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Prof. Dr. Keban ŞAHNAZ

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CONTENTS

CHAPTER 1

MALE REPRODUCTION AND DOXORUBICIN

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Doxorubicin (DOX) is an anthracycline antibiotic derived from *Streptomyces peucetius* and has long been established as a potent antineoplastic agent employed in the treatment of a wide range of malignancies, including leukemia, lymphoma, and various solid tumors (Doroshow et al., 1980; Atessahin et al., 2006; Lebrecht et al., 2007; Gharanei et al., 2013). Its antitumor activity primarily arises from topoisomerase II inhibition, the ability to intercalate into DNA, and the induction of DNA strand breaks, which disrupt replication and transcription processes (Kim et al., 1999; Quiles et al., 2002; Boitani & Puglisi, 2007; Patel et al., 2012). However, these pharmacological properties are also associated with significant off-target toxicities, manifesting as cardiac dysfunction, nephrotoxicity, and notable adverse effects on the reproductive system (Miranda et al., 2003; Minotti et al., 2004; Yeh et al., 2009; Ayla et al., 2011).

The detrimental effects of DOX treatment on male reproductive health have been recognized for a long time. Both clinical and experimental studies have demonstrated that DOX significantly impairs fertility by reducing sperm production, count, and motility (Prahalathan et al., 2005; Howell & Shalet, 2005; Yeh et al., 2007; Hozayen, 2012). Key testicular effects of DOX include disruption of the seminiferous epithelial cycle (Sjöblom et al., 1998), selective damage to rapidly proliferating germ cells such as A1–A4 spermatogonia (Lu & Meistrich, 1979), and the occurrence of chromosomal abnormalities in spermatocytes (Au & Hsu, 1980; Baumgartner et al., 2004). These alterations are closely linked to compromised sperm DNA integrity, which plays a critical role in male infertility (Koch et al., 1989).

The testicular toxicity induced by DOX is primarily mediated through molecular mechanisms such as oxidative stress, lipid peroxidation, and apoptosis. Reactive oxygen species generated during DOX metabolism promote lipid peroxidation in cellular membranes, mitochondrial dysfunction, and oxidative damage to nucleic acids, thereby compromising germ cell viability (Kiyomiya et al., 2001; Minotti et al., 2004; Atessahin et al., 2006; Carvalho et al., 2009; Menna et al., 2010; Trivedi et al., 2011; Hozayen, 2012). Furthermore, elevated proinflammatory cytokine release and activation of caspase-3-mediated apoptosis accelerate germ cell loss and impair sperm quality, ultimately contributing to functional infertility (Shinoda et al., 1999; Baumgartner et al., 2004; Türedi et al., 2015).

In addition, the effects of DOX on DNA synthesis and cellular metabolism extend beyond topoisomerase II inhibition, encompassing diverse biochemical processes such as the modulation of inflammatory mediators, cholesterol biosynthesis, and the generation of reactive nitrogen and oxygen species (Chen et al., 2018; Yun et al., 2019). Reduced activity of enzymes involved in steroidogenesis, including 17α-hydroxylase and 3β-HSD, disrupts the hypothalamic-pituitary-gonadal (HPG) axis, thereby altering testosterone, LH, and FSH levels and leading to hormonal imbalance (Mohan et al., 2021). Moreover, DOX-induced testicular damage has been linked to impairments in autophagy mechanisms, where dysregulation of the mTOR/beclin-1 signaling pathway may result in structural and functional disturbances during spermatogenesis (Dirks-Naylor, 2013; Lv et al., 2020; Christidi & Brunham, 2021). Excessive beclin-1 expression and defective autophagic flux have been associated with compromised blood–testis barrier (BTB) integrity and increased susceptibility of germ cells to apoptosis (Moreira et al., 2019; Christidi & Brunham, 2021).

Despite these findings, no single pharmacological agent has yet been proven to completely prevent DOX-induced testicular toxicity (Jahnukainen et al., 2001). Although various studies have investigated the protective effects of antioxidant and anti-apoptotic agents, the high cellular turnover of the male reproductive system and the unique microenvironment of the testes have limited the overall therapeutic efficacy.

Chemical Structure of Doxorubicin

Doxorubicin possesses a complex chemical structure composed of a tetracyclic anthracycline core linked to an amino sugar moiety called daunorubicin, with these two structural elements largely determining its antineoplastic activity (Weiss, 1992; Minotti et al., 2004). The planar aromatic anthracene ring of the molecule enables intercalation between DNA base pairs, forming a critical component of its cytotoxic mechanism (Tacar et al., 2013). The quinone and hydroquinone rings within the structure contribute to the generation of reactive oxygen species through redox cycling, thereby enhancing oxidative cellular damage (Gewirtz, 1999; Carvalho et al., 2009). Furthermore, the carbonyl group at C-7, the hydroxyl group at C-14, and various hydroxyl and methoxy substituents confer a unique three-dimensional conformation to the molecule, significantly influencing its pharmacodynamic properties (Pommier et al., 2010). These structural features constitute the fundamental biochemical basis for doxorubicin's potent inhibition of the topoisomerase II enzyme (Nitiss, 2009).

Molecular and Cellular Mechanisms of Doxorubicin

Doxorubicin (DOX) is an anthracycline agent whose antineoplastic activity primarily relies on its capacity to interact with DNA and inhibit topoisomerase II. Upon entering the cellular environment, its planar aromatic structure allows DOX to intercalate easily between DNA base pairs, disrupting the helical architecture of DNA and consequently interfering with replication and transcription processes (Pommier et al., 2010). This structural perturbation not only halts cell cycle progression but also induces pronounced cytotoxicity, particularly in rapidly dividing germ cells (Gewirtz, 1999).

DOX also disrupts the catalytic activity of topoisomerase II, thereby interfering with the normal DNA unwinding and re-ligation cycle. Under physiological conditions, topoisomerase II relieves torsional stress in double-stranded DNA by introducing transient breaks. DOX stabilizes the topoisomerase II–DNA complex, preventing the repair of these temporary breaks; as a result, double-strand DNA breaks accumulate and apoptotic signaling pathways are activated (Nitiss, 2009; Montecucco & Biamonti, 2007). This mechanism poses a direct threat to male fertility, as it induces substantial DNA damage, particularly in spermatogonial and spermatocytic cells.

Another fundamental mechanism of DOX toxicity is its ability to induce excessive production of reactive oxygen species (ROS). Through redox cycling of its quinone moiety, DOX generates highly reactive free radicals such as superoxide, hydroxyl radicals, and hydrogen peroxide. These radicals target cellular membranes, mitochondria, and nuclear DNA (Minotti et al., 2004). Given the structurally high content of polyunsaturated fatty acids (PUFAs) in testicular tissue, lipid peroxidation progresses rapidly, leading to severe compromise of testicular cell integrity (Aitken & Roman, 2008).

Mitochondrial effects constitute another critical component of DOX-induced toxicity. DOX disrupts mitochondrial membrane potential, impairing the function of the electron transport chain. In particular, these disruptions reduce ATP production, thereby compromising cellular energy homeostasis (Berthiaume & Wallace, 2007). Cells experiencing energy deficits exhibit heightened oxidative stress, accompanied by rapid accumulation of mitochondrial DNA

(mtDNA) mutations. Given the high energy demands of germ cells, mitochondrial dysfunction leads to significant disturbances in sperm development.

DOX also activates pro-inflammatory pathways, leading to increased cytokine release. Elevations in inflammation markers such as NF-κB and TNF-α promote chronic inflammatory responses within the tissue and exacerbate cellular stress (Octavia et al., 2012). Although testicular tissue is normally considered an immunologically privileged site, exposure to DOX can compromise this barrier and facilitate infiltration of inflammatory cells.

Additionally, DOX strongly induces apoptotic cell death pathways. Increases in the Bax/Bcl-2 ratio, cytochrome c release, and caspase-3 activation indicate active engagement of apoptotic mechanisms (Kalyanaraman, 2020). Elevated apoptosis in germ cells leads to a significant reduction in sperm production.

Recent Studies on Doxorubicin

DOX administration adversely affected testicular function in rats, causing significant body weight loss, reductions in testicular and epididymal weights, decreased sperm count and motility, increased sperm abnormalities, and irregularities in the seminiferous tubules. Additionally, DOX led to a marked reduction in testosterone levels and disrupted LH and FSH balance, while increasing oxidative stress in testicular tissue, weakening antioxidant defense mechanisms, and elevating inflammatory markers. Regarding apoptotic indicators, DOX treatment elevated caspase-3 and Bax expression while decreasing BCL2 and SIRT1 levels, thereby compromising cellular survival. In contrast, concurrent treatment with NCR mitigated DOX-induced weight loss, preserved testicular weight, and improved sperm parameters. NCR also restored seminiferous tubule architecture by reducing cellular degeneration and vacuolization, supported spermatogenesis, and increased the Johnsen score. Moreover, NCR balanced testosterone, LH, and FSH levels to maintain endocrine function, attenuated oxidative stress and inflammatory responses, decreased MDA levels, and restored SOD, GSH, and NO activities to near-normal levels. Regarding apoptotic pathways, NCR reduced caspase-3 and Bax expression while increasing BCL2 and SIRT1 levels, thereby supporting cellular survival and providing a protective effect against DOX-induced testicular damage (Alshuwayer et al., 2025).

Türedi et al. (2015) reported that testicular and epididymal tissues in the control, RES, and DMSO groups exhibited normal histological architecture. In the DOX-treated group, they observed irregularities in the seminiferous basal membrane and epithelium, vacuolization, a reduction in germ cell numbers, and the presence of immature germ cells within the lumens. In the group receiving DOX combined with RES, immature cells were rarely observed in the lumens, and the overall histological appearance closely resembled that of the control group. The Johnsen Tubular Score was found to be decreased in the DOX group compared to controls, whereas it was increased in the DOX + RES group relative to the DOX group. Testicular atrophy index was elevated in the DOX group and reduced in the DOX + RES group. Regarding epididymal sperm parameters, DOX treatment led to decreased sperm concentration and

motility, along with an increased proportion of abnormal sperm. Conversely, DOX + RES administration resulted in increased sperm concentration and motility and a reduction in abnormal sperm rates.

DOX administration in animals led to a significant increase in plasma LPO levels, reductions in LH and testosterone concentrations, decreased sperm count, and elevated proportions of dead and morphologically abnormal sperm. Additionally, DNA damage in epididymal spermatozoa was significantly higher compared to the control group. In contrast, co-administration of nZnO with DOX largely normalized the elevated LPO levels, improved the reduced LH and testosterone concentrations, mitigated the decrease in sperm count and the increase in dead and abnormal sperm, and markedly suppressed DOX-induced DNA damage, thereby exerting a protective effect on reproductive parameters (Badkoobeh et al., 2013).

In a study, treatment of male rats with DOX was found to cause a significant decrease in sperm concentration and motility, accompanied by an increase in the number of dead and abnormal sperm compared to the control group. However, concomitant administration of the aqueous extract of *C. monogyna* fruits resulted in a significant improvement in semen quality and substantially mitigated the toxic effects induced by DOX (Shalizar Jalali & Hasanzadeh, 2013).

DOX administration resulted in marked adverse effects on reproductive and testicular functions, including reductions in FSH, LH, and testosterone levels, as well as decreased amounts of steroidogenic enzymes 17α-hydroxylase and 3β-HSD. Additionally, DOX exposure impaired sperm motility, increased the proportion of immotile sperm, and reduced sperm count, normal morphology, and semen volume. In terms of oxidative balance, DOX decreased the activities of key antioxidant defense components in the testes, including GSH, GST, catalase, and SOD, while elevating MDA and nitrite levels. Concurrently, DOX induced pronounced inflammatory responses, increased caspase-3 activity, reduced Bcl-2 levels, and disrupted autophagy signaling, characterized by decreased mTOR and increased Beclin-1, leading to apoptotic and autophagic disturbances. Histological examination revealed severe disruptions in spermatogenesis, including degeneration of seminiferous tubules, vacuolization, disorganized cellular arrangement, extensive collagen deposition, and fibrotic area expansion. In contrast, lutein pretreatment substantially mitigated these multifaceted deleterious effects of DOX by restoring hormonal levels, preserving steroidogenic enzymes, improving sperm count and quality, enhancing antioxidant defenses, and reducing oxidative stress markers. Furthermore, lutein suppressed inflammation, apoptosis, and autophagy signaling abnormalities by modulating caspase-3, Bcl-2, mTOR, and Beclin-1 levels, while preventing histopathological alterations, collagen accumulation, and fibrotic expansion, thereby preserving testicular structural integrity (Jaiyeoba-Ojigho et al., 2025).

In another study, DOX treatment resulted in a significant decrease in serum testosterone, LH, and FSH levels compared to the control group. The addition of alogliptin, however, led to a notable increase in these hormone levels when compared to rats treated with DOX alone (Kabel, 2018).

Following DOX administration, the proportion of dead sperm and the incidence of abnormal sperm morphology were significantly increased compared to the control group, while total

sperm count and motility were markedly reduced. In contrast, alogliptin treatment in DOX-exposed rats was associated with a significant reduction in dead and abnormal sperm, accompanied by a notable increase in sperm count and motility.

DOX administration significantly disrupted endocrine balance by reducing testicular and epididymal weights, lowering circulating and testicular testosterone levels, and concurrently increasing FSH and LH levels. This was accompanied by declines in sperm count, viability, and motility, as well as increases in various morphological abnormalities. Severe histopathological alterations were also observed, including reduced expression of ghrelin and GHS-R1a in the testes, decreased circulating levels of AG, DAG, and total ghrelin, pronounced seminiferous tubule degeneration, damage to Sertoli and Leydig cells, loss of spermatocytes and spermatids, edema, and decreased luminal sperm counts. DOX's effects on oxidative stress were notable, characterized by elevated ROS and MDA levels alongside significant reductions in antioxidant defense components such as GSH and SOD and decreased Nrf2 expression. Moreover, reductions in steroidogenic proteins StAR and 3β-HSD further impaired hormonal production, while increases in apoptotic markers Bax and cleaved caspase-3 and a decrease in Bcl-2 exacerbated cellular damage. Conversely, AG treatment, both alone and in combination with DOX, increased testosterone levels, reduced FSH and LH concentrations, and significantly improved sperm count and functional parameters. AG administration also enhanced ghrelin and GHS-R1a expression, markedly restored seminiferous tubule architecture, preserved germ cell integrity and Sertoli cells, mitigated oxidative stress by strengthening antioxidant defenses, and elevated Nrf2 levels. Furthermore, AG supported the restoration of steroidogenic proteins and regulated apoptotic markers, substantially attenuating DOX-induced cellular damage; however, when co-administered with brusatol, the majority of these protective effects were abolished (Shati & Khalil, 2023).

