model, testosterone exposure itself may have influenced intestinal permeability either directly or indirectly through inflammatory pathways. Therefore, while zonulin elevation is strongly associated with PCOS induction, it should not be interpreted as a sole or exclusive driver of metabolic dysfunction.

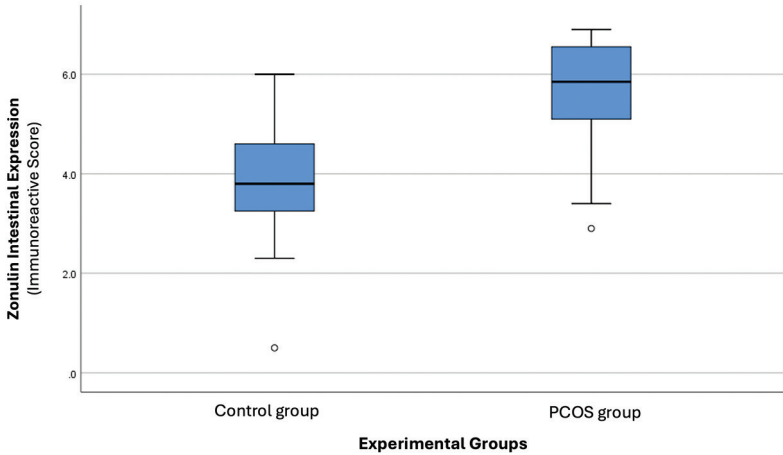


Figure 3. Comparison of intestinal zonulin expression in control and PCOS groups. Boxplots of intestinal zonulin immunoreactive scores in control rats ($n = 20$) and PCOS-induced rats ($n = 20$). The PCOS group demonstrated significantly higher zonulin expression than the control group (Mann–Whitney U test, $p < 0.001$).

Table 2. Direct Effect of PCOS on zonulin expression

Path	STDEV	T-Statistic	p-value	Significance
PCOS → Zonulin	0.073	9.797	0.000	Significant

From a translational perspective, targeting zonulin—either through interventions aimed at restoring gut microbiota composition or by directly modulating zonulin signalling—emerges as a potential strategy to reduce intestinal permeability and alleviate metabolic inflammation. Human intervention studies, including metformin therapy and lifestyle modification, have demonstrated improvements in metabolic status accompanied by reductions in zonulin, while recent reviews have also highlighted pharmacological agents targeting the zonulin pathway as potential therapeutic candidate (Karim et al., 2024). Nonetheless, interventional evidence remains limited and requires further clinical trials (Ibrahim and Ahmeid, 2021; Veres-Székely et al., 2023).

These findings position zonulin as a potential biomarker connecting intestinal barrier dysfunction with the metabolic phenotype of PCOS. However, it remains unresolved whether elevated zonulin causally drives ovarian dysfunction or primarily reflects accompanying metabolic disturbances; targeted interventional studies that specifically lower zonulin would provide stronger causal evidence (Osuka et al., 2019; Ryu et al., 2019). In this study, intestinal permeability was not directly measured in functional assays such as FITC-dextran or lactulose–mannitol tests. Therefore, the observed increase in zonulin expression should be interpreted as an indirect surrogate marker of barrier dysfunction rather than definitive evidence of increased permeability.

The strengths of this study include its controlled experimental design, which enables causal inference between PCOS induction and zonulin elevation, and the use of SEM-PLS to examine mechanistic relationships. Although the association between PCOS induction and zonulin expression could be evaluated with conventional statistical methods, SEM-PLS was employed to provide a model-based approach that allows future integration of additional variables. Nevertheless, the current findings should be interpreted within the context of a simple structural model. However, the study has several important limitations. First, a single PCOS animal model based on androgen induction was used, which reproduces key features such as hyperandrogenism and insulin resistance but does not fully reflect the clinical heterogeneity of PCOS. Second, intestinal permeability was inferred from zonulin expression rather than measured directly using functional permeability assays. Third, this study did not include interventions designed to modulate zonulin expression or intestinal permeability, limiting causal inference regarding its therapeutic potential.

Future studies should incorporate multiple PCOS models to capture phenotypic diversity and include direct permeability assessments to validate zonulin as a functional marker. Interventional approaches targeting the gut barrier, such as zonulin-modulating agents, dietary modifications, or probiotic and prebiotic supplementation, may help clarify whether reducing intestinal permeability improves metabolic and reproductive outcomes in PCOS. Such studies would strengthen the translational relevance of targeting the gut–endocrine axis in PCOS management.

CONCLUSION

This experimental study demonstrated that zonulin expression significantly increased in a PCOS animal model, suggesting a potential alteration in intestinal barrier function. These findings support the role of zonulin as a surrogate marker associated with metabolic features of PCOS; however, direct assessment of intestinal permeability is required to confirm functional impairment.

Authors' contributions

Conceptualization, FU, MIS, and BS; methodology, FU, MIS, KYE, PML, and WW; validation, WW and BS; formal analysis, WW and BS; investigation, FU, KYE, and PML; resources, MIS and EST; data curation, FU and BS; writing—original draft preparation, FU, EST, and BS; writing—review and editing, MIS, KYE, PML, WW, EST, and BS; visualization, BS; supervision, MIS, PML, and WW; project administration, FU. All authors have read and agreed to the published version of the manuscript.

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Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethical statement

All experimental procedures were conducted in accordance with the institutional and international guidelines for the care and use of laboratory animals. The study protocol was reviewed and approved by the Institutional Ethics Committee (No: 3.KEH.150.10.2024). The study protocol was not registered in an external preclinical public registry prior to commencement

Competing interests

The authors declare no competing interests.

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POVEZANOST EKSPRESIJE INTESTINALNOG ZONULINA I SINDROMA POLICISTIČNIH JAJNIKA KOD PACOVA

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Kratak sadržaj

Sindrom policističnih jajnika (PCOS) je složen endokrini poremećaj kojeg karakterišu reproduktivni i metabolički poremećaji često povezani sa insulinskom rezistencijom. Novi dokazi ukazuju da izmenjena funkcija crevne barijere može imati ulogu u patofiziologiji PCOS-a, pri čemu se zonulin smatra markerom povećane crevne propustljivosti. Cilj ovog ispitivanja bio je istraživanje veze između PCOS-a i ekspresije zonulina u crevima, u eksperimentalnom animalnom modelu.

Eksperiment je urađen na ženjkama pacova rase Wistar. Životinje su nasumično raspoređene u kontrolnu grupu i grupu sa eksperimentalno izazvanim PCOS-om. PCOS je indukovao primenom androgena, nakon čega je merena ekspresija zonulina u crevima, i vrednosti upoređene između grupa. Statističkom analizom su procenjene razlike između grupa i veza između indukcije PCOS-a i ekspresije zonulina.

Rezultati su pokazali da je kod pacova sa indukovanim PCOS-om ekspresija zonulina u crevima bila značajno veća nego kod kontrolnih ($5,545 \pm 1,163$, odnosno $3,775 \pm 1,196$; $p < 0,001$). Testiranje je pokazalo da distribucija frekvencija nije bila normalna u grupi sa indukovanim PCOS-om (Shapiro-Wilk $p = 0,027$), dok u kontrolnoj grupi jeste bila ($p = 0,413$), te je primenjena neparametrijska analiza. Mann-Whitney U test potvrdio je značajnost razlika između grupa ($U = 58,0$; $Z = -3,849$), sa velikom veličinom efekta ($r = 0,61$). Strukturno modeliranje (SEM-PLS) pokazalo je značajan direktan efekat indukcije PCOS na ekspresiju zonulina ($T = 9,797$; $p < 0,001$), sa značajnom objašnjavajućom snagom (prilagođeni $R^2 = 0,511$) i prediktivnom relevantnošću ($Q^2 = 0,371$).

Rezultati su pokazali da su pacovi sa PCOS-om imali značajno povećanu ekspresiju zonulina u crevima u poređenju sa zdravom kontrolom. Dalja analiza je otkrila direktnu i značajnu

vezu između indukcije PCOS i povećane ekspresije zonulina, što ukazuje na izmenjenu funkciju crevne barijere, kao što se zaključuje iz povećane ekspresije zonulina u modelu PCOS.

Ovi nalazi ukazuju na to da je PCOS povezan sa povećanom crevnom propustljivošću, što podržava učešće disfunkcije crevne barijere u metaboličkim karakteristikama poremećaja. Ekspresija zonulina u crevima može poslužiti kao potencijalni biomarker koji je povezan sa metaboličkim poremećajima uključujući insulinsku rezistenciju, što impliciraju prethodne studije i komplementarni nalazi. Ovo ispitivanje pruža eksperimentalne dokaze koji ističu značaj crevno-endokrine ose u patofiziologiji PCOS i može doprineti razvoju novih dijagnostičkih ili terapijskih pristupa.

Ključne reči: Sindrom policističnih jajnika, insulinska rezistencija, pacovi, biomarkeri, creva

Short communication

THE OCCURRENCE OF *SALMONELLA* IN BROILER MEAT PRIOR TO ENTER THE MARKET AT ONE FOOD BUSINESS OPERATOR IN SERBIA

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Abstract

The occurrence of *Salmonella* spp. in broiler meat remains a critical public health concern and a major challenge for the food industry. This study systematically examined the occurrence and serovar distribution of *Salmonella* in fresh broiler meat on the Serbian market over a five-year period (2020–2024). A total of 400 samples, including broiler carcasses (n=100), fillets (n=100), leg quarters (n=100), and wings (n=100), were collected from a food business operator's warehouse and analyzed using the VIDAS UP *Salmonella* (SPT) automated screening method and standard ISO 6579-1:2017/A1:2020 protocols.

The results revealed an overall *Salmonella* spp. prevalence of 10% (40/400). Among the positive isolates, *S. Infantis* was identified as the predominant serovar, accounting for 82.5% of cases (n=33), while *S. Enteritidis* was detected in 17.5% (n=7). Distribution analysis across different meat categories showed that broiler wings had the highest contamination rate (15%), followed by whole carcasses (11%), leg quarters (9%), and fillets (5%). The lower frequency observed in fillets indicates that the removal of the skin serves as a critical factor in significantly reducing the overall microbial load.