DOX intoxication in rats manifested as pronounced testicular toxicity, accompanied by increased calcium and magnesium concentrations, elevated acid phosphatase levels, and overexpression of EGFR and K-RAS genes, alongside reduced expression of p53 and JAK-2 genes. Additionally, AKT and PI3K protein levels were significantly elevated following DOX administration. These findings indicate that DOX induces substantial biochemical and molecular imbalances in testicular tissue. In contrast, treatments with TiO_2NP-DOX or lactoferrin-DOX markedly corrected the mineral imbalances and gene/protein expression alterations induced by DOX. Notably, TiO_2NP-DOX was more effective in normalizing calcium and magnesium concentrations and in restoring gene and protein expression. These results suggest that both TiO_2NP-DOX and lactoferrin-DOX exert protective effects against DOX-induced testicular damage, with TiO_2NP-DOX demonstrating superior potential to preserve testicular function through enhanced drug delivery and bioavailability (Abdel-Megeed et al., 2024).

DOX administration markedly impaired both the structural and functional integrity of the reproductive system in experimental animals, accompanied by significant weight loss, reductions in testicular and epididymal weights, and decreased testosterone levels. These adverse effects were further reflected in severe declines in sperm count, motility, and viability, along with an increased proportion of abnormal sperm; concomitantly, reductions in LH and FSH levels and elevated testicular oxidative stress markers were observed. Specifically, DOX exposure increased MDA levels in testicular tissue while suppressing the activities of key

antioxidant enzymes, including SOD, GPx, and CAT, thereby severely compromising both endocrine and spermatogenetic processes. In contrast, L-carnitine administration mitigated DOX-induced weight loss, partially restored testicular and epididymal weights, and significantly corrected the reductions in testosterone, LH, and FSH levels. L-carnitine also improved sperm count, motility, viability, and morphology, supporting spermatogenesis; it reduced testicular MDA levels and enhanced SOD, GPx, and CAT activities, thereby alleviating oxidative damage. Overall, L-carnitine substantially reversed DOX-induced hormonal, cellular, and oxidative disruptions, contributing to the preservation of reproductive function (Aribo et al., 2025).

DOX administration adversely affected reproductive system functions in rats by causing significant reductions in body weight, testicular and epididymal weights, and disruptions in testosterone and FSH levels. These effects were manifested as decreased sperm count and quality, irregularities in the seminiferous tubule basal membrane, the presence of immature and sloughed germ cells within the lumens, interstitial edema, and immature cells in the epididymal lumens. Additionally, reductions in albumin, total protein, and alkaline phosphatase levels, an increase in total cholesterol, decreased testicular GPx activity, and elevated DNA strand breaks were observed. In contrast, treatment with male bee larvae mitigated DOX-induced weight loss, partially restored testicular and epididymal weights, increased testosterone and FSH levels, and improved sperm count and quality. Furthermore, it reduced seminiferous tubule irregularities, limited interstitial edema, decreased the number of immature cells in the epididymis, and significantly attenuated DNA damage, demonstrating antioxidant and reparative effects against DOX-induced testicular toxicity (Ağan et al., 2025).

Conclusion

Doxorubicin (DOX) exerts profound adverse effects on male reproductive health, disrupting both structural and functional integrity of the testes and epididymis. Its administration reduces sperm count, motility, and viability, increases morphological abnormalities, and causes degeneration of seminiferous tubules, alongside hormonal imbalances such as decreased testosterone and dysregulated LH and FSH levels. Mechanistically, DOX induces oxidative stress, mitochondrial dysfunction, inflammation, DNA damage, and aberrant activation of apoptotic and autophagic pathways, collectively compromising spermatogenesis and fertility. Experimental interventions—including antioxidants, antiapoptotic agents, natural extracts, and nanocarrier-based formulations such as TiO_2NP-DOX—have demonstrated protective effects by restoring endocrine function, enhancing antioxidant defenses, regulating apoptosis and autophagy, and preserving testicular architecture. These findings highlight the potential of combinatorial and targeted strategies to mitigate DOX-induced reproductive toxicity. Future research should focus on optimizing protective regimens, evaluating synergistic effects, and exploring advanced drug delivery systems to maintain fertility without compromising chemotherapeutic efficacy.

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CHAPTER 2

TEXT MINING IN HEALTHCARE

Harun YONAR

Introduction

Text mining has gained significant importance due to its potential to extract valuable information from the enormous amount of textual data generated in the healthcare sector alongside increasing digitalization (Allahyari et al., 2017). This approach enables various applications, such as automatically extracting information from patient records, medical publications, and other health-related text documents to provide personalized treatment recommendations to patients and identify public health trends. Essentially, text mining is the process of converting text into a structured format to reveal hidden, important, and meaningful patterns in unstructured text content, laying the groundwork for knowledge discovery by identifying meaningful relationships and patterns in texts. This enables more in-depth analyses of complex issues in the health field and the development of decision support systems, along with quantitative data obtained from textual data (Toplu & Cangür, 2020). In this context, while text mining is considered an interdisciplinary field, it aims to obtain structured data from unstructured text data.

This section discusses the conceptual framework and historical development of text mining, explaining why it has become an increasingly critical tool in the field of health. Text mining processes, the basic techniques used, and its relationship with natural language processing are presented with a holistic approach; the structural characteristics of health data and the methodological, ethical, and technical challenges encountered are discussed in detail. Furthermore, key application areas such as information extraction from electronic health records, clinical decision support systems, disease diagnosis, and prediction are addressed with examples, emphasizing the role of text mining in improving the quality of healthcare services. Thus, the study aims to provide a comprehensive foundation regarding the current state, potential, and limitations of text mining in healthcare.

Definition and Development of Text Mining

This approach uses natural language processing to identify facts and relationships embedded in text corpora, with the aim of converting them into normalized and structured data suitable for analysis or machine learning algorithms (Erdoğan et al., 2022).

Text mining, as a subset of data mining, is the discipline of extracting and analyzing meaningful information from written texts. Often integrated with natural language processing applications, text mining aims to reach conclusions through statistical methods applied to text. The field has developed by drawing on various disciplines such as information access, data mining, machine learning, statistics, and computational linguistics (Talib et al., 2016). Advancements in information and communication technologies have paved the way for text mining, which is now used in many areas, from commercial enterprises to scientific research.

The text mining process involves several stages to extract meaningful information from unstructured text data; these stages typically include text preprocessing, analysis, and visualization of results. This process begins with converting unstructured text into quantitative data and then continues with the application of various machine learning or statistical analysis methods. Text mining analysis, used particularly in the process of obtaining qualified information from natural language texts, has similar system architectures to data mining analysis (Yalçı & Erduran, 2018). Therefore, text mining methods combine natural language processing and data mining techniques to convert unstructured textual data into analyzable structural formats, thereby contributing to decision-making processes by automatically discovering patterns, trends, and correlations from large text collections.

Text Mining Processes and Techniques

Text mining relies heavily on natural language processing to extract meaningful information from unstructured text data. This makes it possible to uncover hidden meanings in texts through linguistic analysis and semantic interpretation. Text mining brings together different disciplines such as linguistics, statistics, data mining, and machine learning to provide a comprehensive framework for analyzing texts. This interdisciplinary approach provides the necessary technical and algorithmic foundations for computer systems to understand and interpret complex structures in natural language (Demir et al., 2019). Beginning with keyword search processes in the 1960s, text mining has made significant progress thanks to the integration of natural language processing algorithms with the development of artificial intelligence (Eti, 2019). Today, text mining applications are actively used in various fields such as author recognition, sentiment analysis, and keyword extraction.

The Importance and Applications of Text Mining in Healthcare

Text mining plays a critical role in analyzing large volumes of unstructured textual data generated in the healthcare sector, such as electronic health records, clinical notes, patient reports, and scientific literature. Thanks to these methods, meaningful insights can be obtained from patient data, enabling early diagnosis of diseases, increasing diagnostic accuracy, and optimizing treatment processes more effectively (Eti, 2019). In particular, text mining-based approaches in clinical decision support systems enhance diagnosis and treatment planning, thereby improving the quality of healthcare services.

Text mining applications are not limited to individual patient management but also offer strategic contributions to increasing the overall capacity of healthcare systems. Predictive modeling techniques have become an important tool in developing health policies by enabling the prediction of injury profiles and medical resource requirements that may arise in disaster and crisis situations. Such analyses are vital in planning emergency response strategies and supporting public health decisions.

Furthermore, text mining offers important contributions in terms of analyzing relationships between medical specialties and improving patient referral processes. Measuring the proximity between treatment areas through clinical texts ensures that patients are directed to the right areas of expertise, reducing the rate of incorrect appointments and increasing patient satisfaction (Kurban, 2021). In this context, the performance of AI-supported appointment and referral systems can also be significantly improved.

Text mining techniques are not limited to clinical fields but are also effectively used in health-related insurance, finance, and service management processes. Applications such as rapid assessment of insurance claims, fraud detection, and analysis of patient feedback and survey data support the efficiency and sustainability of healthcare services. Similarly, text mining approaches integrated with process mining enable more effective management of complex healthcare processes (Erdoğan, 2024).

Along with technological developments, text mining has shown rapid growth in the healthcare field, especially since the 2000s. Today, these methods have a wide range of applications, from epidemiology to biomedical research, personalized medicine, and drug discovery (Yüksel & Tan, 2018; Raparthi, 2023). Thanks to text mining, complex relationships and hidden patterns in large-scale biomedical literature are revealed, researchers' access to information is accelerated, and scientific productivity is supported.

Characteristics and Challenges of Health Data

Text data in the health field presents specific challenges due to its highly unstructured nature, the complexity of medical terminology, and the abundance of abbreviations and synonyms. This situation requires the adaptation of traditional text mining approaches, while

also necessitating the careful adjustment of natural language processing techniques. This complexity further exacerbates fundamental problems encountered by text mining in the healthcare sector, such as data quality and standardization deficiencies. Since it is quite difficult to analyze large and complex healthcare datasets using traditional methods, data mining can reveal new insights in the biomedical and healthcare fields. Text mining applications can be used in a wide range of areas, not limited to topics such as disease risk and drug dosage, but also encompassing the expectations of healthcare professionals (Kumandaş, 2019).

Information Extraction from Electronic Health Records

Obtaining valuable information from free-text data in electronic health records is critical for diagnosis, treatment planning, and epidemiological studies. This process enables text mining techniques to analyze complex medical terms, abbreviations, and unstructured data to support clinical decisions (Chluski & Ziora, 2015). These systems, particularly through clinical decision support systems, reduce clinical error rates by providing evidence-based recommendations to physicians during diagnosis and treatment processes. Furthermore, using natural language processing techniques, patients' symptoms and medical histories can be automatically extracted, contributing to faster and more accurate diagnoses (Zhai & Massung, 2016). In this context, text mining supports public health management by analyzing large amounts of patient data to identify disease patterns, outbreaks, and epidemiological trends (Çetin, 2023).

Clinical Decision Support Systems

These systems use artificial intelligence and machine learning algorithms to quickly and accurately analyze extensive medical data, thereby making significant contributions to the early diagnosis of diseases and the creation of personalized treatment plans (Atılgan, 2023). These systems increase the efficiency of healthcare services by providing benefits such as supporting symptoms, especially in situations where there are a large number of patients, and reducing the number of appointments directed to the wrong treatment areas. Clinical decision support systems require data warehouse-focused databases for the patient's problems in the diagnosis and treatment processes and provide automation, risk, recommendations, and solutions for systems that can be offered during diagnosis or treatment (Çifçi & Hussain, 2018). These systems aim to provide decision support by analyzing patient data and offer physicians the opportunity to discover new patterns along with historical data (Alan & YEŞİLYURT, 2019). Thus, by combining the experience and knowledge of healthcare professionals with AI-supported analysis, they enable more accurate diagnoses and optimized treatment strategies (F. Bulut, 2016). These systems continue to be an intensive area of research for medical data mining applications, especially considering the critical impact of making correct and timely decisions on patient health in critical situations (Akgöbek & Kaya, 2011). Furthermore, AI-based clinical decision support systems contribute to the early detection of diseases and the optimization of treatment processes by supporting healthcare professionals in diagnosis, early detection, and treatment planning. In this context, techniques such as machine learning and natural language processing have been used in the evaluation of patient histories to create auxiliary systems for disease detection (KARAL & Turan, 2021). These systems also help physicians make more informed decisions using patient-specific medical data, improving patient outcomes while reducing care costs (Demirhan, 2018). Clinical Decision Support Systems have been developed in the healthcare field since the 1950s and help integrate new information into patient care by analyzing patient-specific clinical data. The evolution of these systems ranges from knowledge-based architectures such as MYCIN to today's artificial intelligence-supported approaches (Özçelik & Saribekiroğlu, 2025). These developments demonstrate that clinical decision support systems offer significant potential for both improving diagnostic accuracy and personalizing treatment processes (Mirza et al., 2025).

Disease Diagnosis and Prediction

Artificial intelligence and data mining-based clinical decision support systems enable the early detection of potential diseases and the optimization of treatment strategies by analyzing patient data in a multidimensional manner. Early diagnosis shortens treatment times, reduces healthcare costs, and improves patients' quality of life by controlling diseases before they progress (Özata & Aslan, 2015).

Data mining techniques such as decision trees, artificial neural networks, and Bayesian networks are effectively used as key components of clinical decision support systems, particularly in the diagnosis of cardiovascular diseases (Özdemir et al., 2021). Similarly, machine learning and deep learning algorithms, trained on large-scale clinical data sets, can demonstrate performance comparable to, and in some cases superior to, that of specialist physicians in electrocardiogram (ECG) analysis and the diagnosis of certain types of cancer.

The use of AI-supported diagnostic systems in radiology has contributed to a significant reduction in medical error rates by increasing accuracy rates up to 95% in cancer diagnosis. These systems can produce personalized risk scores and treatment predictions by comprehensively evaluating not only image data but also different data sources such as blood test results, biometric measurements, and genetic predisposition.

Unlike traditional, rule-based systems, advanced AI-based clinical decision support systems have the capacity to learn hidden patterns within large and complex data sets and provide predictions based on these patterns. This makes it possible not only to assess current health status but also to identify individuals at high risk for conditions such as chronic kidney disease years before symptoms appear. This approach contributes significantly to the development of preventive and proactive medical practices.

Furthermore, AI-based models can predict the need for hospitalization and help control rising healthcare costs by reducing mortality risks arising from misdiagnosis (Uğraş & Şimşek, 2024). Recent studies show that AI-supported diagnostic technologies have made significant advances in the diagnosis of cancer, cardiovascular diseases, diabetes, and neurodegenerative disorders (Bhatt et al., 2025).

As an example of these developments, artificial intelligence algorithms developed by Google Health for the diagnosis of breast cancer contribute significantly to early and accurate diagnosis by increasing the diagnostic accuracy of radiologists (Kaplan et al., 2024). Such applications clearly demonstrate the transformative potential of artificial intelligence in the field of clinical diagnosis and disease prediction.

Methods and Algorithms Used in Health Text Mining

Artificial intelligence and machine learning algorithms play a central role in analyzing large and complex text-based datasets such as medical reports, patient notes, scientific publications, and epidemiological data. In this context, text mining methods offer significant potential in many healthcare applications, such as disease diagnosis, predicting treatment responses, drug discovery, and tracking epidemics (Tian et al., 2024). These techniques support healthcare professionals' decision-making processes by automatically extracting clinical information from text documents, while also providing data-driven insights that enable researchers to develop new hypotheses. Furthermore, text mining algorithms can contribute to understanding new treatment methods and disease mechanisms by discovering relationships in medical literature (Kaplan et al., 2024). This deep analysis capability offers a new framework for evaluating public health technologies by enhancing the interaction between artificial intelligence and natural intelligence (Tunalıgil, 2024). This framework highlights the potential of artificial intelligence, particularly in public health surveillance systems, promising significant improvements in critical areas such as the early detection and management of

epidemics (McKee et al., 2024; Omale et al., 2025; Tornimbene et al., 2025). These systems have the capacity to detect disease outbreaks in their early stages and rapidly assess public health threats through AI-powered epidemiological surveillance systems (Kaur & Butt, 2025). By analyzing data from various sources such as electronic health records, social media, and news articles, these systems provide relevant insights to public health authorities, enabling effective interventions against disease outbreaks (Anjaria et al., 2023). In this context, machine learning models play a critical role in identifying emerging outbreaks early and enabling timely interventions by detecting correlations within large data sets (Villanueva-Miranda et al., 2025). These integrated approaches significantly contribute to strengthening public health services by enabling the effective monitoring and control of infectious diseases, even in regions with limited health infrastructure (Edet, 2025).

Challenges and Ethical Issues in Health Text Mining

The main challenges in health text mining include processing large and complex data sets, integrating information from different data sources, and resolving semantic ambiguities in natural language. Furthermore, the complexity of medical terminology and the protection of patient privacy pose significant ethical and technical barriers to the development of applications in this field (Hattab et al., 2024). These challenges are also closely related to the ethical, value, and bias issues of artificial intelligence in public health, as the effectiveness and acceptability of AI depend on the proper management of these elements (Bavli & Galea, 2024). Issues such as algorithmic transparency, data security, and discrimination are particularly critical to the widespread adoption of artificial intelligence applications (Hamood et al., 2025). In this context, the integration of AI into public health brings with it important challenges such as data quality, model transparency, and system integration, as well as ethical considerations (Villanueva-Miranda et al., 2025). These challenges raise issues such as data privacy, bias, and human-AI interactions, particularly in the development and implementation of AI-based tools (Tornimbene et al., 2025). In this context, machine learning models present significant challenges that must be addressed in the context of ethical and privacy concerns in public health (Dhanda et al., 2025). These concerns are becoming more pronounced with the proliferation of AI-powered tools such as epidemic early warning systems, as these systems often process sensitive personal data (MacIntyre et al., 2023; Zeng et al., 2020). This situation brings to the forefront issues such as ensuring data privacy, increasing algorithmic transparency, and reducing potential biases (Boatemaa et al., 2024; Morr et al., 2024). Therefore, transparency and feasibility in AI integration, addressing data bias and quality issues, establishing standardized protocols, and adopting human-centered approaches are of great importance (Hattab et al., 2024). Furthermore, practical issues encountered in AI integration, such as cost, adaptation to clinical workflows, and data security, must also be carefully addressed. In this context, numerous obstacles must be overcome for the successful integration of artificial intelligence applications in healthcare, including data quality, model explainability, legal and ethical concerns, and privacy issues (Reddy & Shaikh, 2024). In particular, the analysis of large amounts of personal and medical data by artificial intelligence algorithms carries the risk of malicious use or leakage of data, which can jeopardize individuals' privacy and cause a loss of trust in society (Göde, 2024). Therefore, the transparency and reliability of artificial intelligence systems should not be overlooked for these technologies to be used safely and effectively. Furthermore, the “black box” nature of artificial intelligence, particularly the inability to explain how predictions are generated by complex models such as deep learning, poses a serious trust issue for physicians (Özçelik & Saribekiroğlu, 2025).

Data Privacy and Security

This situation complicates the integration of decision support systems into clinical applications, making it one of the main obstacles to the adoption of artificial intelligence in healthcare. Therefore, advanced encryption techniques, access controls, and strict regulatory frameworks are crucial to ensure the protection of patient data and algorithmic transparency (Panahi, 2024). Furthermore, the processes of collecting, anonymizing, and storing the large data sets required for training artificial intelligence models must be fully compliant with the KVKK and relevant legislation to prevent data security breaches and privacy violations. However, the lack of algorithmic transparency and the 'black box' problem complicate the clinical integration of artificial intelligence systems because physicians cannot understand their decision-making processes (Kaplan et al., 2024). This situation makes it difficult for clinicians and patients to understand the logic behind AI recommendations, making the establishment of trust in AI systems a process that will take many years. Therefore, "explainable artificial intelligence" approaches aim to overcome this trust issue by making the decision processes of artificial intelligence algorithms more transparent and interpretable (Toğa, 2025). Physicians' concerns about the reliability of AI systems and potential errors, the potential to increase their current workload, and legal liability uncertainties are preventing the full adoption of these systems in clinical settings (Göktaş & Grzybowski, 2025; Özçelik & Saribekiroğlu, 2025).

Data Quality and Standardization

This situation, combined with healthcare professionals' lack of training in AI-supported tools and difficulties in adapting to these technologies, further complicates the integration process. The lack of transparency negatively affects the explainability of clinical decisions, complicating both patient safety and legal liability processes (Sarker, 2023). Furthermore, limited access to the high-quality and diverse datasets required for developing artificial intelligence systems can hinder the generalizability and robustness of models, potentially leading to performance declines. This situation makes it difficult for AI models to perform consistently across different patient populations and healthcare organizations due to the heterogeneity and fragmented nature of health data (Nair et al., 2024). These challenges increase reliability concerns, particularly regarding data biases and limited model explainability, in the widespread adoption of AI-supported systems. In this context, explainability and interpretability features are crucial for enhancing the reliability and acceptability of AI models (Reddy & Shaikh, 2024). Therefore, Explainable Artificial Intelligence methods, which make the decision processes of complex artificial intelligence models transparent and gain the trust of clinicians, are emerging as a critical requirement for applications in the healthcare field (Noor et al., 2025). Such models help physicians better understand their diagnostic and treatment decisions while also enabling patients to comprehend the rationale behind these decisions. In this context, methods such as LIME and SHAP explain machine learning model predictions at the local level, quantifying the impact of each feature on the decision (Fahim et al., 2025). These methods, particularly by revealing the inner workings of deep learning models, increase physicians' trust in artificial intelligence and facilitate the adoption of clinical decision support systems. However, the high computational costs of such complex explainability methods (e.g., SHAP and LIME) make their integration into time-sensitive systems challenging.

Methodological Challenges

Furthermore, the fact that these methods require intensive data processing and a significant amount of time to generate explanations poses a major obstacle in environments where real-time clinical decisions are made. Therefore, in the development of artificial intelligence applications in healthcare, it is necessary to focus on fast and effective

explainability mechanisms that are both high-performing and integrable into clinical workflows (Toğa, 2025). In this context, explainable artificial intelligence methods such as LIME and SHAP provide more transparent and understandable results by identifying the important features in the model's decision-making process (Alharthi et al., 2024). LIME works by changing the views of an artificial intelligence model for a specific query or example, while SHAP aims to explain the entire model by assigning importance values to each feature.

Ethical and Legal Regulations

However, methods such as SHAP require significant computational power, which may not be practical, especially in resource-constrained environments. On the other hand, making artificial intelligence models transparent in clinical decision-making mechanisms, i.e., enabling physicians to understand how these models make decisions, is of vital importance in terms of patient safety and ethical responsibility. Specifically, SHAP values provide a combined measure of feature importance based on cooperative game theory and increase model interpretability by quantifying each feature's contribution to individual predictions (Fascia, 2024). Thus, SHAP enables understanding of models' decision processes not only locally but also globally, providing explanations at the level of general trends for the entire patient population and specific predictions for individual patients.

Conclusion

To increase the applicability of these methods in the healthcare field, performance optimization and a comparative analysis of different explainability approaches are important. The integration of explainable artificial intelligence techniques not only improves model performance but also supports more transparent and understandable decision-making processes by enabling the model to be used more reliably in clinical applications. In particular, post-hoc explainable artificial intelligence methods such as SHAP and LIME support the usability of models in clinical applications by making the decision mechanisms of models transparent. When used with complex models such as XGBoost, these methods increase model transparency by revealing the contribution of each feature to the prediction and its local effects in areas such as diabetes diagnosis or heart attack risk prediction (Mane, 2024; Turan, 2025). Studies conducted by Mohanty et al. also emphasize the role of these approaches in increasing transparency and evaluate the benefits of these techniques in healthcare through two case studies. In this regard, methods such as SHAP play an important role in determining which features are the most influential factors on a particular patient's condition by providing “local” explanations for individual predictions. This in-depth analysis enables physicians to better understand decisions generated by artificial intelligence, allowing them to take more informed steps in diagnosis and treatment processes (Metta et al., 2024). As a result, clinicians can trust the model's predictions and combine the insights gained with their own expertise to deliver more accurate and personalized healthcare services. Furthermore, the SHAP technique goes beyond regression coefficients that only show general trends, making the black-box behavior of machine learning models understandable and mathematically explaining the contribution of each feature to the model's final prediction on a solid basis. These approaches increase confidence in clinical practice by making decision processes more understandable, particularly in areas such as risk prediction for various health issues like diabetes and five different diseases. Thus, these methods accelerate the adoption of AI-supported systems by doctors while also strengthening patients' confidence in their treatment processes (Agrawal et al., 2025). Specifically, the integration of data preprocessing steps and hyperparameter optimization enhances the performance of these models, overcoming class imbalances and providing superiority in modeling complex data relationships.

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CHAPTER 3

METHOTREXATE EFFECTS ON MALE REPRODUCTION

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Introduction

Methotrexate (MTX) is an antifolate compound with both chemotherapeutic and immunosuppressive properties that has been widely utilized for decades in the treatment of various cancers and autoimmune disorders (Kozminski et al., 2020; Khan et al., 2012). Acting as a folic acid antagonist, MTX inhibits dihydrofolate reductase, thereby suppressing the synthesis of DNA and RNA nucleotides. This inhibition exerts an antiproliferative effect, particularly on rapidly dividing cells (Sramek et al., 2017; Mikhaylov et al., 2019). Consequently, tissues characterized by a high proliferative rate such as bone marrow, gastrointestinal mucosa, hair follicles, and spermatogenic cells are also susceptible to the cytotoxic effects of MTX (Yüncü et al., 2015; Patel et al., 2014).

The impact of MTX on the testes is of particular concern, as spermatogenic cells undergo frequent division and differentiation, making them highly sensitive to toxic agents (Zhou et al., 2023; Güvenc & Aksakal, 2018). MTX induces degenerative alterations including thinning of seminiferous tubule walls, delayed spermatid maturation, persistent azoospermia, and DNA damage in spermatozoa, thereby impairing male fertility (Yuluğ et al., 2013; Maremanda & Jena, 2017; O'Donnell et al., 1996). Moreover, MTX disrupts hormonal balance by decreasing serum testosterone, FSH, and LH levels (Saad et al., 2018; El-Sheikh et al., 2014).

MTX induced testicular toxicity is closely linked to several intracellular mechanisms. By lowering NADPH levels and depleting glutathione, MTX weakens the cellular defense system against ROS, leading to oxidative stress and DNA damage within germ cells (Vardi et al., 2012; Sherif et al., 2020). The resulting DNA damage triggers the activation of p53, which upregulates pro-apoptotic genes and initiates programmed cell death in germ cells. In addition, MTX exacerbates inflammation, alters energy metabolism, and disrupts cell cycle progression by inhibiting DNA synthesis (Zarei & Shahrooz, 2019; Gutierrez & Hwang, 2017).

During spermatogenesis, maintaining the balance between naturally generated free radicals and antioxidant capacity is essential for testicular homeostasis (Alam et al., 2011; Anbara et al., 2018). The excessive production of ROS and inhibition of antioxidant enzymes induced by MTX disturb this equilibrium, resulting in impaired sperm production and male infertility (Sayılmaz et al., 2016; Koc et al., 2018; Pınar et al., 2018).

The toxic influence of MTX extends beyond malignant tissues, affecting healthy organs with high proliferative potential, particularly the testes (Oufi & Al-Shawi, 2014; Cole et al., 2006). Testicular damage is manifested by reduced sperm count, abnormal morphology, and decreases in seminiferous tubule length and epithelial thickness (Yüncü et al., 2015; Saad et al., 2018; Nouri et al., 2009). Oxidative stress, inflammation, and apoptosis collectively constitute the fundamental mechanisms underlying MTX induced gonadotoxicity (Mansour et al., 2021; Arafa et al., 2024).

The adverse reproductive effects of chemotherapeutic agents such as MTX can ultimately lead to irreversible azoospermia and infertility (Sönmez et al., 2016). MTX induced testicular injury is characterized by compromised membrane integrity, increased lipid peroxidation, and diminished antioxidant capacity (Belhan et al., 2019). Therefore, the gonadotoxic potential of MTX remains a significant concern in both experimental and clinical contexts (Kavram Sarihan et al., 2020; Abdul-Hamid et al., 2023).

In summary, despite its extensive clinical utility owing to its antineoplastic and immunosuppressive actions, MTX exerts deleterious effects on male reproductive health that cannot be overlooked. These testicular toxicities arise through a multifactorial interplay of oxidative stress, inflammatory processes, and apoptotic signaling, collectively resulting in

profound disturbances in spermatogenesis. Hence, protecting fertility and preventing testicular damage in men undergoing MTX therapy are of critical clinical importance.

Development of resistance to methotrexate

The acquisition of resistance to MTX by cells is recognized as a major biological barrier limiting the therapeutic efficacy of the drug. MTX resistance can emerge through a variety of complex biochemical processes. These mechanisms include functional alterations in carrier proteins responsible for drug transport, structural modifications of dihydrofolate reductase (DHFR) one of MTX's primary targets resulting in reduced binding affinity (Dicker et al., 1993; Spencer et al., 1996), overexpression of the DHFR gene (White, 1981), and decreased efficiency in the intracellular conversion of MTX into its polyglutamate forms (Chabner et al., 1985). In certain cell types, even a limited number of high dose MTX exposures over three to four treatment cycles can induce significant resistance (Sobrero et al., 1991).