Furthermore, seasonal trends indicated a rise in *Salmonella* occurrences during warmer months, emphasizing the need for enhanced control strategies during

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summer. These findings underscore the importance of comprehensive facility preparation from veterinary-sanitary and construction-technical perspectives as a prerequisite for effective laboratory monitoring. The results highlight that while current hygiene measures are in place, the high occurrence of *S. Infantis* necessitates more stringent biosecurity and systematic surveillance throughout the broiler meat production chain to mitigate risks to consumer health.

Key words: *Salmonella*, *Salmonella infantis*, *Salmonella enteritidis*, poultry meat, food safety

INTRODUCTION

Salmonella species are among the most significant pathogens associated with foodborne illnesses and rank as the third leading cause of deaths from diarrheal diseases worldwide (Ferrari et al., 2019). With the rapid growth of the poultry industry and the corresponding increase in consumption, maintaining hygiene is becoming crucial to prevent contamination and control foodborne diseases (WHO, 2023) during processing. The most common carriers of foodborne *Salmonella* spp. infections in humans are animal-derived, with eggs, poultry, and pork being the most frequently associated sources (Löfström et al., 2015, Thames et al., 2022). Consuming undercooked, contaminated broiler meat poses the greatest threat to human health. Although rare, the prevalence of human salmonellosis is difficult to diagnose due to non-specific symptoms and intermittent presentation (Rašeta, 2014). Taxonomically, the species *Salmonella enterica* has been identified, including the highly pathogenic serovars *Salmonella enterica* subsp. *enterica* serovar Enteritidis (further *S. Enteritidis*) and *S. Typhimurium*, which have been responsible for more than 78 million cases of illness, 59,153 deaths (Havelaar et al., 2015), and roughly 4,068,000 disability-adjusted life years (DALYs) lost. *Salmonella* serovars in poultry meat and products pose a high risk for consumers. Serovars which constitute the primary security concern for causing foodborne diseases in humans are *S. Enteritidis* and *S. Typhimurium* as globally predominant (Hendriksen et al., 2011). Moreover, other region-specific serovars are reported as sources of human illness, such as *S. Infantis* (Rašeta, 2014) or *S. Indiana* (Xia et al., 2009). Several studies have reported the existence of this pathogen, highlighting the role of elimination and control for the safety of the human health sector (Shaji et al., 2023; Toloee et al., 2025; Brasileiro et al., 2025). Subsequent changes in dominant foodborne serovars are anticipated over time (Foley et al., 2008), indicating that surveillance of the emergence and distribution of serotypes for improving efforts on salmonellosis control is necessary.

Slaughterhouses are a principal site of contamination because *Salmonella* spp., when transmissible by intestinal matter at the time of processing, can be passed through equipment, transport containers, water, and human contacts. Processing

processes, such as bleeding, scalding, plucking, and evisceration, increase the risks of cross-contamination (Boubendir et al., 2021).

Therefore, the aim of this study was to provide data regarding the burden of *Salmonella* spp. in fresh broiler meat products (broiler carcasses and primal cuts) intended for human consumption after the application of routine control measures and prescribed food safety procedures. The study particularly focused on the occurrence of *Salmonella* spp. in products that had already passed standard production and inspection processes, thereby contributing to the identification and assessment of residual public health risks associated with broiler meat consumption. In addition, the collected data are expected to contribute to a comprehensive risk assessment of *Salmonella* presence throughout the broiler meat production and distribution chain. However, since the study included only one food business operator, the findings should be interpreted with caution and should not be directly extrapolated to the national level without considering the broader epidemiological context of *Salmonella* spp. occurrence in broiler meat.

MATERIAL AND METHODS

The presence of *Salmonella* was systematically examined over a five-year period (2020-2024) as part of the food business operator's self-control plan, involving a total of 400 samples of fresh broiler meat. Testing for the presence of *Salmonella* was conducted on fresh packaged broiler meat in the finished product cold storage room at the FBO premises, immediately prior to distribution to the market. Samples were systematically collected over a five-year period, consistent with the FBO self-control plan. Sampling was performed on a bi-weekly basis, with one to two samples obtained per interval, depending on the type and availability of the finished product in the cold storage room. A detailed overview of the sampling volume dynamics by month is presented in Supplementary table S1. Each sample comprised five individual units to enhance representativeness, in compliance with the Guidelines for the Application of Microbiological Criteria for Food (Serbian Regulation, 2011; 2024). Sampling was performed in FBO warehouse for packaged fresh poultry, immediately prior to shipment. The objective was to determine the occurrence of *Salmonella* at the FBO's dispatch point. The FBO implements all legally prescribed control measures and maintains a fully operational food safety management system (HACCP). Furthermore, the broilers from which the meat was obtained exhibited no clinical signs of disease, nor did they show reduced weight gain during the fattening period. Sampling was conducted at the stage of finished product storage within the FBO dispatch cold store. This represents the point of entry into the distribution chain; subsequent market handling necessitates appropriate storage within the defined shelf-life under 'cold chain' conditions. The established contamination level will not be eliminated during distribution; conversely, it may only increase due to potential logistical failures.

Sampling was performed in accordance with domestic (Serbian regulation 2019, 2024) and EU (European regulation 2002, 2005). The carcass and primal cuts were sampled in its original packaging, after cooling, immediately before being sent to the dispatch cold store. The following parts were sampled and tested: broiler carcasses (n=100), broiler fillets (n=100), broiler leg quarters (n=100), and broiler wings (n=100). These primal cuts, along with the whole carcass, were selected as they represent the most widely sold fresh broiler meat products in retail. It is the standard production practice of the FBO to utilize the remaining parts of the broiler carcass (broiler backs with pelvis and broiler necks) in the production of mechanically separated meat (MSM). All birds were slaughtered in accordance with the provisions of the Animal Welfare Act (Serbian regulation 2009; European regulation 2009) and relevant bylaws (Serbian regulation 2010). The slaughter process ensured that the animals were deprived of life exclusively in an unconscious state, strictly adhering to both national and international guidelines (Raj and Tserveni-Gousi, 2000) for the ethical treatment and welfare of animals during slaughter. Every positive isolate was subjected to serotyping.

When taking and preparing samples for testing, the relevant ISO (International Organization for Standardization) standards and Codex Alimentarius guidelines are applied as reference methods. For the detection of *Salmonella* spp., the method SRPS EN ISO 6579-1:2017/A1:2020 is utilized—Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* - Part 1: Detection of *Salmonella* spp. - Amendment 1: Broader range of incubation temperatures, amendment to the status of Annex D, and correction of the composition of MSRV and SC (ISO 6579-1:2017/Amd 1:2020).

The detection of *Salmonella* antigens in the samples was performed using the Enzyme-Linked Fluorescent Assay (ELFA) method (Rohonczy et al., 2014), specifically via the VIDAS UP *Salmonella* (SPT) assay on VIDAS series analyzers (Manufacturer BioMérieux) (Cetin et al., 2019). This automated, rapid, and sensitive screening method utilizes specific antibodies for the detection of *Salmonella* in food and environmental samples, providing high reliability within approximately 48 hours.

All positive samples identified by the VIDAS immunoanalyzer were subcultured onto differential media, such as Xylose Lysine Deoxycholate (XLD) agar (Merck KGaA, Darmstadt, Germany) and Rambach agar (Merck KGaA, Darmstadt, Germany), for the isolation of *Salmonella* using standard microbiological procedures (Carrique-Mas et al., 2009). The inoculated plates were incubated for 24 hours at 37 °C. On Rambach agar, *Salmonella* typically forms distinct red colonies. On XLD agar, characteristic colonies appear red with black centers, although some isolates may present as yellow colonies with black centers (Hess et al., 2023). Further identification of the isolates was performed by serotyping using polyvalent and monovalent antisera according to the White-Kauffmann-Le Minor scheme (Issenhuth-Jeanjean et al., 2014).

Statistical analysis was performed to evaluate the association between broiler meat sample type, environmental temperature, and the occurrence of *Salmonella* spp. Categorical data were analyzed using the chi-square (χ^2) test of independence to assess the relationship between broiler meat sample type and the presence of *Salmonella* spp. A significance level of $p < 0.05$ was applied throughout the study.

To evaluate differences in the distribution of *Salmonella* serovars, a binomial test was performed comparing the proportions of *S. Infantis* and *S. Enteritidis* among positive samples.

The relationship between environmental conditions and *Salmonella* occurrence was assessed by correlation analysis between the average monthly ambient temperature and the number of *Salmonella*-positive samples. Pearson's correlation coefficient (r) was used for normally distributed data, while Spearman's rank correlation coefficient (ρ) was applied when normality assumptions were not met.

Simple linear regression analysis was additionally performed to evaluate the effect of average monthly temperature on the occurrence of *Salmonella*-positive samples. The number of positive samples was treated as the dependent variable, while average monthly temperature ($^{\circ}\text{C}$) was included as the independent variable.

Binary logistic regression analysis was used to assess the effect of broiler meat sample type on the likelihood of *Salmonella* contamination. The dependent variable was the presence of *Salmonella* spp. (0 = negative, 1 = positive), while broiler meat sample type was included as the independent variable, with broiler fillet used as the reference category. Results were expressed as odds ratios (OR) with 95% confidence intervals (CI). All statistical analyses and graphical visualizations were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) with the RegressIt add-on for regression analyses. Results were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Out of 400 samples examined, 40 tested positive for *Salmonella*; among these, *S. Infantis* was identified in 82.5% of cases ($n=33$), while *S. Enteritidis* was detected in 17.5% of cases ($n=7$). The distribution of positive samples varied across different broiler meat categories. The highest frequency was observed in wings (15%), followed by carcasses (11%) and leg quarters (9%), while fillets showed the lowest contamination rate (5%).

The results of positive samples across the investigated categories of fresh poultry meat are presented in Table 1.

Table 1 shows significant differences in contamination depending on the type of sample.

Table 1. Positive cases across the investigated sample types of fresh broiler meat

Sample types of fresh broiler meat	n (samples)	Positive samples
		(%)
Broiler fillet	100	5
Broiler leg quarters	100	9
Broiler carcass	100	11
Broiler wings	100	15
Total	400	10

Based on the data from Table 1 (fillet: 5 positive / 95 negative; broiler leg quarters: 9/91; carcass: 11/89; wings: 15/85), a chi-square test of independence was performed to assess whether contamination differed significantly among broiler meat sample types. The analysis revealed a statistically significant association between broiler sample type and the presence of *Salmonella* spp. ($\chi^2 = 8.72$, $df = 3$, $p = 0.033$), indicating that contamination was not uniformly distributed across different types of broiler meat. A clear numerical trend of increasing contamination was observed from fillets (5%) to wings (15%). Logistic regression results are presented in Table 2.