Research has demonstrated that mdr1 transgenic mice exhibit resistance to a wide range of chemotherapeutic agents, including doxorubicin, vinblastine, taxol, etoposide, and actinomycin D, while remaining sensitive to MTX. This observation suggests that P-glycoprotein does not play a direct role in the efflux of MTX from cells (Mickisch et al., 1991). However, in 3T6 mouse fibroblast cells, which possess a deficient MTX transport system, P-glycoprotein has been shown to mediate MTX resistance. This finding indicates that when MTX crosses the cell membrane via passive diffusion, it may potentially serve as a substrate for P-glycoprotein (De Graaf et al., 1996).

Studies on Methotrexate

In a study investigating the protective effects of proanthocyanidins (PAC) against MTX induced testicular damage, significant decreases in SOD and GPX activities were observed in the MTX treated group. Conversely, the PAC+MTX group demonstrated a restoration of these enzymatic activities, reaching levels comparable to the control group. MDA levels were significantly elevated in the MTX group, while a partial reduction was observed in the PAC+MTX group, with no significant difference between the PAC and PAC+MTX groups. Histological analyses revealed that MTX exposure led to thinning and vacuolization of seminiferous tubules, degeneration and nuclear shrinkage in spermatogonia, as well as interstitial edema and congestion. In the PAC+MTX group, tubular damage was alleviated, spermatogenesis was largely preserved, and interstitial edema remained limited. Seminiferous tubule diameter and testicular epithelial thickness, both reduced in the MTX group, approached normal values in the PAC+MTX group. MTX administration also resulted in thickening of the membrana propria, increased collagen deposition, and irregularities in the basal lamina; degeneration of Sertoli cells, expansion of endoplasmic reticulum cysts, and apoptotic changes in mitochondria were detected. In the interstitial compartment, fragmented Leydig cells and lipid droplets were observed (Yüncü et al., 2019).

In a study evaluating the effects of vitamin B17 on MTX induced reproductive toxicity and oxidative stress, side effects such as body weight loss, decreased activity, lethargy, and yellowing of the fur were observed in MTX treated animals. Relative body and testicular weights were significantly decreased in the MTX group compared to the control and VitB17 groups, but these parameters increased in the VitB17 or post-treatment groups compared to MTX. MTX treatment led to significant decreases in serum total testosterone, LH, FSH, and prolactin levels in rats. Conversely, these hormone levels were increased in the VitB17+MTX

and MTX+VitB17 groups compared to the MTX group. When oxidative stress indicators were examined, testicular TBARS levels increased in the MTX group, while CAT, GSH, and SOD activities decreased. In the groups treated with or after VitB17, TBARS levels decreased and CAT, GSH and SOD levels increased significantly (Felemban et al., 2020).

In another study, MDA levels were significantly elevated in the MTX treated group compared to the MTX+VitB1 group, whereas high-dose VitB1 administration resulted in the lowest MDA concentrations. TAC was lowest in the MTX group and highest in the MTX+high dose VitB1 group. Morphometric analyses revealed reductions in testicular capsule thickness, seminiferous tubule diameter, germinal epithelial thickness, cell layer count, and primary spermatocyte number in MTX treated animals, along with germinal epithelial disorganization and widened intertubular spaces. In VitB treated groups, these structural impairments were alleviated in a dose-dependent manner, reflecting tissue recovery. Functional indices, including the Regeneration Index, Spermiogenesis Index, Tubular Differentiation Index, and Johnsen Score, were reduced in the MTX group but increased following VitB1 treatment, with the most pronounced restoration observed in the 100 mg/kg VitB1 group (Al-Rikabi et al., 2025).

In a study examining the protective role of pyrrolidine dithiocarbamate (PDTC) against MTX induced testicular injury, MTX treated groups exhibited significant reductions in body and total testicular weights, as well as decreased serum levels of FSH, LH and testosterone. Histological analyses revealed that seminiferous tubule diameter and germinal epithelial thickness were diminished in the MTX group, while the interstitial area was expanded. Disorganization of germinal series cells, vacuolization due to cell loss, detachment from the basal membrane, and the presence of immature cells within the tubule lumen were observed. Additionally, the interstitial region showed cellular loss, vascular congestion, and edema. Administration of PDTC alongside MTX markedly mitigated these structural alterations: reductions in seminiferous tubule diameter and germinal epithelial thickness were prevented, and interstitial expansion was restored to near-normal levels (Delen & Uz, 2021).

In another study, MTX administration led to a significant decline in serum testosterone levels compared to the control group. Testicular tissue exhibited elevated MDA content, while SOD and CAT activities, along with serum total antioxidant capacity, were markedly reduced. MTX treatment also increased testicular IL-6 and miRNA-29a expression, whereas CDC42 expression was downregulated. Histological evaluation revealed pronounced vacuolar degeneration in Sertoli and spermatogenic cells, nuclear pyknosis, thickening of the basal lamina, and interstitial vascular congestion with edema. The Johnsen score indicated a significant impairment of spermatogenesis in the MTX group. Furthermore, MTX exposure resulted in upregulation of NF-κB P65 and p53 expression, highlighting the activation of apoptotic and inflammatory pathways (Waly et al., 2023).

In the study by Taleahmad et al. (2024), MTX administration resulted in significant increases in ROS, MDA, nitrite, IL-6, and TNF-α levels compared to the control group. Concurrently, activities of antioxidant enzymes, including GSH, SOD and CAT, as well as serum testosterone levels, were markedly reduced. Histological analyses also revealed pronounced damage to the seminiferous tubules in the MTX treated animals (Taleahmad et al., 2024).

In the study by Mohammed et al. (2021), no significant differences in body or testicular weights were observed among the experimental groups. However, in the MTX treated group, seminiferous tubules exhibited pronounced disruption, reduced diameter, thinning of the germinal epithelium, and loss of Sertoli cells. The Johnsen score was significantly decreased, and interstitial edema alongside thickening of the tunica albuginea was noted. Administration of adipose-derived mesenchymal stem cells (ADMSCs) largely preserved tubular architecture,

restored the arrangement of Sertoli and germ cells, increased tubule diameter and epithelial thickness, and improved the Johnsen score. Fluorescence microscopy confirmed homing of MSCs within the testicular tissue, and transmission electron microscopy revealed well-organized tubule walls, normal ultrastructure of germ and Sertoli cells, and interstitial tissue comparable to controls in the ADMSC group. MTX treatment reduced testicular stem cell factor (SCF) expression, whereas ADMSC administration increased SCF levels relative to MTX. Serum testosterone levels were lower in both the MTX and ADMSC groups compared to controls, but ADMSC treatment significantly elevated testosterone relative to MTX, showing a positive correlation with SCF. Oxidative stress markers indicated decreased TAC and GSH, along with increased MDA in MTX and ADMSC groups; ADMSC therapy enhanced TAC and GSH while reducing MDA levels. SCF demonstrated positive correlations with TAC and GSH and a negative correlation with MDA, while serum testosterone was positively associated with TAC and GSH and negatively with MDA, suggesting that oxidative stress markers largely account for changes in testosterone levels (Mohammed et al., 2021).

In another study investigating the effects of MTX, the expression levels of steroidogenic genes, including StAR, CYP11a1, and HSD17B3, were significantly reduced, accompanied by decreased serum levels of FSH, LH, and testosterone. Testicular tissues showed elevated levels of MDA, NO, and MPO, while antioxidant markers, including GSH, gGPx, and SOD, were diminished. Proinflammatory mediators such as TNF-α, IL-1β, IL-6, and NF-κB were increased, whereas the anti-inflammatory cytokine IL-10 was decreased. Apoptotic indicators, including Bax and cleaved caspase-3, were upregulated, while Bcl2 expression was downregulated. Additionally, MTX enhanced p53 expression and reduced phosphorylated Akt (p-Akt) levels. Co-administration of edaravone reversed these alterations: enzymatic activities normalized, germinal epithelium and Leydig cells were preserved, steroidogenic gene expression and hormone levels increased, antioxidant levels were elevated, proinflammatory markers decreased, IL-10 increased, Bax and cleaved caspase-3 were reduced, Bcl2 expression increased, p53 expression decreased, and p-Akt levels were restored. Administration of edaravone alone in healthy rats did not induce significant changes in enzymatic activity, steroidogenesis, oxidative stress, inflammation, or apoptotic markers (Hassanein et al., 2023).

In a reported study, MTX administration led to reductions in testicular weight and serum testosterone levels compared to the control group, along with increases in testicular TNF-α, IL-1β, and MDA, and decreases in TAC and GSH levels. Histopathological analyses revealed disorganized seminiferous tubules, a marked reduction in germinal epithelial cell number, detached cells with pyknotic nuclei, and arrested spermatogenesis in the MTX group. MTX also decreased eNOS immunoexpression and increased p53 levels. Pretreatment with nicorandil substantially mitigated these biochemical and histological alterations: testicular weight and serum testosterone levels increased, TNF-α, IL-1β, and MDA levels decreased, TAC and GSH levels improved, seminiferous tubules and germinal epithelium were restored, eNOS expression increased, and p53 levels declined. High-dose nicorandil produced more pronounced improvements than the low dose. Both Johnsen and Cosentino scores confirmed that nicorandil significantly alleviated MTX induced testicular injury (Abdelzaher et al., 2020).

In a study investigating the effects of MTX, administration of MTX resulted in the highest levels of TOS, TT, NT, and IMA, which were corroborated by histopathological analyses. Seminiferous tubule lumens in the MTX group contained immature germ cells, invaginations, and premature shedding of cells from the spermatogenic series. Caspase-3, -8, and -9 immunoreactivities were elevated in spermatogonia, primary spermatocytes, early and late spermatids, as well as Leydig cells. Additionally, high concentrations of HSP70 were observed in spermatogenic series cells, Sertoli cells, and Leydig cells. Immunoreactivity of KISS1 was reduced in spermatogenic series and Leydig cells, whereas CPT1C expression was markedly

increased. Resveratrol treatment attenuated MTX induced oxidative damage and caspase immunoreactivity, decreased HSP70 levels, and restored KISS1 and CPT1C expression, thereby promoting structural and functional recovery in spermatogenic and Leydig cells (Sarman et al., 2023).

In another study, MTX administration led to significant reductions in body weight, sperm count, and sperm viability, along with a dramatic decrease in sperm motility compared to the control group. Histological examination of testicular tissue revealed degeneration in most seminiferous tubules, shedding of germ cells into the lumens, expansion of the interstitial space, and a reduction in Leydig cell numbers. Treatment with alpha-pinene improved body weight and sperm count, partially preserved sperm motility and viability, and, when combined with MTX, promoted structural recovery characterized by increased sperm presence within seminiferous tubules, reduced interstitial space, and normal Leydig cell distribution. MTX also disrupted oxidative stress parameters, evidenced by decreased TAS, increased TOS and OSI, reduced Nrf2 mRNA expression, and low Hmox1 expression. In the Alpha-pinene + MTX group, Nrf2 expression was upregulated, OSI was partially reduced, although Hmox1 levels remained lower than controls. MTX elevated inflammatory and apoptotic markers, including TNF-α, IL-1β, caspase-3, and the BAX/BCL-2 ratio, while alpha-pinene treatment partially normalized these parameters, and the MTX + Alpha-pinene combination produced pronounced improvements (Kabartan et al., 2025).

In the MEX-treated group, serum testosterone levels were significantly reduced, and testicular tissue exhibited necrosis, interstitial edema, thinning of the germinal epithelium, and decreased sperm count within the seminiferous tubule lumens. Testicular levels of MDA, IL-6, and miR-29a, along with NF-κB p65, TNF-α, and p53 expression, were elevated, whereas CAT, SOD, serum TAC, and expression of SIRT1, FoxO3a, and Nrf2 were diminished. Pretreatment with REBA markedly attenuated MEX-induced damage by increasing serum testosterone levels, enhancing sperm counts in seminiferous tubules, and restoring germinal epithelium thickness. REBA also reduced testicular MDA, IL-6, and miR-29a levels, increased CAT, SOD, and serum TAC, and upregulated SIRT1, FoxO3a, and Nrf2 expression, while downregulating NF-κB p65, TNF-α, and p53. These effects collectively mitigated oxidative stress, inflammation, and apoptotic signaling induced by MEX (El-Shitany et al., 2025).

MTX administration significantly reduced testicular weight and serum testosterone levels compared to the control group. Histopathological examination revealed germinal epithelium loss in seminiferous tubules, detachment of the basal membrane, and vascular dilation with edema in the interstitial space. Spermatogenesis was markedly impaired, as indicated by decreased Johnsen scores and increased Cosentino damage scores. Paeonol treatment in MTX exposed rats substantially improved testicular weight and serum testosterone levels, largely restored the germinal epithelial layer in seminiferous tubules, and reduced Cosentino scores, although minor structural alterations persisted in some tubules. MTX induced oxidative stress was evident through increased testicular MDA and NO levels and decreased GSH and SOD activities; paeonol pretreatment effectively reversed these changes by lowering MDA and NOx and enhancing GSH and SOD levels. Moreover, TNF-α and caspase-3 expression were elevated in both germinal and interstitial cells in the MTX group, whereas paeonol co-administration significantly attenuated the expression of these markers (Morsy et al., 2020).

Conclusion

MTX, despite being an indispensable agent in clinical practice due to its antineoplastic and immunosuppressive properties, exerts significant toxic effects on the male reproductive system. The high mitotic activity of spermatogenic cells increases their susceptibility to MTX, leading to structural alterations in testicular histology, disruption of hormonal balance, and severe impairments in spermatogenesis. Enhanced oxidative stress, weakened antioxidant defense mechanisms, intensified inflammatory responses, and activation of apoptotic pathways represent the primary molecular mechanisms underlying MTX induced testicular injury. Consequently, sperm count, motility, and morphological integrity are adversely affected, testosterone production declines, and the risk of infertility rises. Future research focusing on pharmacological or herbal agents capable of mitigating MTX induced gonadotoxicity is essential for developing novel strategies to preserve male fertility.

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CHAPTER 4

EVALUATION OF COMMON REED AS FORAGE AND SILAGE: POTENTIAL, CHALLENGES, AND SOLUTION APPROACHES

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1. Introduction

The demand for animal protein is increasing day by day due to the growing world population, urbanization, and rising living standards (Saeed, 2015; Büyükkılıç Beyzi et al., 2023). According to FAO [Food and Agriculture Organization] data (2018), the world population is estimated to reach 9.7 billion by 2050. This makes the provision of high-quality roughage a priority for sustainability in animal production (FAO, 2018; Makkar, 2018). This is because roughage used in the nutrition of ruminants is not only a source of energy and protein, but also has a number of vital functions such as maintaining rumen function, keeping rumen pH at the desired level, optimizing chewing movements, and maintaining rumination behavior (Beauchemin & Yang, 2005; Aschenbach et al., 2011; Guo et al., 2022). As in many parts of the world, Turkey also experiences a shortage of quality roughage, especially during late autumn and winter. Closing this gap is becoming increasingly difficult each year due to declining pasture quality, rising forage production costs, and the effects of climate change (Temel et al., 2023; Temel et al., 2025).