Table 2. Logistic regression analysis of *Salmonella* spp. occurrence relative to product type

Variable	β (Coeff.)	S.E.	Wald	<i>p</i> -value	Odds ratio (OR)	95% CI for OR
Broiler fillet	/	/	/	/	1.000 (Ref.)	/
Leg quarters	0.628	0.587	1.144	0.285	1.872	0.592-5.881
Broiler carcass	0.855	0.569	2.258	0.133	2.351	0.771-7.152
Broiler wings	1.210	0.541	5.003	0.025*	3.353	1.161-9.682
Constant	-2.944	0.459	41.134	< 0.001	0.053	/

Legend: β : Regression coefficient; S.E.: Standard Error; Wald: Wald chi-square statistic; CI: Confidence Interval.

* Statistically significant difference ($p < 0.05$)

The binary logistic regression model identified the sample type as a significant predictor for *Salmonella* contamination ($p < 0.05$). Using broiler fillet as the reference group (OR = 1.00), broiler wings showed a statistically significant increase in the odds of contamination (OR = 3.35, $p = 0.025$). Although broiler carcasses and leg quarters displayed higher odds of contamination (OR = 2.35 and OR = 1.87,

respectively), these differences did not reach statistical significance within the 95% confidence interval. To present the logistic regression results more transparently and clearly, a Forest Plot was utilized. This graph illustrates which factors increase the risk, indicated by values positioned to the right of the reference line (1.0).

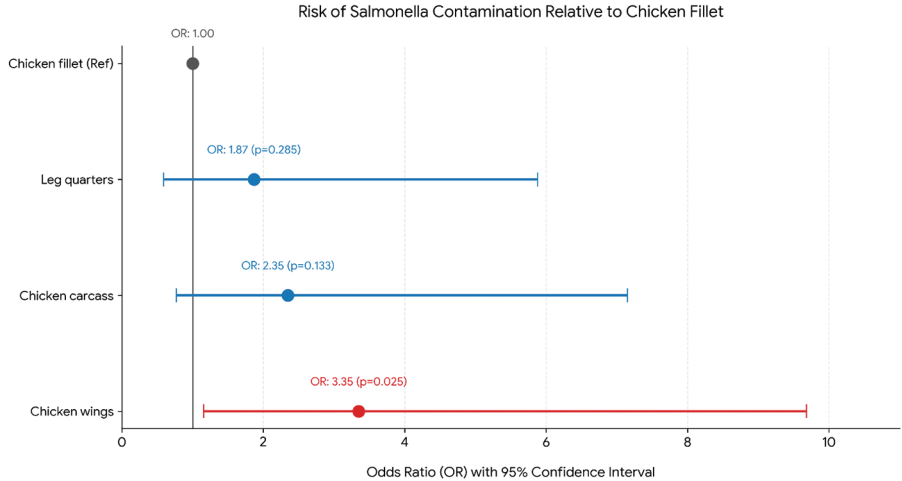


Figure 1. Visualization of Risk (Odds Ratio)

The logistic regression model demonstrates that the broiler sample type is a significant predictor of *Salmonella* contamination ($p<0.05$). Broiler wings are identified as a sample with the highest risk of contamination. Samples from broiler wings were 3.35 times more likely to be contaminated compared to skinless fillets (OR=3.35; 95%CI: 1.16-9.68; $p=0.025$). Other categories: while carcasses (OR=2.35) and leg quarters (OR=1.87) showed numerically higher *Salmonella* occurrence than fillets, their results did not reach the threshold of statistical significance ($p>0.05$) primarily due to the wide confidence intervals associated with the sample size. Reference Point: The vertical line at 1.0 represents the baseline risk (broiler fillet). Any interval that does not cross this line (like broiler wings) is considered statistically significant.

The serotyping results of the positive cases across the investigated sample types are presented in Figures 2–5.

Figures 2-5 illustrate the temporal distribution of pathogens. Seasonal trend is present in all figures, a discernible increase in the frequency of positive samples is observed across all figures during the summer months (May, June, and July). While *S. Infantis* remains endemic throughout the year, *S. Enteritidis* exhibits sporadic occurrence, with peak detection typically recorded between May and June (Figures 2 and 3).

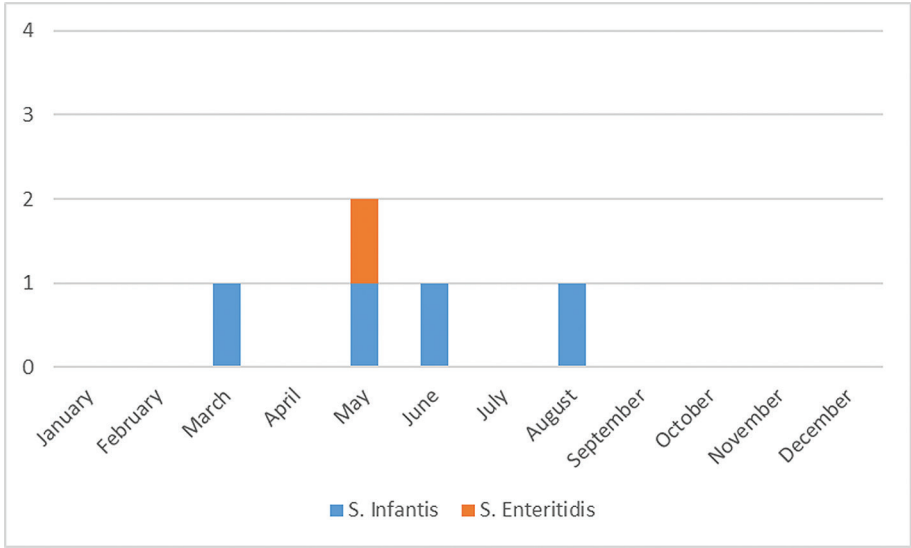


Figure 2. *Salmonella* serovars occurrence in relation to the month of the year from broiler fillet in period 2020-2024.

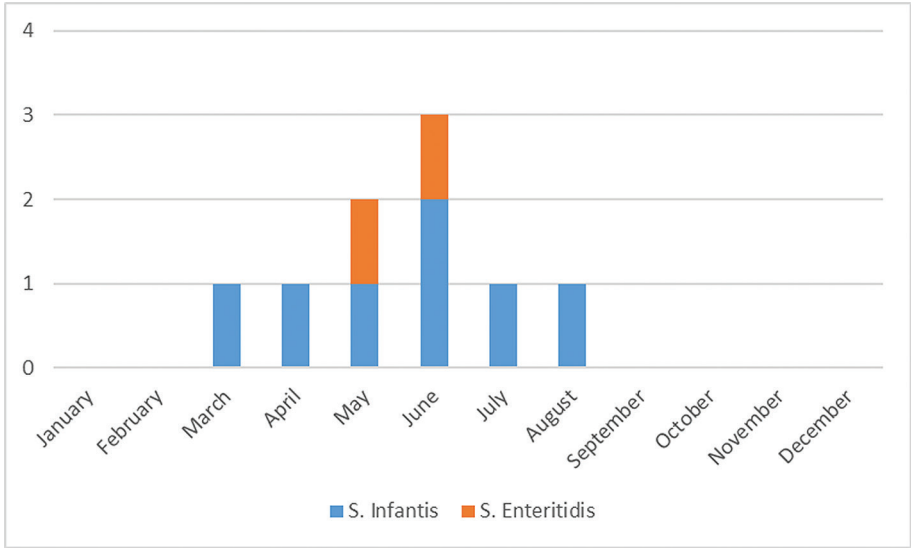


Figure 3. *Salmonella* serovars occurrence in relation to the month of the year from broiler leg quarters in period 2020-2024.

Comparing the prevalence of the serovars *S. Infantis* and *S. Enteritidis*, out of 40 positive samples, 33 were identified as *S. Infantis* and 7 as *S. Enteritidis*. The binomial test (or Chi-square) resulted in a p-value < 0.0001, indicating a highly significant difference in serovar distribution.

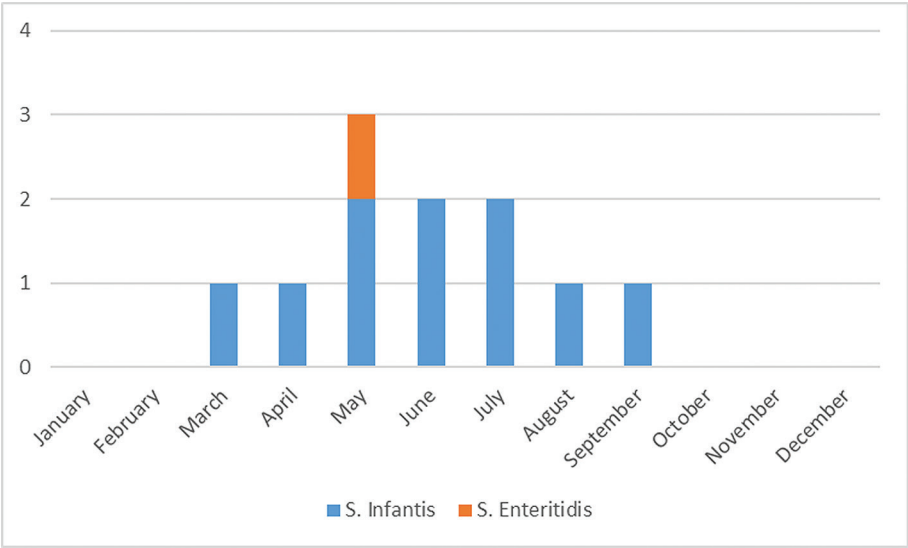


Figure 4. *Salmonella* serovars occurrence in relation to the month of the year from broiler carcass in period 2020-2024.

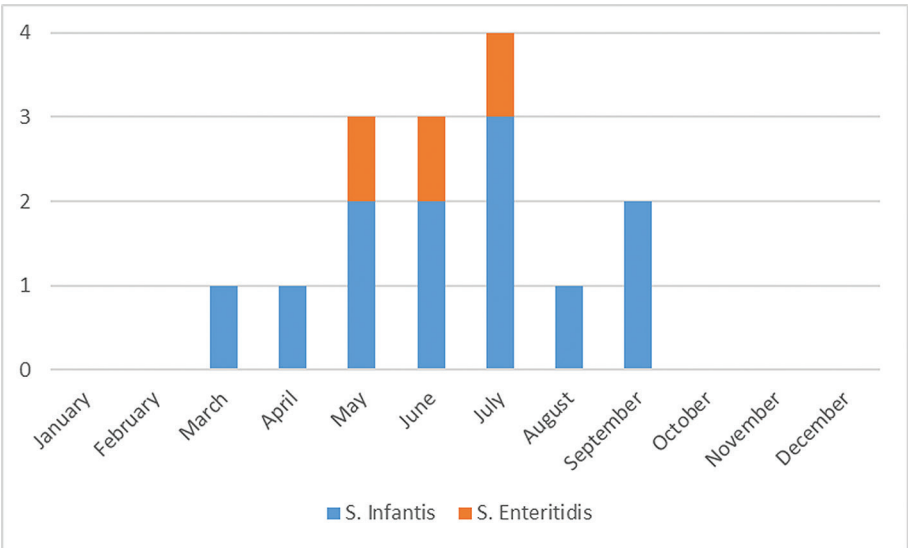


Figure 5. *Salmonella* serovars occurrence in relation to the month of the year from broiler wings in period 2020-2024.

By comparing the obtained results of *Salmonella* occurrence in fresh broiler meat with the official monthly average temperature data for the period 2020–2024, provided by the Republic Hydrometeorological Service of Serbia (RHMZ, 2024), a statistically significant positive correlation was found between average medium temperature and the occurrence of both *S. Infantis* and *S. Enteritidis* across the fresh broiler meat sample types. Results are presented in Table 3.

Table 3. Results of *Salmonella* prevalence and average medium air temperature

<i>Salmonella</i> strain	Coefficient (Temperature)	<i>p</i> -value	Interpretation
S. Infantis	0.1161	< 0.001	Highly significant; occurrence increases as temperature rises
S. Enteritidis	0.1409	0.044	Significant; also increases with temperature, though fewer overall cases

The regression coefficient (0.116) indicated a positive association between average ambient temperature and *Salmonella* occurrence, demonstrating that the frequency of positive samples increased with rising temperature. Both serovars showed peak occurrence during the warmest months (May to September; 18.5–24.5 °C). During the coldest months (January, February, November, and December; below 9 °C), positive detections decreased to nearly zero across almost all sample types. Broiler wings and carcasses consistently exhibited the highest numbers of *S. Infantis*-positive samples during warmer periods compared to fillets and leg quarters (Figures 2–5).

Salmonella can contaminate broiler carcasses at any point along the production chain, starting from breeder farms and extending to retail outlets (Zeng et al., 2021). *Salmonella* spp. is present throughout all stages of production and distribution and cold chain maintenance (0–4 °C) effectively inhibits the proliferation of the pathogen, but it does not achieve inactivation or elimination (Gonçalves-Tenório et al., 2018).

Through using the indicator microorganisms—specifically total viable count (TVC), *Enterobacteriaceae* count (EC), and the occurrence of *Salmonella* spp. monitored on carcasses—the risk-based classification of slaughterhouses should be established according to process hygiene performance (Mrdović et al., 2018). Comparing results a study conducted in China reports slaughterhouses as main contamination sources 62,9%, followed by retail 54,7% and farm level with only 7.0% *Salmonella* spp. prevalence (Ren et al., 2016). Such a high prevalence in slaughterhouses necessitates an evaluation of primary production characteristics, as well as an assessment of cross-contamination, which is inevitable on high-capacity poultry slaughter lines. The presence of *Salmonella* spp. in the gastrointestinal tract of broilers can be asymptomatic, meaning that clinical symptoms may be absent (Chlebicz and Śliżewska, 2018); consequently, during evisceration and the opening of the body cavity, the pathogen can contaminate the carcass surface if the integrity of the gastrointestinal tract is compromised. This also accounts for the lower *Salmonella* occurrence observed in broiler fillets in the present research (5%), as the skin—which serves as a primary carrier of the microbial load—is removed during the preparation of the broiler breast.

The contamination level of fresh broiler meat identified in the present study (10%) was higher: 7,1% (Gonçalves-Tenório et al., 2018), 9% (Kim et al., 2025) and lower than similar studies: 18% (Brasileiro et al., 2025); 18,1% (Regalado-Pineda et al., 2020); 26,4% (Wang et al., 2014, Sun et al., 2021); 32% (Thames et al., 2022); 32,55% (Di Taranto et al., 2025); 40% (McKee, 2011), 46,1% (Lunara Santos Pavelquesi et al., 2023) and 50% (Yang et al., 2011). Pathogen control across the poultry production and distribution chain must be rigorous in order to mitigate contamination risks (Brasileiro et al., 2025), while cross contamination can occur (Xiao et al., 2019). Research conducted in Switzerland revealed a 6% *Salmonella* prevalence, with *S. Infantis* emerging as the dominant serovar (Barmettler et al., 2025); similar trends were observed in Italy (Di Taranto et al., 2025) and Belgium (Zeng et al., 2021). In a study conducted in Japan, *S. Infantis* was identified in 32.1% of the cases (Ishihara et al., 2020). In China *S. Enteritidis* was the most dominant serovar (Zhou et al., 2025), while in Iran *S. Thompson* (Dillal et al., 2014).

Seasonal patterns in *Salmonella* contamination exist in many food products (Williams et al., 2014). In the context of current global climatic shifts, extreme weather events are of particular concern as they may facilitate the dissemination of *Salmonella*. Elevated temperatures promote accelerated growth rates, leading to a higher microbial load on broiler meat during the summer months (Mahmud et al., 2011, Naurin et al., 2013, Williams et al., 2014, Ishihara, 2020).

Seasonal trends indicated a rise in *Salmonella* occurrences in slaughterhouses during the warmer months, which is similar to findings of this research, which emphasizes the necessity for improved control strategies. These results underscore the crucial need for biosecurity measures on farms and the enforcement of stringent hygiene practices in processing facilities to mitigate *Salmonella* contamination in poultry production. Broiler fillet was selected as the reference category (OR = 1) due to its lowest observed contamination rate (5%). Broiler wings were the only sample type where the increase in risk was statistically significant ($p=0.025$). Specifically, the odds of a wing sample testing positive for *Salmonella* were 3.35 times higher than those for fillets. This significant increase in risk is likely associated with the combined effect of high skin surface-to-mass ratio and the anatomical positioning of wings during the suspension phase of the processing line. While carcasses and leg quarters showed numerically higher risks (OR 2.35 and 1.87, respectively), their confidence intervals were wider and crossed the reference line (1.0). This indicates that a larger sample size would be required to confirm statistical significance for these sample types.

A comparison of serovar proportions revealed that the prevalence of *S. Infantis* (82.5%) was highly significantly higher ($p<0.001$) compared to *S. Enteritidis* (17.5%). This finding indicates a strong adaptation of *S. Infantis* within this specific production chain, suggesting its endemic presence within the facilities or among suppliers. In early 2023 *S. Enteritidis* was now co-occurring, indicating either a shift in the microbial landscape or a collapse of control in peak summer months.

Looking at the colder months (November–February) versus the warmer months (May–August), a clear seasonal increase in pathogen occurrence was observed during warmer periods. The specific temperature cut-off points (7°C for inactivity and 17°C for *S. Enteritidis* emergence) serve as valuable data for risk assessment in either food safety or environmental monitoring.

Occurrence of *Salmonella* serotypes and environmental conditions are presented in Supplementary tables S1. The main conclusion is the great positive relationship of average monthly temperature with detection of *S. Infantis* and *S. Enteritidis*. By decreasing the ambient temperature drop to 7 °C, the detection of *Salmonella* spp. is almost 0. *S. Infantis* remains constant once temperature is in excess of 10–12 °C with a high correlation coefficient ($r=0.75$) that temperature is the primary force for its global distribution. *S. Enteritidis* occurs in the dataset when temperatures go beyond 17.5°C, with highest occurrence at 28.4 °C in 2024. Over the 5-year period, signs of contamination seem to rise. In 2020, only 2 positive samples were generated throughout the length of the year. By 2024, this number rose to 13 positive samples. Difference between the earlier years (2020–2022) was represented by *S. Infantis* alone.

The results of this study are indicative of a positive association between temperature and *S. Infantis* and *S. Enteritidis* in fresh broiler meat. Seasonal variations in Serbia, as represented by RHMZ 2020–2024 data, played a vital part in the evaluation and assessment of microbial safety for poultry specimens in Serbia. In particular, the rise of average medium temperature, more than 18 °C at the end of spring (May) and 24.5°C during summer months (July and August), increases *Salmonella* frequency. The period 2020–2024 has been marked by record-breaking temperatures, with 2024 officially recorded as the warmest year since 1888, featuring mean temperatures up to 2.7 °C above the historical average. Climatic influence is evident and this correlation is attributed to elevated ambient temperatures, which facilitate the accelerated proliferation of *Salmonella* spp. within production facilities and throughout the transport chain. This observation is consistent with the previously known biological behaviour of *Salmonella* spp., which respond to mesophilic environments. In addition, the almost complete lack of both strains during December–February (when the ambient temperature drops to about 2.4°C) indicates that low ambient temperature is its own natural deterrent and high temperature can disrupt the cold chain.

CONCLUSION

The presence of *Salmonella* spp. in fresh broiler meat and retail cuts represents a significant public health concern. The findings of this study revealed that *Salmonella* spp. occurrence in fresh broiler meat at a single FBO, immediately prior to market distribution and following all control measures, amounts to 10%. Among these cases, *S. Infantis* was the dominant serovar at 82.5%, as opposed to *S. Enteritidis* at

17.5%. The wide variability in the contamination rates—from 5% in skinless fillets to 15% in broiler wings—suggests the skin and inverted position of the carcass during the process as the main sources of microbial load. Additionally, the seasonal peaks (in summer) showed that warmer ambient temperatures greatly aid pathogen growth.

This paper verifies that both serovar distribution and environmental conditions play a very substantial role in determining the microbiological safety of broiler meat.

Although the broiler meat tested positive for *Salmonella* spp., broilers remained asymptomatic, showing no clinical signs of disease or growth impairment during the fattening period. The bacteria colonized the gastrointestinal tract of the birds, subsequently leading to carcass contamination during the slaughter process. Given the high intensity of commercial broiler meat production, currently implemented control measures are insufficient to completely guarantee the absence of *Salmonella* on the surface of retail broiler meat.