The drought and shrinking of arable land caused by global climate change are further increasing the importance of alternative local roughage sources (Büyükkılıç Beyzi et al., 2023; Güllap et al., 2024; Rzaig et al., 2025). In this context, *Phragmites australis* (common reed), a wetland plant that naturally produces high biomass in wetlands, may attract increasing attention as an alternative roughage source in the coming years (Büyükkılıç Beyzi et al., 2023; Temel et al., 2023; Kazemi et al., 2024). This species, which has a wide distribution area; Its ability to grow in extreme conditions such as swamps, lake shores, drainage canals, coastlines and saline soils, its high biomass production, rapid spread and its perennial rhizome structure give it significant potential as a dominant species in ecosystems (Temel et al., 2023; Güllap et al., 2024).

In many regions, common reeds are left unused or considered invasive, and even attempts are made to eradicate them (Asano et al., 2018). However, recent studies show that with the appropriate harvesting time, suitable additive use, and correct silage techniques, this plant can be a valuable roughage source in ruminant nutrition (Asano et al., 2018; Kadi et al., 2018; Buğdaycı et al., 2025). While the use of wetland plants as fresh or dry hay may be limited due to their high water content, low water-soluble carbohydrate (WSC) levels, high lignification, and high buffering capacity, their conversion to silage can significantly reduce these limitations (Asano et al., 2018; Temel et al., 2025).

The quality of *Phragmites australis* silage largely depends on the use of additives (Kazemi et al., 2024; Temel et al., 2025). Recent studies have shown that molasses, barley, urea, formic acid, lactic acid bacteria (LAB), whey, cellulase enzymes, and various biological inoculants significantly improve the fermentation quality and digestibility of thatch silage (Asano et al., 2018; Saeed et al., 2019; Kazemi et al., 2024; Temel et al., 2025). Thanks to these additives, the pH of the silage decreases faster, undesirable fermentation products (such as butyric acid) decrease, lactic acid production increases, and aerobic stability improves (Saeed et al., 2019; Buğdaycı et al., 2025).

In light of all these findings, *Phragmites australis* is considered to be a valuable resource in terms of both sustainable ruminant feeding strategies and environmental management. In this study, the biological characteristics, chemical composition, yield, silage process, and additives used in the common reed plant will be examined comprehensively; furthermore, its effects on digestive parameters and animal performance will be evaluated, and its potential as an alternative roughage source in ruminant feeding will be discussed.

2. Biological and Agronomic Characteristics of *Phragmites australis*

Phragmites australis is a perennial grass species with a cosmopolitan distribution, found worldwide in tropical, subtropical, and temperate climate zones (Figure 1). The plant naturally grows in environments with diverse hydrological conditions, including drainage channels, swamps, lake and river banks, lagoons, wetlands, saline areas, and puddles. The ecological success story of common reed is based on a series of physiological and morphological adaptation mechanisms. With a wide ecological tolerance, common reed can thrive in both freshwater and saltwater ecosystems; it can grow in soils with organic matter content ranging from 1% to 97%; and it can adapt to pH levels ranging from acidic (2.9) to basic (>8.5) (Büyükkılıç Beyzi et al., 2023; Temel et al., 2023; Güllap et al., 2024).

Figure 1. *Phragmites australis*.

Note. Image adapted from "*Phragmites australis1*" (Wikipedia, 2025). https://upload.wikimedia.org/wikipedia/commons/f/fa/Phragmites_australis1.jpg

This extensive adaptability is closely related to the plant's strong rhizome structure and rapid vegetative propagation capacity. *Phragmites australis* can reach a height of 3-9 m morphologically, spread over large areas in colonies thanks to its horizontal and vertical rhizome networks, produce shoots 1-3 times a year, and a high percentage of above-ground biomass is formed from these shoots. The rhizome network provides the plant with both the

advantage of carbon storage and the ability to survive under stressful conditions (drought, salinity, low temperature). Therefore, common reed has a double importance as it can become dominant in ecosystems as an invasive species and can be used as a valuable resource for biomass production (Büyükkılıç Beyzi et al., 2023; Temel et al., 2023; Temel et al., 2025). Temel et al. (2023) reported a dry forage yield of 16.32-28.35 tons/ha, while Asano et al. It was reported by (2015) that the annual dry grass production is 1 kg KM m^{-2}.

3. Chemical Composition and Nutritional Value of *Phragmites australis*

The chemical composition of *Phragmites australis* can vary widely depending on numerous factors such as growth stage, environmental conditions, soil structure, harvest time, water level, and the nutrient richness of the ecosystem in which the plant is located (Asano et al., 2018; Buğdaycı et al., 2025). Although common reed is generally considered limited for ruminant feeding due to its high fiber content (NDF [Neutral detergent fiber], ADF [Acid detergent fiber]), high lignification level, and low WSC, it can achieve acceptable nutritional value when harvested at the appropriate time and ensiled with suitable additives (Asano et al., 2017; Liu et al., 2025; Rzaig et al., 2025). Young shoots generally have a high water content, but relatively high HP levels and lower lignin content (Aydoğan & Demiroğlu Topçu, 2022; Temel et al., 2023). However, as the plant matures, the HP (hydrocarbon) content decreases, as does many other plants. Fiber fractions increase. Lignin (ADL [Acid detergent lignin]) content rises, negatively affecting digestibility (Johnson et al., 1999; Baran et al., 2002; Büyükkılıç Beyzi, & Sırakaya, 2019).

In the literature, HP content is reported to be between 9-20%, total digestible nutrients (TDN) between 40-55%, and NDF between 60-75%. The digestibility of common reeds harvested late may be quite low (Tanaka et al., 2016; Büyükkılıç Beyzi, & Sırakaya, 2019; Büyükkılıç Beyzi et al., 2023). Therefore, harvest time is a critical factor in the usability of common reeds as feed (Asano et al., 2018; Buğdaycı et al., 2025). The chemical composition values reported in the literature show a wide variation. Dry Matter (DM): 18-40%, HP: 3.4-18%, Crude Cellulose: 30-42%, NDF: 60-78%, ADF: 35-50%, ADL: 6-12%, Crude Ash: 5-15%, WSC contents: generally 3-7% (below the critical level for silage) (Kadi et al., 2018; Büyükkılıç Beyzi, & Sırakaya, 2019; Aydoğan & Demiroğlu Topçu, 2022; Büyükkılıç Beyzi et al., 2023). These values indicate that common reed is a high-fiber and low-protein forage, especially in its mature stage (Kazemi et al., 2024; Buğdaycı et al., 2025; Rzaig et al., 2025).

High fiber and lignin content limits the metabolic energy (ME) and net energy lactation (NEL) values of common reed. Some reported energy values are as follows: ME: 8.2-9.2 MJ/kg DM and NEL: 4.3-4.5 MJ/kg DM. These values are lower than most meadow grasses and corn silages (NRC 2001; Kadi et al., 2018; García-Chávez et al., 2022; Büyükkılıç Beyzi et al., 2023). Therefore, common reed may be an attractive alternative in ruminant rations not as a primary energy source, but as a supplementary roughage due to its inexpensive biomass production. For this reason, common reed can be evaluated in ruminant feeding, especially in the form of supplementary silage, in regions with a high roughage deficit (Temel et al., 2025).

4. Agronomic Practices and Harvesting Techniques

Because common reed typically grows naturally in wetlands, its agricultural production is limited; however, controlled harvesting practices are implemented in some countries (Büyükkılıç Beyzi et al., 2023; Buğdaycı et al., 2025). Mechanical mowing and harvesting methods can affect the plant's fiber structure and moisture content. Rapid silaging after mowing improves fermentation quality, while delays can lead to protein loss and undesirable fermentation (Asano et al., 2018; Borreani et al., 2018; Temel et al., 2023; Liu et al., 2025).

Managing this plant in wetlands is also important from an environmental sustainability perspective. Regular mowing and silage production prevent overspread of common reed while also providing environmental benefits (Güllap et al., 2024; Rzaig et al., 2025). In this context, the biological and agricultural characteristics of *Phragmites australis* have a twofold impact, creating both advantages and management challenges for silage production (Güllap et al., 2024; Liu et al., 2025).

Harvest time is one of the most critical factors in the evaluation of *Phragmites australis* as forage. Morphological and chemical changes that occur throughout the plant's developmental stages directly affect both total biomass yield and forage quality. Therefore, determining the correct harvest time is of great importance for the effective use of common reed in ruminant feeding (Güllap et al., 2024; Buğdaycı et al., 2025).

In the northern hemisphere, the juvenile stage occurs in May–June. During this period, DM: 15-25%, water content is very high (75-85%). During this period, the HP ratio is relatively high (HP: 10-18%), NDF and ADF are low, SF is high, digestibility is >60%, plant tissues are soft (higher appetite and consumption), and ensiling is easier. Drainage and compaction problems may occur during ensiling. The mid-mature stage occurs in July–September. During this period, DM: is at 25-40%. During this period, the HP ratio (HP: 3-10%) begins to decrease. NDF and ADF levels rise to moderate levels. Lignification becomes more pronounced. This is the period where the balance between feed quality and biomass is most harmonious. It is the optimum period in terms of both quality and ensiling. However, biomass yield is higher during this period. Therefore, while the younger period provides higher quality feed, the mature period offers higher yields. The plant has aged, lignification has increased, and water content has decreased. Ensiling is easier; however, the quality is lower (Asano et al., 2018; Buğdaycı et al., 2025).

5. Forage and Silage Potential of *Phragmites australis*

Phragmites australis may exhibit limiting characteristics when used as green fodder or hay. The plant's high structural carbohydrate content, lignification, and low digestibility are the main factors restricting its use as fodder (Liu et al., 2024; Liu et al., 2025; Temel et al., 2025). Silage is a technology that allows for the safe long-term storage of high-moisture forage materials through controlled fermentation in an anaerobic environment (Saeed & Al-Sultani, 2017; Rzaig et al., 2025; Tuovinen et al., 2025). Wetland plants like *Phragmites australis* are among the materials that are difficult to ensile due to their high water content, low wsc, high buffering capacity, and extensive lignification. However, when converted to silage form, many of these drawbacks can be mitigated. The silage process can soften the plant's fiber structure, making it easier for ruminants to consume and preserving nutrients during fermentation (Saeed et al., 2019; Rzaig et al., 2025; Tuovinen et al., 2025).

5.1. Ensiling Mechanism and Biochemical Processes

Silage production initiates pH reduction and desired fermentation by activating LABs in an anaerobic environment. During this process, the WSC in the plant form a substrate for lactic acid production (Saeed et al., 2019). Silage quality is a multifaceted process dependent on the plant, environmental conditions, silage technique, and the use of additives. Each of these factors becomes even more critical in *Phragmites australis* silage because the natural structure of the common reed (high moisture, low SSC, high buffering capacity, lignified stem structure) makes the silage process difficult (Kazemi et al., 2024; Liu et al., 2025). Since the natural SSC content of common reed is low, the use of easily soluble additives (molasses, barley) and inoculants can frequently improve the fermentation efficiency of the silage. Partial hydrolysis

of lignin and cellulose occurs during silage production, resulting in a slight decrease in NDF and ADF levels. Thus, the rumen microflora can more easily break down the plant fibers, increasing the yield of digestible energy. Furthermore, silage can be stored for longer periods compared to fresh or dried hay, and can stabilize seasonal biomass fluctuations (Kazemi et al., 2024; Buğdaycı et al., 2025; Temel et al., 2025).

5.2. Energy and Protein Content

Phragmites australis silage is essentially a low-energy-density roughage and is usually included in the ration in combination with concentrate feeds (Kadi et al., 2018; Kazemi et al., 2024). While its protein content is high in young shoots, it can be low in mature plants (Valizadeh et al., 2015; Büyükkılıç Beyzi et al., 2023; Kazemi et al., 2024). Therefore, supplementing the silage with urea, other NPN (non-protein nitrogen) sources, or different protein sources to optimize its protein balance can improve silage quality, energy, and protein ratios (Temel et al., 2025). The effect of silage on animal performance depends not only on its chemical composition but also on the quality of fermentation, the use of additives, and the storage conditions of the silage. Canary grass silage prepared under optimal fermentation conditions can increase feed utilization by meeting the energy and protein needs of ruminants (Saeed, 2015; Temel et al., 2023).

5.3. Solution Strategies for Ensiling *Phragmites australis*

Numerous solution strategies have been proposed in the literature to address the problems in silage production of *Phragmites australis*. Energy additives (molasses, barley, corn) accelerate lactic acid production by increasing the necessary WSC level for fermentation (Güllap et al., 2024; Temel et al., 2025). LAB inoculants, especially homofermentative species (such as Lactobacillus plantarum), reduce proteolysis by providing a rapid pH drop. Heterofermentative species (such as Lactobacillus buchneri) increase the aerobic stability of the silage. In addition, it has been reported that spore-forming bacteria such as Bacillus subtilis increase digestibility by producing fiber-degrading enzymes, and whey and other biological additives both support fermentation and offer an economical alternative. These additives can significantly reduce the limitations of *Phragmites australis* silage, enabling its safe use in animal feed (Liu et al., 2025).

6. Additives Used to Improve Silage Quality

The use of additives in *Phragmites australis* silage has been reported as mandatory in almost all studies (Asano et al., 2018; Büyükkılıç Beyzi et al., 2023; Kazemi et al., 2024; Buğdaycı et al., 2025; Temel et al., 2025). The main reason for this is that the common reed plant naturally has a chemical composition that does not allow for good fermentation. Low WSC content, high buffering capacity, high moisture content, and hard-lignified tissues lead to insufficient lactic acid production, delayed pH reduction, increased protein degradation, and clostridial fermentation in silages made without additives (Asano et al., 2018; Temel et al., 2025). These additives aim to balance the limiting characteristics of the plant, such as low WSC content, insufficient natural LAB density, and high fiber content. Additives can generally be classified as energy sources, NPN sources, microorganism inoculants, enzymes, and chemical preparations (Saeed & Al-Sultani, 2017; Asano et al., 2018; Büyükkılıç Beyzi et al., 2023; Temel et al., 2025).

The most commonly used carbon sources include molasses (3-5% dry matter), crushed barley (8-12% dry matter), crushed corn (10-15% dry matter), and starch-based additives.

Carbon additives are the most successful group of additives in common reed silage. Many studies have reported that the combination of barley and molasses significantly improves fermentation characteristics. These provide the necessary energy source for the production of lactic acid by the common reeds. Energy sources increase the level of WSC required for fermentation in the silage. This accelerates lactic acid production and rapidly lowers the pH. The rapid pH drop prevents proteolysis and unwanted fermentation, thus improving silage quality. The literature reports that applying molasses at a rate of 3-5% accelerates the pH drop and reduces NH_3-N production (Güllap et al., 2024; Kazemi et al., 2024; Buğdaycı et al., 2025; Temel et al., 2025).