Author's contribution:

Mladen Rašeta, Becskei Zsolt: Conceptualization, methodology, and study design. Mladen Rašeta, Jelena Jovanović, Mirjana Lukić: Investigation, sampling, and laboratory microbiological analysis. Elmin Tarić, Becskei Zsolt, Boris Mrdović: Formal analysis, data curation, and software-assisted visualization of seasonal trends. Snežana Paskaš: Literature review and validation of results. Mladen Rašeta, Becskei Zsolt: Writing – original draft preparation. Mirjana Lukić, Jelena Jovanović, Snežana Paskaš: Writing – review and editing. Mladen Rašeta: Project administration and final supervision of the manuscript. All authors have read and agreed to the published version of the manuscript

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Ethical statement

This study did not involve experimental research on live animals. All procedures were conducted in accordance with applicable institutional and ethical standards. ARRIVE guidelines are not applicable to this study.

Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

No potential conflict of interest was reported by the authors

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NALAZ *SALMONELLA* U MESU BROJLERA PRE ISPORUKE NA TRŽIŠTE U JEDNOM SUBJEKTU U POSLOVANJU HRANOM U SRBIJI

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Kratak sadržaj

Prisustvo *Salmonella* spp. u mesu brojlera ostaje bitan problem za javno zdravlje i veliki izazov za prehrambenu industriju. U ovom radu je sistematski ispitana pojava i distribucija serovarijeteta *Salmonella* u svežem mesu brojlera na srpskom tržištu tokom pet godina (2020–2024). Ukupno 400 uzoraka, uključujući trupove brojlera (n=100), grudi (n=100), batake sa karabatakom (n=100) i krila (n=100), prikupljeno je iz skladišta jednog subjekta u poslovanju hranom i analizirano korišćenjem automatizovane metode skrininga VIDAS UP *Salmonella* (SPT) i standardnih protokola ISO 6579-1:2017/A1:2020.

Rezultati su pokazali ukupnu prevalenciju *Salmonella* od 10% (40/400). Identifikovana su dva serovarijeteta: *S. Infantis*, u 82,5% (n=33), i *S. Enteritidis* u 17,5% (n=7) pozitivnih slučajeva. Analiza distribucije u različitim delovima trupa brojlera pokazala je da su krila imala najveću stopu kontaminacije (15%), zatim celi trupovi (11%), bataci sa karabatakom (9%) i grudi (5%). Najniža učestalost zabeležena u mesu grudi ukazuje na to da je uklanjanje kože ključni faktor u značajnom smanjenju ukupnog mikrobnog opterećenja.

Sezonski trendovi koji ukazuju na učestaliju pojavu salmonele u toplijim mesecima ističu potrebu za poboljšanim strategijama kontrole tokom leta. Ovi nalazi naglašavaju važnost sveobuhvatne pripreme objekata sa veterinarsko-sanitarnog i građevinsko-tehničkog stanovišta kao preduslova za efikasan laboratorijski monitoring. Rezultati pokazuju da, uprkos sprovođenju postojećih higijenskih mera, značajna prevalencija *S. Infantis* zahteva strožu biološku bezbednost i sistematski nadzor u celokupnom lancu proizvodnje mesa brojlera kako bi se smanjile pretnje po zdravlje potrošača.

Ključne reči: *Salmonella*, *Salmonella Infantis*, *Salmonella Enteritidis*, meso živine, bezbednost hrane

Case report

TREATMENT OF MALIGNANT FIBROUS HISTIOCYTOMA IN A DONKEY WITH *CATHARANTHUS ROSEUS* EXTRACT - A CASE REPORT

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Abstract

Malignant fibrous histiocytoma (MFH) is a pleomorphic sarcoma of unknown etiology that rarely occurs in equines (horses and donkeys). In September 2021, a case of an 8-year-old female donkey with a tumor mass in the right eye was admitted to Animal Care (ACE) Hospital in Luxor, Egypt. The case underwent both clinicopathological and radiological examination. Based on microscopic examination, the morphological diagnosis was malignant fibrous histiocytoma. Radiological assessment (X-ray) revealed a non-metastatic tumor mass. An aqueous extract of *Catharanthus roseus* leaves and flowers was (ESC16) prepared by cold maceration and characterized by GC-MS analysis. The tumor was treated with 0.9 ml of an intratumoral injection of extract ESC16 and received one

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injection every other day for 50 days due to the large size of the tumor mass. The tumor showed macroscopic regression over the study period. To our knowledge, this is the first time that MFH in a donkey has been treated with *C. roseus* extract. ESC16 showed efficacy in treating MFH in that female donkey. Thus, it may be concluded that the intralesional injection of aqueous extract of *C. roseus* could be a promising antitumor agent for equine MFH in the Egyptian field.

Keywords: Malignant fibrous histiocytoma, donkeys, *Catharanthus roseus*, eyes, histopathology, radiology, gas chromatography, mass spectrometry.

INTRODUCTION

Malignant fibrous histiocytoma (MFH) is a pleomorphic sarcoma of unknown etiology that rarely occurs in equines (horses and donkeys) (Hamir, 1989; Schneider et al., 1999a; Valentine, 2006; Geburek et al., 2007). MFH is often manifested as a single solid or soft tissue mass with a diameter ranging from one to several centimeters. It is characterized by invasive growth and is not well separated from the surrounding tissue. Although MFH can develop anywhere on the body, it most frequently affects the neck, upper hind legs, knees, and proximal parts of all limbs (Geburek et al., 2007). Fibroblastic cells, histiocytic round or polygonal, and multinucleated giant cells make up MFH under a microscope (Hatano et al., 1999).

A survey conducted in the Pacific Northwest of the USA revealed that the incidence of MFH was 1.3% in all equine cutaneous neoplasms (Knottenbelt et al., 2015). Having developed from undifferentiated mesenchymal cells, it was known as an undifferentiated sarcoma and a giant cell tumor (Schneider et al., 1999b). Reliable and available information about MFH in Egypt is scarce. Previous investigations have denied an age- or breed-specific predilection for tumor development (Gilger, 2013). Other research found this tumor type in animals over 10 years of age with a male propensity; six cases of equine MFH were reported at ages ranging from 3 to 12 years, with a mean age of 6.8 years (Hamir 1989; Knottenbelt et al. 2015). The most common complication reported was recurrence in the ocular structures, which occurred in 10% to 51% animals, or in approximately 30% on average (Plummer et al., 2007). Many surgical and therapeutic interventions were utilized, including surgical excision of the third eyelid, keratectomy, enucleation, or exenteration (Elce et al., 2011); cryotherapy; radiofrequency hyperthermia; radiotherapy; topical and intralesional chemotherapy with cisplatin or 5-fluorouracil, Bacillus Calmette-Guerin (BCG) cell wall extract; and carbon dioxide laser ablation (Rabo et al., 2000; Chahory et al., 2002; Montiani-Ferreira et al., 2008; Cian et al., 2016).

C. roseus is a perennial herb species of the *Apocynaceae* family, originally native to Madagascar (Goyal et al., 2008). Its seeds, stems, leaves, and flowers contain various bioactive chemicals, including alkaloids, particularly vincristine and vinblastine (Kabesh et al., 2015; Sharma et al., 2019), steroids, phenolic compounds, flavonoids, saponins, terpenes, and anthocyanins (Ferrerres et al.,

2008; El Enshasy, 2019). Owing to its phytoconstituents that possess a wide range of important pharmacodynamical properties, *C. roseus* extract can be used to combat various diseases, for example, Hodgkin's disease, malaria, and diabetes. Vincristine and vinblastine, two essential anticancer agents, are solely present in this plant species, which generates significant interest in cancer therapy (Sharma et al., 2019; Chunarkar-Patil et al., 2024).

Here, a case of a female donkey histologically diagnosed with MFH in Luxor, Egypt, is presented. As a treatment option, an extract of *Catharanthus roseus* was prepared, used for intralesional injection, and monitored to evaluate its therapeutic efficacy. Given the clinical regression of the tumor, a successful treatment of MFH is reported.

CASE PRESENTATION

Anamnestic Data

In September 2021, an 8-year-old female donkey was admitted to Animal Care Hospital (ACE) in Luxor, Egypt, for further diagnosis and treatment. The animal had a tumor mass in the right eye, which was perceived by its owner a month and a half before the presentation as increasing in size over time. The owner stated that the donkey was a hard-working animal, housed in a sunny area, exposed to solar radiation. Palpation of the mass revealed its solid, soft consistency. An ocular neoplasm was suspected and confirmed by clinicopathological examination. Ophthalmic examination of the affected eye revealed a 5 x 3 cm tumor mass originating from the palpebral conjunctiva of the lower eyelid, with purulent discharge and epiphora leading to blackening skin under the eye, and moderate swelling of the mandibular lymph node (Figure 1). For histopathological examination, an 8-mm biopsy sample was collected. Moreover, X-ray images were captured before and after treatment.

Histopathological examination

Tissue biopsies from the affected eye with ocular neoplasm were collected and fixed in 10% neutral, buffered formalin, processed routinely in paraffin wax to form blocks, sectioned into 5- μ m-thick slices, and stained with hematoxylin and eosin (H&E). The second biopsy was obtained after the swelling had resolved. The evaluation of mitotic count (MC) via Aperio image scope for counting the mitotic figures was performed as follows: the hotspot area of high mitotic activity, free from necrosis, hemorrhage, or inflammation was identified, and a contiguous field of view (FOV) in an area of 2.37 mm² defined; at the highest power for the scanned slide, 22 FOV were selected, and the mitotic figures (MF) were counted within the area (Romansik et al., 2007).



Figure 1. A female 8-year-old donkey with malignant fibrous histiocytoma (MFH) in the right eye, admitted to the hospital in September 2021.

Radiological assessment

Radiography was used to determine if the tumor had metastasized into the bones of the eye socket. The examination was done with an Agfa computed radiography (CR) apparatus. An X-ray plate was held against the side of the donkey's face while a camera on a tripod took the pictures. Since the donkey is very sensitive about its head, the animal was sedated with xylazine to keep its head perfectly still. Several pictures were taken from different angles to "see around" the eye: lateral, oblique, and dorsoventral views.