Urea is used to increase the protein content of silage and support microbial protein production. However, it should be applied carefully as excessive use can increase ammonia accumulation and reduce silage quality. The optimal dosage varies depending on the plant's HP content and fermentation time. Protein-based and nitrogen-containing additives (sunflower meal, soybean meal, alfalfa hay, urea) can be used to balance the low HP level of common reed: These additives increase the nutritional value of silage; however, they serve the purpose of rationally enhancing value rather than directly improving fermentation. Whey stands out as an economical alternative due to both its natural source of LAB and its fermentation-supporting effect (Saeed et al., 2019; Kazemi et al., 2024; Buğdaycı et al., 2025; Rzaig et al., 2025; Temel et al., 2025).

Apart from these, the most preferred additives are biological inoculants. Their purpose is to control fermentation, increase LA production, and reduce aerobic spoilage. The most commonly used LAB species are reported to be homofermentative species (Lactobacillus plantarum), heterofermentative species (Lactobacillus buchneri), and Bacillus subtilis. However, due to the low WSC content, inoculants alone may not be sufficient. Therefore, it has been reported that carbon source + inoculant combinations may be ideal. Spore-forming bacteria such as Bacillus subtilis increase digestibility by producing fiber-degrading enzymes during silage. In addition, enzymes (cellulase, hemicellulase) and chemical additives (propionic acid, formic acid) have also been used. Enzymes increase the WSC level by breaking down the cell wall. Chemical additives suppress unwanted microorganisms (Asano et al., 2018; Liu et al., 2024; Liu et al., 2025; Temel et al., 2025).

Each additive group requires different dosages and application strategies. Energy additives and NPN sources are generally mixed homogeneously during silage preparation. LAB inoculants can be applied in liquid or dry form depending on the moisture content of the plant and fermentation conditions. Enzyme preparations, on the other hand, are usually added to the plant material at the beginning of silage fermentation. Correct additive strategies both improve fermentation quality and optimize the effect of silage on animal performance. In the literature, it has been reported that combinations of energy additive + LAB + enzyme significantly improve silage quality compared to additives applied alone (Saeed et al., 2019; Temel et al., 2023; Liu et al., 2025; Temel et al., 2025).

7. Potential Use of *Phragmites australis* in Animal Feeding

Literature reports that common reed silage can be successfully applied to ruminant rations in cattle and Awassi lambs (Asano et al., 2017; Saeed & Al-Sultani, 2017). Although *Phragmites australis* silage has a low energy density, when used with the right additives and intensive feed combinations, it does not negatively affect animal performance (Asano et al., 2017; Buğdaycı et al., 2025). In fact, some studies have observed an increase in feed consumption and stability in rumen health (Uzatici et al., 2022). Silage is a critical roughage source for ruminants, especially during winter months and periods when pastures are limited.

The low availability and seasonal absence of fresh grass increase the importance of silage use. In addition, silage allows animals to receive nutrients in a more balanced way and reduces feed waste (Temel et al., 2023; Güllap et al., 2024; Temel et al., 2025).

7.1. Effects of *Phragmites australis* on Animal Performance

The effect of common reed silage on animal performance varies depending on factors such as the additives used, silage quality, animal type, and the energy-protein balance of the ration. Literature indicates that studies conducted on cattle, sheep, and rabbits show that common reed silage formulated with appropriate additives does not negatively affect animal performance (Asano et al., 2017; Saeed & Al-Sultani, 2017; Kadi et al., 2018).

Silage from *Phragmites australis* is an alternative roughage source that can be obtained at low cost from highly productive wetlands. However, the chemical composition and digestibility characteristics of common reed have some limitations compared to other traditional forage crops. Therefore, the use of common reed silage in ruminant rations varies depending on numerous parameters such as silage quality, additive use, animal species, and animal production level (Asano et al., 2017; Buğdaycı et al., 2025; Temel et al., 2025).

Due to its high fiber content, low HP, and limited metabolic energy content, *Phragmites australis* should be considered more as a supplementary roughage rather than a primary energy and protein source (Asano et al., 2017; Rzaig et al., 2025; Razij et al., 2025). The most significant limiting factor in *Phragmites australis* silage consumption is the high NDF and ADF levels. The high lignin content makes it difficult for rumen microorganisms to break down the cell wall. Fibrous and lignified tissues slow down rumen transit speed and create a rumen fullness effect (Temel et al., 2023; Büyükkılıç Beyzi et al., 2023). Due to its high NDF content, common reed silage shifts the rumen microflora towards cellulose bacteria. Therefore, *Phragmites australis* silage is beneficial for providing fiber balance to highly concentrated feed rations (Saeed, 2015; Aydoğan & Demiroğlu Topçu, 2022; Wang et al., 2022).

8. Studies on *Phragmites australis* in Different Countries

The use of *Phragmites australis* as silage has been a subject of interest for researchers in various countries around the world. This plant, which grows in wetlands, is considered by many growers as an untapped biomass; however, researchers have reported that it may have significant potential in animal nutrition thanks to silage techniques using various additives (Saeed, 2015; Asano et al., 2018; Liu et al., 2024; Buğdaycı et al., 2025).

Research conducted in Türkiye presents reports on the use of combinations of molasses, barley, and LAB (Low Algae) to improve the quality of common reed silage. These studies report that the use of common reeds in animal feed can help alleviate roughage shortages in rural areas and also make positive contributions to the environmental management of wetlands (Temel et al., 2023; Güllap et al., 2024; Buğdaycı et al., 2025). In Japan, common reeds are also largely known as an underutilized biomass resource. However, thanks to silage techniques using additives, it is now seen as a significant potential alternative roughage source in terms of both environmental sustainability and animal production (Asano et al., 2018; Güllap et al., 2024; Kazemi et al., 2024). Studies, particularly on Japanese Black cows, have reported that using *Phragmites australis* silage as a base for up to 80% of the ration with appropriate feed combinations meets protein and energy requirements (Asano et al., 2017). In Iraq, *Phragmites australis* silage enriched with urea and palm honey has been investigated as a solution to feed shortages and reported as an economical alternative (Saeed, 2015). In Algeria, common reed leaves have been used as a fiber source in rabbit common breeding, and positive effects on digestible energy and live weight gain have been observed (Kadi et al., 2018). In China, millions

of tons of common reed stalk waste are being utilized through silage technologies, both for animal feed and for controlling wetland waste (Liu et al., 2024). Applications in different countries reveal that *Phragmites australis* has universal potential for use as fresh, dried hay and silage (Saeed, 2015; Asano et al., 2017; Kadi et al., 2018; Kazemi et al., 2024, Liu et al., 2024; Buğdaycı et al., 2025).

Phragmites australis silage, when used with the right technology and additives, can be an economical and sustainable roughage alternative in ruminant feeding. Especially with improved fermentation quality through additives, the consumption of common reed silage can reach acceptable levels. Digestibility can be increased. It can support rumen function. Performance losses can be minimized. However, its use should be limited in high-yielding animals and supplemented with an energy source. Common reed silage is a strategic supplementary feed, particularly for farms experiencing roughage shortages (Temel et al., 2023; Kazemi et al., 2024; Liu et al., 2025; Temel et al., 2025).

9. Conclusion and Recommendations

Phragmites australis is a natural forage source with a wide distribution in wetlands and high biomass production. In areas with severe regional roughage shortages, common reed offers a strategic alternative due to its high biomass production capacity, low production cost, and wide accessibility. Because it grows in natural conditions, it requires no planting or cultivation costs, providing an economic advantage by reducing operating expenses. However, the chemical composition and structural characteristics of common reed, particularly its high NDF, ADF, and ADL ratios, low WSC, and high moisture content, make it difficult to utilize as silage and hay. Therefore, the effective use of common reed in ruminant feeding depends on appropriate harvesting time, silage technology, additive use, and correct ration formulation.

When common reed silage is prepared without additives, it often results in undesirable outcomes such as insufficient fermentation, high pH, low lactic acid production, high ammonia (NH_3-N), butyric acid formation, and aerobic stability problems. In this situation, animal consumption and performance are negatively affected. However, when carbon sources (barley, molasses), biological inoculants (Lactobacillus plantarum, Lactobacillus buchneri, Bacillus subtilis), enzymes such as cellulase/hemicellulase, and certain chemical additives are used, fermentation improves significantly; pH decreases, NH_3-N decreases, and aerobic stability reaches acceptable levels. In terms of ruminant performance, high-quality common reed silage can be successfully incorporated into rations prepared with appropriate levels of concentrate feeds, especially in small ruminants, low and medium-yielding dairy cows, and beef cattle. However, considering the energy requirements of high-yielding animals, common reed silage should be used not as a primary energy source, but rather as a supplementary component to balance the fiber in the ration. In addition, harvesting common reeds at appropriate times will provide environmental benefits such as controlling overgrowth in wetlands, protecting water quality, and maintaining ecosystem health.

In conclusion, *Phragmites australis* can be considered a significant alternative in ruminant feeding with appropriate silage techniques and additive strategies. Given its high volume of biomass production and low cost, it could play a strategic role in reducing feed gaps and promoting sustainable roughage production in the future.

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CHAPTER 5

TUMOR MICROENVIRONMENT AND TUMOR MICROBIOME IN VETERINARY ONCOLOGY

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INTRODUCTION

The tumor microenvironment (TME) comprises the tumor microbiome and components that surround tumor cells, such as stroma, immune cells, and blood vessels. Recent studies have observed bacterial communities within tumor tissue. It has been emphasized that the tumor microbiome affects tumor biology (Ciernikova et al., 2022). Tumor cells acquire the capacity to survive and disseminate by engaging with both cellular and non-cellular elements in the tumor microenvironment. The tumor microenvironment encompasses the cellular and noncellular components that surround and actively interact with tumor cells, such as fibroblasts, macrophages, T and B cells, myeloid-derived suppressor cells (MDSC), dendritic cells, pericytes, adipocytes, neutrophils, endothelial cells, and stem cells (cellular components), and the extracellular matrix, cytokine and chemokine networks, signaling molecules, growth factors, extracellular vesicles, DNA, and RNA (noncellular components). It dynamically shapes tumor behavior, growth, metastasis, and treatment response (Baş Topcu, 2022; Liu et al., 2024; Rizzi et al., 2025). Hypoxia, chronic inflammation, and immunosuppression in the TME are key determinants of tumor progression and treatment resistance. Chronic inflammation is a fundamental biological process contributing to tumor progression and treatment resistance by promoting accumulation of pro-inflammatory cytokines and immunosuppressive cells within the tumor microenvironment (Baş Topcu, 2022; Liu et al., 2024). Böhm et al. (2025) report that inflammation is one of the fundamental biological causes of tumorigenesis and that this process is substantially modulated by the microbiota. The notion that tumors are biological systems that develop dynamic, adaptive strategies in response to environmental pressures suggests that microbial communities in the TME may be among the selection pressures acting on tumors. They also suggest that intratumoral ecological competition encompasses microbial communities and that microbial metabolites significantly influence the selection of tumor clones (Böhm et al., 2025). Under healthy conditions, the microenvironment protects against tumorigenesis and invasion, whereas under abnormal conditions, it can support tumor development (Wang et al., 2017).

The tumor microbiome is defined as the collection of bacteria, viruses, fungi, and other microorganisms present in the tumor or its peritumoral region. Microbial communities are hypothesized to contribute most directly to tumor biology via immunological regulation, metabolite production, and direct cellcell interactions (GaleanoNino et al., 2022). Through complex host-microbiota interactions, microbial metabolites can contribute to tumor progression by regulating oncogenic pathways and immune responses (Ciernikova et al., 2022). Furthermore, polymorphic microbiomes have been discussed as a potential means of distinguishing tumor characteristics (Lythgoe et al., 2022). In contrast to other species, dogs develop several spontaneous tumors that exhibit many of the same characteristics as human ones, such as genetic, immunological, and microbiological features, and are widely considered good models for comparative oncology research (Filippo et al., 2024; Rizzi et al., 2025; Santiago-Rodriguez, 2024). For example, the gut microbiome similarity between dogs and humans has been reported to be approximately 60-65% (Santiago-Rodriguez, 2024). Tumor-associated microbiota, including the gut microbiome, have a significant impact on the TME. Current studies indicate that the gut microbiota plays a role not only in tumors of the digestive system but also in metastasis to distant organs (Liu et al., 2024). Understanding the role of the microbiome in veterinary oncology is important both for improving animal health and for informing research on human health.

The Tumor Microbiome in Dogs

Dogs develop spontaneous tumors that are histologically and clinically similar to human tumors and that possess a similar tumor microenvironment (e.g., colorectal tumors, lymphomas, mammary tumors, and mast cell tumors) (Aluai-Cunha et al., 2023; Kleber et al., 2022; Santiago-Rodriguez, 2024; Zamarian et al., 2020). In dogs and cats, the gastrointestinal and respiratory systems and the skin microbiota are the most extensively studied areas (Suchodolski, 2011). As in humans, the canine microbiome has been studied in relation to inflammatory diseases, malignancies, and oncological treatments (Kleber et al., 2022; Pilla and Suchodolski, 2020; You and Kim, 2021). Compared with healthy dogs, dogs with tumors have lower levels of beneficial bacteria (e.g., Bacteroides species), whereas some pathogenic species (e.g., Staphylococcus species) have been reported to be increased in aggressive tumors (Bae et al., 2023). Changes in the gut microbiota of both healthy and diseased dogs exhibit significant similarities to those observed in humans. Research on colorectal tumors in dogs has identified, compared with healthy dogs, increases in bacterial groups such as Enterobacteriaceae, Bacteroides, Streptococcus, Helicobacter, Peptostreptococcus, and Porphyromonas, and decreases in beneficial bacteria such as Faecalibacterium, Ruminococcaceae, Slackia, and Clostridium cluster XI. Certain bacterial species are found in human colorectal tumors. Furthermore, a high prevalence of oral-origin bacteria, such as Fusobacterium, Peptostreptococcus, and Porphyromonas in canine and human feces has been associated with colorectal tumors (Santiago-Rodriguez, 2024). The oral microbiome, in addition to the gut microbiome, appears to contribute to colorectal tumor growth. For example, high levels of oral bacteria, including Fusobacterium, Peptostreptococcus, Parvimonas, and Porphyromonas, have also been reported in the fecal microbiome of humans with adenomas or colorectal tumors (Flemer et al., 2018). Some researchers have strongly associated several bacterial species, including Streptococcus sp., Fusobacterium sp., *Capnocytophaga gingivalis*, Peptostreptococcus sp., *Porphyromonas gingivalis*, and Prevotella sp. with oral tumors because these bacteria can increase cell proliferation, inflammation, and production of oncogenic substances (Karpinski, 2019; Lisjak et al., 2023). These data suggest that the digestive system microbiome in dogs may contribute to tumor progression.

Lymphoma is one of the most common types of tumors in dogs. Dogs with tumor types such as lymphoma experience significant, rapid changes in their gut microbiota (Mahiddine et al., 2022). These changes can affect both tumor biology and host immune response. In gut microbiota studies, several taxa, including the phylum Actinobacteria and the species *Streptococcus lutetiensis*, *Corynebacterium amycolatum*, *Peptostreptococcus canis*, and *Proteus mirabilis*, were more abundant in dogs with lymphoma than in healthy dogs (Mahiddine et al., 2022; Santiago-Rodriguez, 2024). Conversely, studies have reported greater abundance of species such as *Blautia schinkii*, *Clostridium spiroforme*, and *Roseburia intestinalis* in healthy dogs (Santiago-Rodriguez, 2024). These differences imply that microbial alterations may contribute to the pathogenesis of lymphoma.

The development of canine mammary tumors also exhibits microbial trends similar to those observed in human breast tumors. One study examined tumor tissue, oral and fecal microbiomes in dogs with canine mammary tumors (CMT). This study reported that Bacteroides levels in the gut the microbiome of CMT-affected dogs showed a significant increase compared with the microbiome in healthy dogs, whereas Ralstonia levels were markedly decreased. Furthermore, CMT tissues exhibited decreased intratumoral microbial richness, as measured by alpha diversity indices (ACE and Chao1), compared with adjacent normal tissues (Zheng et al., 2022). These findings suggest that dysbiosis of the gut and oral microbiota may contribute to the development of canine mammary tumors. Zamarian et al. (2020) examined the microbiota

profile in skin samples from dogs with mast cell tumors (MCT). Researchers observed significant differences between the tumor surface and healthy skin. The main phyla observed on the MCT-affected skin surface were Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria; among these, the relative abundance of Firmicutes was significantly increased compared with healthy skin (mean: 30% vs. 21%; $p = 0.03$). The same study reported that members of the family Corynebacteriaceae were significantly more abundant on the tumor surface than on healthy skin (mean 6.5% vs. 2.4%; $p = 0.05$) (Zamarian et al., 2020). These results suggest that the presence of skin tumors affects the microbial population in the tumor microenvironment. Furthermore, Zamarian et al. (2020) reported a decrease in bacterial diversity in MCT tumor tissue.

These findings indicate that the presence of tumors in dogs alters the gut and tissue microbiota and that some oral or gut bacteria may serve as tumor-specific markers. In summary, tumor-specific microbial markers have been identified in dogs: bacteria of oral origin predominate in colorectal tumors, specific pathogenic bacteria predominate in lymphoma, and distinct patterns at the phylum and family levels are observed in deep-tissue skin tumors (Santiago-Rodriguez, 2024; Zamarian et al., 2020). These findings indicate that the presence of tumors in dogs disrupts the balance of the microbiome.

The Tumor Microbiome in Cats

Studies on the tumor microbiome in cats are limited. One important study in cats compared small-cell gastrointestinal lymphoma (SCGIL) with inflammatory bowel disease (IBD) and examined mucosal bacterial colonization. The number of Fusobacterium spp. in the ileal and colonic mucosae of cats with SCGIL was significantly higher than that in cats with IBD. Similarly, researchers observed a significant increase in the abundance of Fusobacterium in noninvasive areas of the intestinal mucosa in cats with SCGIL. Furthermore, compared with cats with IBD, cats with SCGIL exhibited a significant increase in mucosal $CD11b^{+}$ myeloid cells and in mucosal NF-κB activation. Researchers have reported a positive correlation between this increase and the Fusobacterium load (Garraway et al., 2018). These data suggest that Fusobacterium species may exert a pro-inflammatory effect in feline intestinal lymphomas, potentially supporting tumor progression by increasing immune cell infiltration.

Few studies have investigated microbes associated with cutaneous or other tumors in cats. However, it has been suggested that changes in the microbiota may also occur in common feline skin tumors, such as squamous cell carcinomas and fibrosarcomas. In summary, tumor microbiome research in cats is an emerging field. Although current data are limited, the relationship between the microbiota and the immune response in gastrointestinal lymphoma has been proposed as a model (Garraway et al., 2018). Changes in the microbiota are under investigation in cats with gastrointestinal lymphomas, nasal cavity tumors, and other malignancies. It has also been suggested that the gut microbiota in cats may contribute to tumor progression through pathways similar to those observed in dogs and humans (Aluai-Cunha et al., 2023). However, additional original studies are needed to draw definitive conclusions. As in dogs, the relationship between the tumor microenvironment and the microbiota in cats remains to be explored in veterinary oncology.

The Tumor Microbiome in Horses

Few studies have investigated tumor microbiomes in horses. Microbiome research in horses has primarily focused on digestive system health and disease. While the equine gut microbiome generally plays an important role in health and disease, limited data are available regarding the equine tumor microenvironment. Although studies have examined gastrointestinal disorders

(such as colic and ileus) in horses in relation to microbiota and metabolic changes, tumor-specific investigations are lacking. However, in a study using an equine model of phenylbutazone (NSAID)-induced intestinal inflammation, the microbiome, metabolome, and host exfoliome (RNA profile of shed cells) were analyzed simultaneously. A connection among the microbiota, host gene expression, and the metabolome was demonstrated in NSAID (phenylbutazone)-induced intestinal damage. NSAID treatment caused changes in the intestinal microbiome and metabolome of horses. Some of these alterations correlated with oxidative stress and changes in gene expression associated with endoplasmic reticulum stress pathways in specific bacterial genera. Environmental stressors drive adaptive changes in gene expression; in particular, oxidative stress shapes tumor genomic behavior (Whitfield-Cargile et al., 2024). These results suggest that microbial metabolites and metabolic disturbances in horses may affect host cell signaling pathways.

Although no microbial studies of equine tumors have been conducted to date, available findings indicate that changes in the digestive system microbiome of horses can alter host gene expression. No specific publications address the tumor microbiome in horses; however, evidence indicate that the equine microbiome can elicit inflammatory and stress responses by modulating systemic gene expression (Whitfield-Cargile et al., 2024). Future studies could investigate the role of the microbiome, particularly in equine melanoma or other equine tumor models. Currently, most data on the microbiome in veterinary oncology come from dogs, with limited data from cats and none from horses. In summary, tumor cells and the microenvironment adaptively alter gene expression in response to environmental stressors such as oxidative stress, hypoxia, and drug pressure. These alterations have been interpreted as leading to increased tumor aggressiveness, metastasis, or treatment resistance, thereby shaping genomic behavior.

Tumor Microenvironment and Immune Components

The tumor microenvironment (TME) consists of blood vessels; fibroblasts (tumor-associated stromal cells); immune cells, including lymphocytes and myeloid-derived inflammatory cells, that surround tumor cells and actively interact with them; and an extracellular matrix (ECM) that provides structural and biochemical support (Rizzi et al., 2025; Sadeghi et al., 2024; Wang et al., 2018). The tumor microenvironment plays an active role in tumor growth, metastasis, angiogenesis, and response to treatment.

The TME comprises tumor-associated fibroblasts and stromal cells, T and B lymphocytes, macrophages, dendritic cells, neutrophils, mast cells, myeloid suppressor cells, natural killer (NK) cells, extracellular matrix, cytokines and chemokines, growth factors, and DNA, RNA, and metabolites. These include resistance to cell death, sustained proliferative signaling, evasion of growth suppressors, activation of invasion and metastasis, uncontrolled cell proliferation, and induction of angiogenesis (de Visser and Joyce, 2023; Hanahan and Monje, 2023; Rizzi et al., 2025). It has been emphasized that tumor-associated macrophages (TAMs), dendritic cells, regulatory T cells (Tregs), cytotoxic T lymphocytes ($CD8^+$ T cells), and myeloid-derived suppressor cells (MDSCs) can play both supportive and inhibitory roles in tumor development. With respect to the tumor, these cell populations regulate both the pro-tumor and anti-tumor roles of the immune system (Rizzi et al., 2025). This complex network of cells and secretions is thought to facilitate tumor immune evasion, tumor growth, and modulation of the response to treatment.

Immune cells within the tumor microenvironment (TME), particularly T cells and tumor-associated macrophages (TAMs), play a critical role in tumor progression and treatment responses (Liu et al., 2024). Tumor-associated macrophages are important components of the

TME and generally promote tumor progression, angiogenesis, and metastasis (Baş Topcu, 2022; Schweer et al., 2023). The TME contains cytotoxic $CD8^+$ T cells, which target and kill tumor cells, and immunosuppressive regulatory T cells (Tregs). Treg cells exhibit immunosuppressive functions (through the production of IL-10 and TGF-β) that prevent tumor cells from being recognized by the immune system (Balkwill et al., 2012; Baş Topcu, 2022). Classically activated M1 macrophages exert antitumor effects through secretion of proinflammatory cytokines such as TNF-α and IFN-γ. In contrast, the vast majority of TAMs are M2 macrophages, which tend to produce immunosuppressive cytokines such as IL-10 and TGF-β. Immune checkpoint inhibitors can support reprogramming toward M1-type macrophages by relieving immune suppression and increasing the production of proinflammatory cytokines (IL-6, IL-12, and TNF-α). The binding of surface PD-L1 to PD-1 on T cells is a key mechanism driving T-cell exhaustion (Tsai et al., 2014; Baş Topcu, 2022; Anand et al., 2023; Duan et al., 2020). Inflammatory cytokines and chemokines in the tumor microenvironment also influence tumor progression. For example, pro-inflammatory cytokines such as IL-6, GM-CSF, TNF-α, and IL-8 may be produced during the early stages of tumor development. IL-6 and GM-CSF induce differentiation of monocytes into M2-type protumoral macrophages, whereas cytokines such as TGF-β and IL-10 can inhibit immune responses. Tumor-associated fibroblasts support angiogenesis by secreting factors such as VEGF and FGF-2and can limit immune cell infiltration by remodeling the extracellular matrix (Tsai et al., 2014; Rizzi et al., 2025). *Fusobacterium nucleatum* is an important model microorganism frequently used to study tumor-associated microbial mechanisms. Such microorganisms can suppress the antitumor immune response by binding to TIGIT, an inhibitory receptor expressed on T cells. *Helicobacter pylori* infection causes a shift in the balance between $CD4^+$ and $CD8^+$ T cells during gastric tumor development (Liu et al., 2024). M1-phenotype TAMs attack tumor cells by producing pro-inflammatory cytokines such as TNF-α and IFN-γ, whereas M2-phenotype TAMs support tumor growth by secreting IL-10 and TGF-β (Liu et al., 2024; Rizzi et al., 2025). Microbial factors, particularly F. nucleatum, may promote macrophage differentiation toward the M2 phenotype via the miR-1322/CCL20 axis (Liu et al., 2024). Similarly, excessive accumulation of Tregs and MDSCs enhances immunosuppression and facilitates tumor immune escape. All these interactions may play a critical role in tumor progression and in shaping the response to treatment.

In veterinary oncology, studies on the immune microenvironment of spontaneously occurring tumors in dogs and cats are increasing. For example, studies indicate that lymphocytes and TAMs in canine and feline tumors vary with disease progression. However, compared to human oncology, TME studies in veterinary oncology remain limited. Nevertheless, animal models are important for discovering new immunotherapy targets (Rizzi et al., 2025).

Microbial Metabolites and Cytokine Profiles

The microbiome affects the tumor microenvironment not only through its components but also through the metabolites it produces, thereby significantly regulating the immune response. Short-chain fatty acids (SCFAs) help prevent various chronic inflammatory conditions by exerting anti-inflammatory effects that regulate immune activity (Zhang et al., 2022; Leonov et al., 2023). SCFAs can reduce the production of myeloperoxidase and reactive oxygen species, directly affect neutrophils, and promote their apoptosis (Huang et al., 2011; Leonov et al., 2023). SCFAs help regulate intestinal homeostasis and control epithelial proliferation and differentiation. They also enhance intestinal barrier integrity by strengthening epithelial tight junctions (Dekaboruah et al., 2020; Taliboğlu and Kıran, 2024). Metabolites such as butyrate, propionate, and acetate (SCFAs) can modulate immune responses and cell proliferation. Microbially derived butyrate can exhibit immunomodulatory functions by inducing conversion

to regulatory T (Treg) cells (Bae et al., 2023). These Treg cells increase IL-10 production, which in turn contributes to the suppression of chronic inflammation and carcinogenesis (Smith et al., 2013; Salman et al., 2015; Genç and Hacıbekiroğlu, 2017). By inhibiting histone deacetylases and stimulating the IL-12 response, SCFAs increase the activity of $CD8^+$ cytotoxic T cells and CAR T cells, thereby triggering production of interferon-gamma (IFN-γ) and tumor necrosis factor (TNF), which exert antitumor effects (Luu et al., 2018; He et al., 2021; Taliboğlu and Kıran, 2024). Pro-inflammatory mediators produced by M1 macrophages, such as TNF-α, reactive oxygen species (ROS), and inducible nitric oxide synthase (iNOS) exhibit antitumor activity (Anand et al., 2023). Stimulation of Toll-like receptors (TLRs) on the cell surface by bacterial components, such as LPS, triggers the release of pro-inflammatory cytokines (IL-1, IL-6, IL-8, TNF-α, IFN-γ), the anti-inflammatory cytokine IL-10, and chemokines (CXCL9, CCL2, CXCL10) (Anand et al., 2023; Cao et al., 2024; Taliboğlu and Kıran, 2024). Various inflammatory mediators released by tumor-infiltrating cells, such as IL-1, IL-6, and IL-8, promote a tumor microenvironment supporting tumor cell proliferation, motility, invasion, and epithelial-mesenchymal transition (EMT) (Gilbert and Slingerland, 2013; Tsai et al., 2014; Baş Topcu, 2022). Tumor-associated macrophages (TAMs or M2 macrophages) support a pro-tumor environment by producing anti-inflammatory cytokines such as IL-10 and TGF-β. This situation facilitates tumor cell escape from the immune response (immune evasion) and promotes metastasis (Baş Topcu, 2022; Anand et al., 2023). Virulence factors secreted by F. nucleatum, such as adhesin A (FadA), trigger an inflammatory response that promotes epithelial-mesenchymal transition (EMT), cell invasion, and metastasis (Alon-Maimon et al., 2022; Taliboğlu and Kıran, 2024). Helicobacter pylori secretes the CagA protein, which accelerates cell division by activating mitotic signaling pathways. TGF-β is a cytokine released by M2 macrophages, whereas IL-1β is predominantly produced by M1 macrophages. They promote tumor growth and support tumor stem cells (Anand et al., 2023; Liu et al., 2024).