***C. roseus* extract preparation**

C. roseus leaves and flowers were collected from the garden of the Animal Care Hospital (25°40'45.3"N 32°39'13.3"E) (Figure 2). The plant parts were screened, washed, dried, and ground into a fine powder with a high-speed mixer (Moulinex Egypt, Al-Araby Co., Egypt). The material that passed through an 80-mesh sieve was retained for use. The plant species was authenticated by the Botany Department of the Faculty of Science, Aswan University. The aqueous extract of *C. roseus* leaves and flowers was prepared by maceration of 150 g of dried powder in 1 L of distilled water at 37°C for 72 h (Da Silva et al., 2018). The supernatant was filtered using Whatman filter paper No. 1, and its moisture was removed in a rotary evaporator.

The extract was sterilized by autoclaving at 220°C for 1 h. The resultant extract was named ESC16.

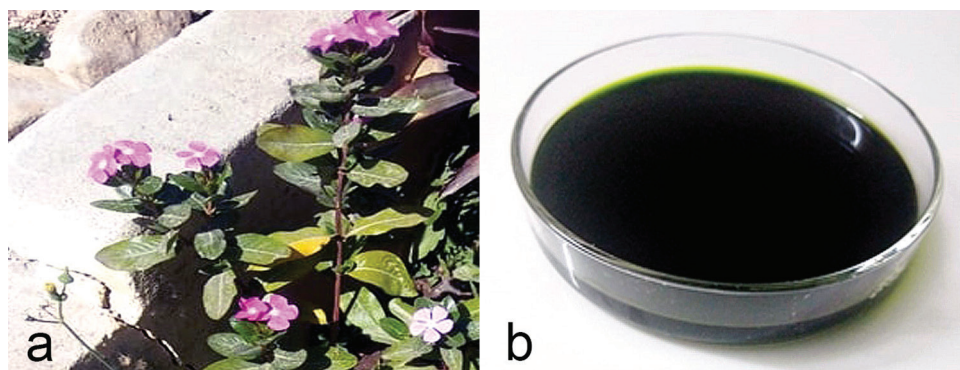


Figure 2. a) *Catharanthus roseus* plant. b) *Catharanthus roseus* aqueous extract

GC-MS analysis

The GC-MS analysis was carried out using a gas chromatography-mass spectrometry instrument TRACE GC Ultra Gas Chromatograph (Thermo Scientific Corp., USA), coupled with a thermo-mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC-MS system was equipped with a TR-5 MS column (30 m x 0.32 mm i.d., 0.25 μ m film thickness). The analyses were carried out using helium as carrier gas at a flow rate of 1.0 mL/min and a split ratio of 1:10 in the following temperature program: 60°C for 1 min, rising at 4.0°C/min to 240°C, and held for 1 min. The injector and detector were held at 210°C, and diluted samples (1:10 hexane, v/v) of 1 μ L of the mixtures were always injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 40-450. The identification of the chemical constituents of the essential oil was deconvoluted using AMDIS software (www.amdis.net) and identified based on their retention indices (relative to n-alkanes C8-C22), mass spectrum matching to authentic standards (when available), the Wiley spectral library collection, and the NIST library database.

Treatment protocol and follow-up

The tumor was irrigated with 100 ml of saline solution, followed by the intratumoral injection of 0.9 ml of ESC16. One injection was administered every other day for 50 days due to the large size of the tumor mass. After the treatment was finished, the animal remained in the hospital for an additional month and was monitored for any signs of recurrence. In addition, a long-term follow-up

was obtained through telephone contact with the owner, who also provided the photographs of the animal.

GC-MS findings

GC-MS analysis revealed that *C. roseus* extract contained fifteen compounds (Figure 3 and Table 1). The retention time, molecular weight, and molecular formulas of the compounds are presented. The results detected the presence of alcohols, esters, acids, steroids, nitrogen-derived compounds, and other secondary metabolites. The highest peak was obtained for the following compounds: 9,12,15-octadecatrienoic acid, methyl ester, (Z, Z, Z)-, 6,9,12,15-docosatetraenoic acid, methyl ester, with a retention time of 23.44.

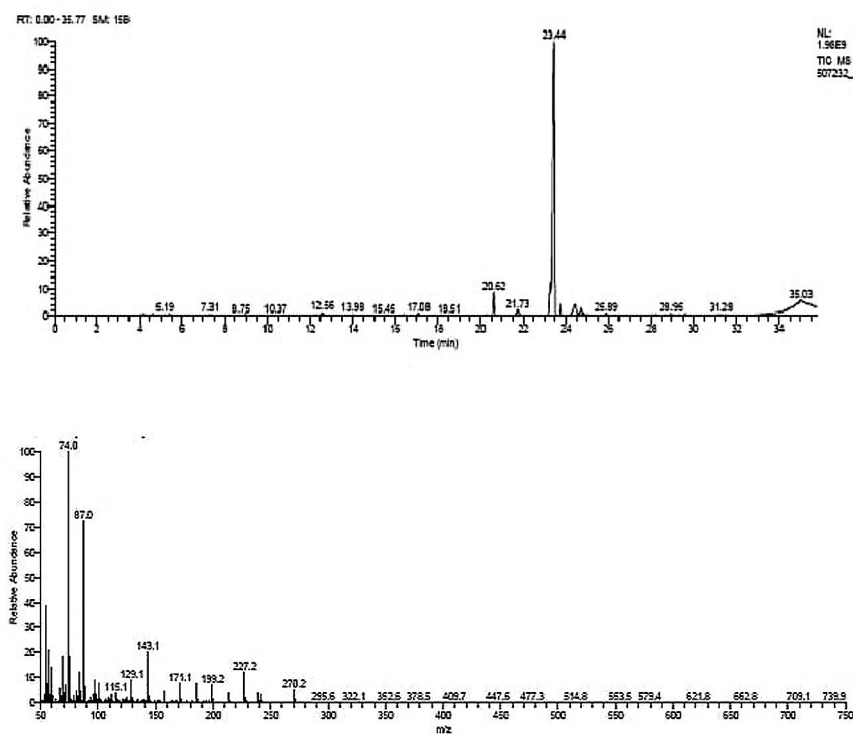


Figure 3. Chromatogram of *Catharanthus roseus* aqueous extract

GC-MS analysis of *C. roseus* revealed certain chemical compounds that have anticancer and antioxidant effects, such as 9,12,15-octadecatrienoic acid, methyl ester, (Z, Z, Z)- [Linolenic acid, methyl ester].

Table 1. GC-MS analysis of *C. roseus* aqueous extract

No	Compound	Molecular Formula	Rt	Molecular Weight	Area %
1	Hexadecanoic acid, methyl ester	C17H34O2	20.62	270	4.50
2	n-Hexadecanoic acid	C16H32O2	21.74	256	1.32
3	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	C19H34O2	23.26	294	4.01
4	Methyl 9-cis,11-trans-octadecadienoate	C19H34O2	23.26	294	4.01
5	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	C19H32O2	23.45	292	81.91
6	6,9,12,15-Docosatetraenoic acid, methyl ester	C23H38O2	23.45	346	81.91
8	Methyl stearate	C19H38O2	23.77	298	2.16
9	Trans-13-Octadecenoic acid	C18H34O2	24.43	282	3.03
10	Oleic acid	C18H34O2	24.43	282	3.03
11	Cis-Vaccenic acid	C18H34O2	24.43	282	3.03
12	Octadecanoic acid	C18H36O2	24.73	284	1.33
13	4h-1-benzopyran-4-one, 2-(3,4 dihydroxyphenyl)-6,8- Di-á-d glucopyranosyl-5,7- Dihydroxy-[6,8-DI-C-á-GLUCOSYLLUTEOLIN]	C27H30O16	35.03	610	1.74
14	1-Heptatriacotanol	C37H76O	35.03	536	1.74
15	03027205002 flavone 4'-oh,5-oh,7-di-o-glucoside	C27H30O15	35.03	594	1.74
16	Rhodopin	C40H58O	35.03	554	1.74

Clinical findings

On day 10, the tumor mass began to shrink; on day 20, it diminished, as did the swelling of the lymph node. On day 30, the tumor decreased in size by half. On day 40, the tumor was barely detected, and on day 50, it completely disappeared (Figures 4).

Histopathological findings

The microscopic examination of the tumor biopsy sample revealed a hypercellular, densely packed neoplasm (Figure 5). The mitotic figures were identified in two areas of 2.37 mm² and revealed numerous mitotic figures, both typical and atypical ones (Figure 5a). The average number of mitotic figures across 22 contiguous FOVs in each area was 2.14. The neoplastic cells exhibited two different cellular patterns: spindle-shaped cells with a fascicle pattern and polygonal cells with a cluster aggregation pattern (Figure 5b).



Figure 4. a) Tumor disappearance after 50-day treatment protocol with intralesional injection of *Catharanthus roseus* 0.9 % aqueous extract. b) At the five-year follow-up (April 2026), no tumor recurrence was found.

The neoplastic cells were of two types: polygonal and spindle. The spindle cell fibroblasts were arranged in irregular patterns of interconnected fascicles with occasional storiform patterns and dense sheets (Figure 5c) and contained hyperchromatic nuclei (Figure 5d). These cells, admixed with a pleomorphic population of histiocytes and multinucleated tumor giant cells (MGCs) with abundant eosinophilic cytoplasm, vesicular nuclei, and prominent nucleoli, showed many xanthoma cells laden with lipid vacuoles. Generally, the histiocytes exhibited anisokaryosis, nuclear hyperchromasia, and mitoses. The inflammatory reaction of mononuclear cells was evident between neoplastic cells (Figures 5e and 5f). The phytotherapeutic treatment of the tumor mass resulted in its regression and markedly affected the neoplastic cells. The microscopic evaluation of the tumor mass after phytotherapy revealed hypocellularity, massive necrosis of neoplastic cells (Figure 5g), apoptosis of histiocytes, and necrosis of fibroblasts (Figure 5h). In addition, there were no xanthoma cells or tumor giant cells in the treated tumor. Neutrophil infiltration was observed between the neoplastic cells.

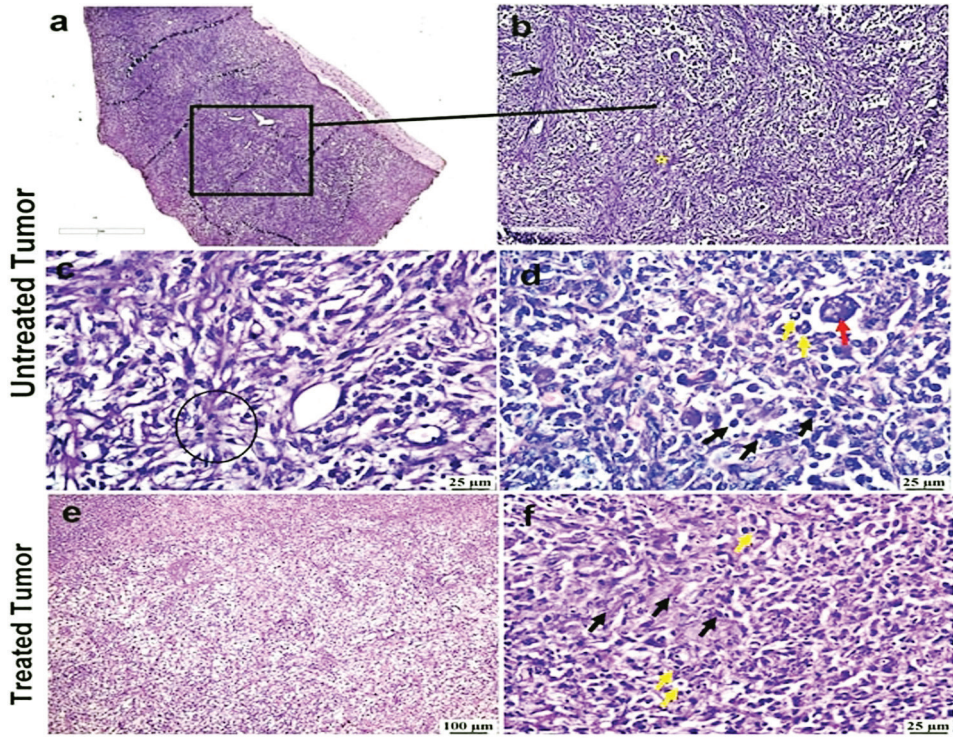


Figure 5. Histopathologic section of malignant fibrous histiocytoma, excised untreated (a-f) and treated tumor mass (g-h) showing dense cellular closely packed neoplastic cells a) with typical (black & red lines) and atypical multipolar mitotic figures (yellow line). Two different histological patterns of arrangement, irregular, interconnected, fascicles (black arrows) and clustered group population of polygonal cells (yellow arrows) (b). The spindle-shaped fibroblasts showed a storiform growth pattern (c), with spindle hyperchromatic nuclei (black arrows) associated with an inflammatory reaction in the stroma (d), The second type of pleomorphic histiocytes admixed with multinucleated giant cells (e), the neoplastic cells showing anisokaryosis, karyomegaly, and vesicular nuclei, prominent nucleoli, mitoses were evident (black arrows), xanthoma cells (yellow arrows) (f). Hypocellularity of the tumor mass (g) with necrosis of fibroblasts and apoptosis of histiocytes (black arrows) associated with an inflammatory reaction (h).

DISCUSSION

Malignant fibrous histiocytomas (MFH), now known as undifferentiated pleomorphic sarcomas (UPSs), are a group of soft tissue sarcomas that have been reported in many species, including humans, horses, dogs, cats, mice, rats, rabbits, birds, a pig and a cow (Al-Agha and Igbokwe 2008; Reesink et al. 2009). The MFH of soft parts, also referred to as a giant cell tumor, is considered rare in the horse (Marryatt, 2003). This tumor was reported in the hindquarters of

Arabian horses and mules (Hamir, 1989; Meuten, 2020) and in the forelimb quarter of a horse mare (Geburek et al. 2007). Also, previously conducted surveys confirmed the development of MFH in soft tissue in different body parts: the neck, limbs, mammary tissue, muzzle, shoulders, and trunk (Knottenbelt et al., 2015). Mammary tumors are uncommon in mares; however, MFH was documented in the mammary gland of a 6-year-old mare (Reesink et al., 2009). Ocular MFHs were recorded previously in dogs (Smith et al., 1976), cats (Demir et al., 2023), and a man (Ryan et al., 2012). The presented case revealed the development of MFH at an older age, as it was recorded at 8 years, which corresponds with previous studies (Hamir, 1989; Marryatt, 2003). Hamir (1989) stated that there is no sex bias in the development of MFH. In this case, MFH was reported in a donkey as previously documented (Valentine, 2006). Geburek et al. (2007) used conventional and laser surgery to treat MFH in the forelimb of a Quarter Horse mare. Generally, post-surgical recurrence of MFH was a variable outcome with a high rate after surgical excision. Similarly, Bellitto et al. (2023) used a harmonic scalpel to excise a large fibrosarcoma in the distal equine limb.

The tumor's histopathology validated the diagnosis of MFH. Histologically, the tumor was hypercellular and of an infiltrative growth. The neoplastic cells were composed of fibroblasts admixed with a pleomorphic population of histiocytes and multinucleated tumor giant cells with abundant eosinophilic cytoplasm and vesicular nuclei. Generally, the histiocytes exhibited anisokaryosis and nuclear hyperchromasia, and frequent mitosis. Regarding the histopathology of this tumor type, a similar microscopic description was recorded by (Marryatt et al (2003).

In the present case, we showed an 8 years-old female donkey afflicted with MFH that agrees with previous reports documented old age of animals affected with MFH (Hamir, 1989; Schneider et al., 1999a; Mair and Couto, 2006; Valentine, 2006; Knottenbelt et al., 2015). The lesion originated from the palpebral conjunctiva of the lower eyelid of the right eye, and it had a soft and solid consistency. Eye infection associated with malignant cell tumor is uncommon, according to previous studies (Geburek et al. 2007). Moreover, the lesion was assessed via radiography, which did not reveal any metastasis in the surrounding areas (Figure 6).

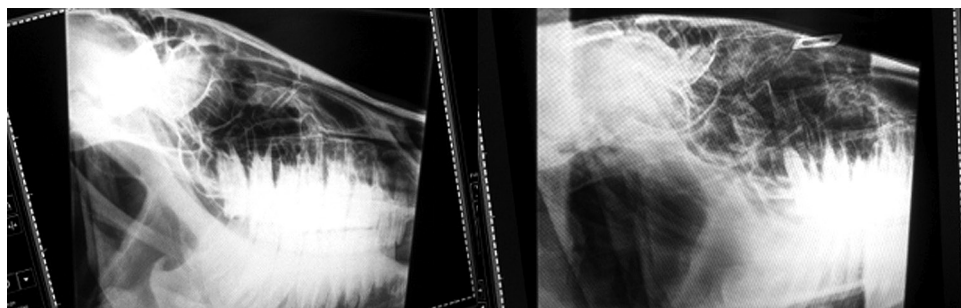


Figure 6. Radiographs revealed a non-metastatic tumor mass, as the tumor had not invaded the skull.

Catharanthus roseus (*C. roseus*) is an important medicinal plant that grows in many countries. Since *Catharanthus roseus* ("Annual Vinca") is well known for its anticancer activity (El Enshasy, 2019; Chunarkar-Patil et al., 2024), an aqueous extract of its leaves and flowers was prepared to assess its efficacy against MFH when administered via intra-tumoral injections. *C. roseus* produces the anticancer bisindole alkaloids vinblastine and vincristine, which are derived from the union of the monomeric terpenoid indole alkaloids catharanthine and vindoline (Zhu et al., 2015). The extract was characterized using GC-MS analysis. GC-MS analysis identified 15 compounds in the extract of *C. roseus*. GC-MS analysis of *C. roseus* extract showed the presence of hexadecanoic acid, methyl ester [C17H34O2], 9,12-Octadecadienoic acid (Z,Z)-, methyl ester [C19H34O2], Methyl 9-cis,11-trans-octadecadienoate [C19H34O2], 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- [C19H32O2], 6,9,12,15-Docosatetraenoic acid, methyl ester [C23H38O2].

Bansal et al. (2023) reported that the presence of hexadecanoic acid, methyl ester, as bioactive in Anther Callus of *C. roseus* L. Methyl 9-cis,11-trans-octadecadienoate is a polyunsaturated fatty acid (fatty acid ester of linoleic acid) that has anti-inflammatory and anti-carcinogenic properties (Cifuni et al., 2023). This fatty acid and its derivatives are known to exert potent antioxidant activities (El Hosry et al., 2023; Radajewska et al., 2023). 9, 12, 15-octadecatrienoic acid, methyl ester, (z, z, z)-] is an essential polyunsaturated fatty acid molecule. It was reported that 9,12,15-octadecatrienoic acid can inhibit caspase-3. It has anticancer, antibacterial, anti-inflammatory, and antioxidant properties (Vohra and Kaur, 2011; Yamunadevi et al., 2011; Akpuaka et al., 2013; Rani et al., 2021). The extract from *C. roseus* cell cultures exhibits its most potent anticancer effects through the combined action of multiple bioactive compounds rather than any single component. Studies suggest these compounds act synergistically, enhancing their impact on cancer cells. This synergistic interaction, also noted in other plants, is currently being explored as a potential strategy for cancer therapy (Bhuyan et al., 2018).

Macroscopically, following treatment, the tumor mass has undergone regression without complications. The microscopic appearance showed hypocellularity of the tumor mass, massive necrosis involving the neoplastic cells; apoptosis of histiocytes and coagulative necrosis of fibroblasts. In addition, there were no xanthoma cells or tumor giant cells in the treated tumor. Neutrophil infiltration was observed between the neoplastic cells. It showed the direct cytotoxic effect of *C. roseus* extract on cancerous cells, resulting in tumor regression. Other previous treatments have some disadvantages; for example, cisplatin-based intralesional chemotherapy is toxic and is a hazard to personnel exposure (Théon, 1998), systemic chemotherapy is costly and leads to adverse reactions and results in potential tumor recurrence (Geburek et al., 2007). Laser treatment and its thermal effects are considered effective for inhibiting tumor growth in horses (Weigand and Gerhards, 1996). After seven weeks, a regression in the size of the lesional area was noted, as the tumor mass was initially large. Interestingly, no recurrence or relapse was reported in the female

donkey after intralesional treatment with *C. roseus* extract either during the one-month stay in the hospital or follow-up via telephone contact with the owner and photographs of the animal. Radiography confirmed that the MFH didn't extend to the hard tissues (Figure 6). MFH, in this case, was confirmed, and its metastasis to the surrounding bone was assessed through clinical and histopathological examination, and diagnostic imaging, as reported in similar cases (Reesink et al. 2009), such as in soft tissue sarcomas (Bellitto et al., 2023).

CONCLUSION

The current paper demonstrated that the aqueous extract of *Catharanthus roseus* had anti-tumor activities. *C. roseus* extract showed efficacy in the treatment of malignant fibrous histiocytoma in a donkey. Thus, it could be concluded that the intralesional injection of aqueous extract of *C. roseus* may be a potential therapeutic agent; however, this is a preliminary finding that requires further studies.

Authors' contributions

HS, AAM, AAS, FFM, MAE, and AA have equally contributed to the case description and manuscript preparation.

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Data availability

All data generated during this study are included in this published article.

Ethical statement

The tissues were fixed and processed routinely as per any clinical ophthalmic case. This study did not influence the recommended therapy in any way. The owner provided consent for his donkey to participate in this study and for the publication of this case report and any accompanying images.

Competing interests

The authors declare no competing interests.

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TRETMAN MALIGNOG FIBROZNOG HISTIOCITOMA KOD MAGARCA EKSTRAKTOM BILJKE *CATHARANTHUS ROSEUS*– PRIKAZ SLUČAJA

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Kratak sadržaj

Maligni fibrozni histiocitom (MFH) je pleomorfni sarkom nepoznate etiologije, koji je redak kod ekvida (konja i magaraca). U septembru 2021, osam godina stara magarica sa tumorskom masom na desnom oku dovedena je u Bolnicu za životinje (ACE) u Luksoru, Egipat. Urađen je klinički i radiološki pregled životinje. Na osnovu mikroskopskog pregleda dijagnostikovao je maligni fibrozni histiocitom. Radiografijom je ustanovljena tumorska masa bez metastaza. Vodeni ekstrakt lišća i cvetova biljke *Catharanthus roseus* (ESC16) pripremljen je hladnom maceracijom i analiziran primenom GC-MS. Tumor je tretiran primenom 0,9 ml ekstrakta ESC16 koji je zbog veličine tumorske mase aplikovan intratumorskom injekcijom svaki drugi dan tokom 50 dana. Tokom perioda praćenja tumor se makroskopski smanjio. Po saznanju autora rada, ovo je prva primena ekstrakta *C. roseus* u lečenju MFH kod magaraca. ESC16 se pokazao efikasnim u tretmanu MFH kod ove magarice. Na osnovu svega navedenog, može se zaključiti da vodeni ekstrakt biljke *C. roseus* primenjen u obliku injekcija u tumorsko tkivo može predstavljati obećavajući antitumorski agens protiv MFH kod ekvida u egipatskim uslovima.

Ključne reči: Maligni fibrozni histiocitom, magarci, *Catharanthus roseus*, oči, histopatologija, radiologija, gasna hromatografija, masena spektrometrija.

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Notes for Contributors

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The journal publishes high-quality research in veterinary medicine, biotechnology, and related interdisciplinary fields, with particular emphasis on animal health, public health, food safety, and the One Health approach.

Starting from May 2026, significant changes have been introduced in the manuscript preparation and submission process. Authors are strongly advised to carefully review these updated requirements. Additionally, all necessary documents and templates required for manuscript preparation and submission are available in the “**AVM Submission Toolkit 2026**”, which can be downloaded from the following link: https://niv.ns.ac.rs/e-avm/AVM-Manuscript_Submission_Toolkit-2026.zip.

All submissions must comply strictly with the guidelines below. Manuscripts that do not conform to the journal’s requirements may be returned prior to peer review.

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The journal publishes the following types of manuscripts:

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- **Review Articles**
- **Short Communications**
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Materials and Methods	Required	Not applicable	Required	Optional
Results	Required	Not applicable	Combined with Discussion	Not applicable
Discussion	Required	Included in Main Text	Combined with Results	Required
Conclusion	Required	Required	Required	Required
Acknowledgements (if applicable)	Optional	Optional	Optional	Optional
References	≤ 40	≤ 60	≤ 15	≤ 15
Total Word Limit	< 5000	< 6000	< 3000	< 2500

Note: Word limits apply to the main text and exclude references, tables and figure legends.

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All manuscripts must include the required declarations immediately after the Conclusion and before the References section, following the exact order presented below:

- **Author Contributions**
- **Funding**
- **Ethical Statement (where applicable)**
- **Data Availability Statement**
- **Conflict of Interest**
- **Acknowledgments (optional; include in this position if applicable)**
- **ORCID iDs**

These statements form an integral part of the manuscript structure. Submissions that do not include all required declarations in the correct position may be returned to authors prior to peer review.

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- **Cover Letter**
- **Authors Declaration Form (3-in-1: Authorship, Contributions, Competing Interests, and Copyright & License Agreement)**
- **Ethical Approval Document (for experimental animal studies)**
- **ARRIVE 2.0 Checklist (for experimental animal studies)**
- **Owner Consent Confirmation (for studies involving client-owned animals)**
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Archives of Veterinary Medicine applies the **Harvard (author–date) referencing system**. Detailed instructions for formatting references are provided in the **AVM Submission Toolkit 2026**, which can be downloaded from the following link: https://niv.ns.ac.rs/e-avm/AVM-Manuscript_Submission_Toolkit-2026.zip This guide contains comprehensive instructions for preparing in-text citations and reference lists in accordance with the journal's referencing style and editorial standards.

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Authorship and Contributions

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Before starting the submission process, authors must ensure that:

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- The manuscript complies with formatting and structural requirements.
- All mandatory end-of-manuscript statements are included in the correct order.
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Manuscripts that do not meet these criteria may be returned for technical correction or rejected without external review (desk rejection). Manuscripts passing the initial screening are sent to at least two independent expert reviewers. Reviewers are selected based on subject expertise, academic competence, and absence of conflicts of interest.

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Based on reviewers' reports, the Editor may reach one of the following decisions:

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If reviewers provide substantially divergent recommendations, the manuscript may be sent to an additional reviewer for further evaluation. The final decision regarding acceptance or rejection rests with the Editor-in-Chief. Authors may be invited to revise their manuscript. A detailed point-by-point response to reviewers' comments must accompany all revised submissions. Authors who believe that a decision has been made in error may submit a justified appeal to the Editor-in-Chief. Appeals are evaluated objectively and may involve consultation with members of the Editorial Board. All participants in the peer review process are expected to adhere to the journal's ethical standards and confidentiality principles.

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Dmytro Masiuk, Oleksiy Sheptukha, Victor Nedzvetsky: CURRENT APPROACHES TO MODULATION OF INTESTINAL BARRIER FUNCTIONING IN PIGLETS: A LITERATURE REVIEW.....	1
Marko Koprivica, Tatjana Pustahija, Snežana Medić, Mirjana Štrbac, Miodub Ristić: EPIDEMIOLOGY OF HAEMORRHAGIC FEVER WITH RENAL SYNDROME IN THE AUTONOMOUS PROVINCE OF VOJVODINA, SERBIA, 1992–2023.....	36
Cut Nila Thasmi, Husnurrisal Husnurrisal, Tongku Nizwan Siregar, Hafizuddin Hafizuddin, Amalia Sutriana, Herrialfian Herrialfian, Faisal Jamin, Darniati Darniati, Shakira Ramadhani, Naila Nuha Najdha, Alya Fadilah, Syeicha Le Adzra: ANTIBACTERIAL AND ANTIBIOFILM ACTIVITIES OF ECO-FRIENDLY HYDROGEL <i>MORINGA OLEIFERA</i> –CHITOSAN FORMULATION AGAINST <i>ESCHERICHIA COLI</i> AND <i>STAPHYLOCOCCUS AUREUS</i>	54
Vesna Milićević, Slaviša Stojković, Sofija Šolaja, Dimitrije Glišić, Snežana Ristevski, Dragan Ristevski, Gordana Ristić, Vladan Miljković: DECLINE OF MATERNAL ANTIBODIES AGAINST SWINE INFLUENZA A AND PRRS IN PIGLETS FROM DIFFERENT SOW VACCINATION PROGRAMS.....	76
Asma Dahmani, Safia Zenia: CLINICAL AND EPIDEMIOLOGICAL INVESTIGATION OF FELINE CHRONIC GINGIVOSTOMATITIS AND PERIODONTAL DISEASE IN BLIDA, NORTH OF ALGERIA...	90
Razika Boukert, Dahia Saidj, Sara Boukert, Baaissa Babelhadj, Mira Chikhaoui, Amir Abderrauof Benotmane, Başak Şentürk, Eylül Ece Islek Camadan, Buse Kevser Karadeniz, Gizem Sele, Tanil Kocagöz, Naima Sahraoui: PREVALENCE, RISK FACTORS AND MOLECULAR CONFIRMATION OF <i>EIMERIA</i> SPP. IN ONE-HUMPED CAMELS (ALGERIA).....	107
Temitope Morenikeji Oladipo, Olusola Lawrence Ajayi, Joel Temidayo Odion, Fakilahyel Musa Mshelbwala: CLINICOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL EVALUATION OF MAMMARY TUMOURS IN INTACT FEMALE DOGS.....	123
Fatimah Usman, Masagus Irsan Saleh, Kemas Yusuf Effendi, Peby Maulina Lestari, Widjiati Widjiati, Elvandy Suwardy Tjan, Bella Stevanny: ASSOCIATION BETWEEN INTESTINAL ZONULIN EXPRESSION AND POLYCYSTIC OVARY SYNDROME IN RATS.....	142
Mladen Rašeta, Boris Mrdović, Jelena Jovanović, Mirjana Lukić, Snežana Paskaš, Elmin Tarić, Becskei Zsolt: THE OCCURRENCE OF <i>SALMONELLA</i> IN BROILER MEAT PRIOR TO ENTER THE MARKET AT ONE FOOD BUSINESS OPERATOR IN SERBIA.....	156
Hana Salama, Asmaa A. Metwally, AbdulRahman A. Saied, Faten F. Mohammed, Mohamed A. El-Saied, Asmaa Ali: TREATMENT OF MALIGNANT FIBROUS HISTIOCYTOMA IN A DONKEY WITH <i>CATHARANTHUS ROSEUS</i> EXTRACT - A CASE REPORT.....	176