Microbial metabolites and cytokines are primarily regulated through the host's cellular signaling pathways and immune cell activation. Components derived from pathogens (MAMPs) are recognized by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs). The recognition of bacterial antigens by TLRs and NLRs leads to the activation of the NF-κB signaling pathway. The activated NF-κB pathway triggers the expression of pro-inflammatory cytokines, chemokines, and genes associated with tumor development (Taliboğlu and Kıran, 2024). *F. nucleatum* enhances carcinogenesis by activating NF-κB via Toll-like receptor 4 (TLR4) signaling (Aluai-Cunha et al., 2023). Activation of TLRs also stimulates STAT3 signaling. The active STAT3 pathway promotes the production of immunosuppressive factors that limit anti-tumor immune responses. The Salmonella protein AvrA activates the STAT3 pathway, leading to the transcription of genes that support intestinal tumorigenesis (Taliboğlu and Kıran, 2024). The FadA protein from *F.* nucleatum binds to E-cadherin, activating β-catenin signaling. This activation triggers the expression of transcription factors and oncogenes that stimulate proliferation (Liu et al., 2024; Taliboğlu and Kıran, 2024).

Butyrate inhibits histone deacetylases, leading to epigenetic regulation. Butyrate also exhibits anti-inflammatory effects by activating the GPR109a receptor (Canani et al., 2012; Liu et al., 2024). For example, butyrate, an intestinally derived SCFA, has been shown to regulate mast cell activity in P815 cells, a model of experimental mast cell tumor (Zamarian et al., 2020). Secondary bile acids (such as deoxycholic acid) are produced by the gut microbiota and can inhibit the NF-κB pathway by activating the TGR5 receptor. This process affects dendritic cells, thereby influencing T cell differentiation (Liu et al., 2024). Microbial composition also influences the inflammatory cytokine profile. For instance, intraperitoneal administration of trimethylamine N-oxide (TMAO), a metabolic byproduct of the gut microbiota, diminishes tumor growth and increases effector T-cell levels in the tumor microenvironment (Ciernikova

et al., 2022). These microbial metabolites alter the tumor immune microenvironment and influence the efficacy of immunotherapy by reprogramming the metabolic and biosynthetic pathways of immune cells (Baş Topcu, 2022). Such metabolites can affect inflammation and tumor aggressiveness by influencing epigenetic changes at the cellular level or by modulating NF-κB signaling pathways. Metabolites also affect the energy utilization and activation status of immune cells.

The tumor microenvironment contains abundant cytokines. Tumor-associated cells secrete cytokines such as TNF-α, IL-6, IL-8, IL-10, and TGF-β. These cytokines promote tumor cell proliferation, angiogenesis, and metastasis by increasing inflammation. For example, increased levels of IL-6 and IL-8 in canine mammary tumors have been associated with tumor progression and metastasis (Baş Topcu, 2022; Anand et al., 2023). In a study of cats with SCGIL, significant increases in $CD11b^{+}$ cells and NF-κB expression, alongside an increase in Fusobacterium, were observed (Garraway et al., 2018). This study indicates that microbial stimulation triggers the production of mesenchymal and inflammatory cytokines. Additionally, microbial toxins or PAMPs (pathogen-associated molecular patterns) can trigger the release of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β from tissue cells. M1 macrophages promote a Th1-type immune response by secreting pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-6, and IL-12 and exhibit anti-tumor activity, clearing tumor cells (Anand et al., 2023; Schweer et al., 2023). M2 macrophages, on the other hand, produce anti-inflammatory and immunosuppressive cytokines such as IL-10 and TGF-β, thereby supporting angiogenesis, tumor growth, metastasis, and tissue repair (Liu et al., 2020; Anand et al., 2023; Rizzi et al., 2025). Inflammation has been reported to be one of the fundamental biological drivers of tumorigenesis, and this process is heavily modulated by the microbiota (Böhm et al., 2025). The immunological microenvironment of a tumor is shaped by microbial metabolites and the balance of pro- and anti-inflammatory cytokines. Dysbiosis adjacent to the tumor can increase pro-e signaling, thereby facilitating tumor progression. Conversely, a balanced microbiome can support anti-inflammatory responses, such as IL-10-mediated induction of Tregs.

In summary, the tumor microenvironment functions as a battlefield, shaped by microbial metabolites and cytokines. On the one hand, beneficial metabolites (butyrate) and pro-inflammatory cytokines (IFN-γ) support M1 macrophage-mediated immune responses that aim to eliminate tumor cells; on the other hand, pathogenic metabolites (FadA, CagA) and immunosuppressive cytokines (TGF-β, IL-10) activate genetic and cellular signaling pathways that enable the tumor to evade immunity (via M2 macrophages) and spread, thereby shifting this balance in favor of the tumor (Tsai et al., 2014; Anand et al., 2023; Taliboğlu and Kıran, 2024).

Microbiota: Host Gene Expression and Signaling Pathways

The interaction between the microbiota and the host (human or animal) occurs through a series of complex gene expression and signaling pathways that modulate tumor development, progression, and response to treatment. These interactions are mediated by microbial metabolites, structural components (such as LPS), and virulence factors. Pathogenic or oncogenic bacteria contribute to carcinogenesis by activating signaling pathways that typically trigger chronic inflammation and cell proliferation.

NF-κB Signaling Pathway: The nuclear factor kappa B (NF-κB) pathway regulates inflammatory responses and the expression of genes associated with tumor growth. Toll-like receptors (TLRs) and NOD-like receptors (NLRs) are examples of pattern recognition receptors (PRRs). They identify bacterial antigens and activate the NF-κB signaling pathway (Taliboğlu and Kıran, 2024). Activation of the NF-κB signaling pathway inhibits apoptosis by upregulating

the transcription of cyclin D1 and Bcl-2. This signaling pathway triggers the expression of pro-inflammatory cytokines, chemokines, and adhesion molecules (Buchholz et al., 2005; Taliboğlu and Kıran, 2024). The FadA protein secreted by F. nucleatum upregulates the expression of components of the NF-κB pathway, thereby triggering the inflammatory response (Taliboğlu and Kıran, 2024). Lipopolysaccharide (LPS), secreted by some Gram-negative bacteria, activates phospho-p21-activated kinase 1 (P-PAK1) via TLR4, leading to the phosphorylation and nuclear accumulation of β-catenin (Bennedsen et al., 2022).

Wnt/β-Catenin Signaling Pathway: The Wnt/β-catenin signaling pathway is a fundamental regulator of epithelial transformation, cell proliferation, and tissue homeostasis. When the Wnt protein binds to its receptor, the destruction complex (including the tumor suppressor APC, GSK3-β, and β-catenin) is disassembled, leading to increased cytoplasmic β-catenin levels, facilitating its translocation to the nucleus and enhancing transcription. The FadA protein of *F.* nucleatum binds to E-cadherin on the epithelial cell surface, activating β-catenin signaling. This activation increases the expression of oncogenes and transcription factors, such as c-Myc and Cyclin D1. The AvrA factor of Salmonella enterica serovar Typhimurium affects the Wnt/β-catenin pathway in mice, reducing Wnt1 protein levels and inducing expression of Wnt2, Wnt3, Wnt6, Wnt9a, and Wnt11. Enterotoxigenic *Bacteroides fragilis* (ETBF) induces nuclear β-catenin signaling and increases cell proliferation by secreting fragilysin, which disrupts E-cadherin (Bennedsen et al., 2022; Taliboğlu and Kıran, 2024).

STAT Signaling Pathways (STAT3): STAT (Signal Transducers and Activators of Transcription) signaling pathways, particularly STAT3, are closely associated with tumor progression and immunosuppression. Activation of TLRs and NLRs stimulates STAT3 and NF-κB signaling. The active STAT3 pathway promotes the production of immunosuppressive factors that limit anti-tumor immune responses. The AvrA protein expressed by *Salmonella spp.* activates the STAT3 pathway, leading to the transcription of genes that support cell proliferation and intestinal tumorigenesis (Taliboğlu and Kıran, 2024). The FadA protein from *F. nucleatum* activates signaling pathways, such as ERK and STAT (Aluai-Cunha et al., 2023).

In particular, metabolites and intercellular communication trigger transcriptional changes in host cells. A study in horses indicated that alterations in the microbiota and metabolites during NSAID-induced intestinal inflammation were associated with the expression of genes linked to oxidative stress and endoplasmic reticulum (ER) stress in the intestinal mucosa (Whitfield-Cargile et al., 2024). This integrated analysis revealed that specific bacterial genera activate stress response genes in host cells. In the presence of tumors, microbial components can similarly influence various signaling pathways. For example, tumor-associated bacteria have been reported to alter transcriptional pathways involved in chronic inflammation, metastasis, and DNA repair (Galeano Nino et al., 2022). In clinical models, microbiome modifications have been shown to interact with pathways influencing tumor prognosis, including TGF-β, MAPK, and Wnt/β-catenin (Canani et al., 2012).

Interactions between host cells and the microbiota alter signaling pathways and gene expression profiles. Microbial metabolites direct gene activity through epigenetic modulation. For example, gut-derived butyrate can increase the expression of tumor suppressor genes by inhibiting histone deacetylases (Canani et al., 2012). In addition, microbial components activate host defense pathways via TLRs. Through this pathway, oncogenic signaling pathways such as NF-κB, STAT3, MAPK, and PI3K/Akt can be activated. Microbial components can directly affect host cell signaling pathways. The FadA protein of *F.* nucleatum increases the expression of oncogenic transcription factors by activating the E-cadherin/β-catenin pathway. The CagA and VacA toxins of *H. pylori* affect multiple signaling pathways, including ERK/MAPK, PI3K/Akt, NF-κB, Wnt/β-catenin, and STAT3 (Liu et al., 2024). Deoxycholic acid induces

DNA damage and the senescence-associated secretory phenotype (SASP) in hepatic stellate cells, leading to the release of inflammatory cytokines. The effects of these metabolites may differ by tumor type, with some inhibiting and others promoting tumor growth (Liu et al., 2024).

Clostridioides difficile is considered a pathobiotic bacterium (a bacterium with the potential to cause disease) and is frequently detected in animals with intestinal diseases, such as chronic enteropathies (CE) and small cell lymphoma (SCL), particularly in dogs. Tumor-suppressive mechanisms typically act by inducing apoptosis, inhibiting cell proliferation, and maintaining immune surveillance. The dysbiosis caused by *C. difficile* can indirectly weaken these mechanisms. In one study, the prevalence of C. difficile was 0% in healthy dogs but 81.8% in diseased dogs (CE and SCL). An increase in pathobionts, such as *C. difficile*, during disease states is often associated with a decrease in beneficial (commensal) bacteria, particularly SCFA-producing bacteria (e.g., *Faecalibacterium* and *Clostridium hiranonis*) (Kaga et al., 2024). Members of this genus, including *C. difficile*, *C. perfringens*, and *C. septicum*, produce numerous life-threatening toxins. This toxin-producing ability has been reported to contribute to tumorigenesis by increasing the frequency of DNA mutations and destabilizing the intestinal bacterial population (Aluai-Cunha et al., 2025). In conclusion, microbiota-host interactions can influence tumor cell proliferation, apoptosis, and migratory capabilities by modifying various signaling pathways. Furthermore, microbial toxins (colibactin, B. fragilis toxin, cytolethal distending toxin, among others) can trigger pathological genomic alterations that initiate carcinogenesis by directly causing DNA breaks or disrupting cell-cycle control.

Response to Treatment

The microbiota plays an important role in shaping the response to tumor treatments. Despite the importance of canine models in translating immunotherapy findings to humans, the literature in this area remains limited. Studies in human oncology have demonstrated that the composition of the microbiome influences the effectiveness of immune checkpoint therapies, such as PD-1/PD-L1 inhibitors. Tumor-associated microbiota can influence both tumor progression and the response to immunotherapy by modulating immune surveillance mechanisms (Galeano Nino et al., 2022). The use of broad-spectrum antibiotics before or after anti-PD-1 therapy may affect survival time. This underscores the pivotal importance of microbial composition in immunotherapeutic responses. Fecal microbiota transplantation (FMT) can restore treatment responses in melanoma patients who are resistant to immune checkpoint blockade. Similar developments have been observed with probiotics: specifically, CBM588, which contains Clostridium butyricum, has been shown to increase response rates and prolong disease control in patients with renal cell carcinoma receiving anti-PD-1 therapy (Liu et al., 2024). Similar mechanisms may also apply in dogs.

Chemotherapy, on the other hand, has direct toxic effects on the microbiome, which may have clinical consequences. Significant dysbiosis was observed in the gut microbiota of dogs with lymphoma during the first week of treatment with the CHOP protocol, which includes vincristine and prednisolone. The study found a significant increase in the microbiota dysbiosis index (DI) after chemotherapy, accompanied by a notable decrease in beneficial bacterial populations, including Peptacetobacter hiranonis and Enterococcaceae. These alterations modified fecal lipid profiles, resulting in decreased levels of arachidonate, nervonate, and campesterol. The data suggest that chemotherapy-induced gastrointestinal symptoms in dogs may be associated with microbiota composition and metabolic disruptions (Aragon et al., 2025). In this context, monitoring the microbiota during treatment allows for the prediction and management of dysbiosis-induced toxicities.

Consequently, microbiome-modifying interventions (e.g., probiotics, prebiotics, transplantation) in veterinary oncology have been suggested to reduce chemotherapy-induced toxicities or enhance the efficacy of immunotherapy (Aragon et al., 2025; Galeano Nino et al., 2022). However, these approaches have not yet been extensively evaluated in large-scale clinical trials involving dogs, cats, and horses.

Future Perspectives and Conclusions

In veterinary oncology, the tumor microbiome and the tumor microenvironment are important and rapidly emerging research areas for understanding tumor biology and developing new treatment protocols. Recent studies indicate that alterations in the microbiota significantly influence tumor development and therapeutic responses. Research indicates that the microbial composition of tumor tissues and surrounding areas in dogs, cats, and other animal species differs significantly from that of healthy tissues. In canines, alterations in gut and tissue microbiota have been observed in association with tumor presence; specific bacterial groups may correlate with tumor progression, immune infiltration, and response to treatment. In cats, limited evidence of an association between gastrointestinal lymphoma and Fusobacterium has emerged. Although data on this subject in horses are scarce, interactions between the digestive system microbiome and host gene expression influence overall health and may therefore represent potential targets for tumor research.

The role of the microbiome in tumor pathogenesis is not yet fully understood; for example, the effects of pro-oncogenic bacteria, immunosuppressive metabolites, or microbial products need to be examined in detail. In the future, high-resolution metagenomic and single-cell analyses aim to comprehensively map both the tumor-intrinsic microbiome and the gut microbiome. This will enable the development of new tumor biomarkers, targeted therapies, and personalized immunotherapy approaches. From a One Health perspective, microbiota-tumor relationships in veterinary species have translational potential to elucidate animal health and human tumor biology. Furthermore, microbiome-based biomarkers have considerable potential for early diagnosis and prognostic evaluation.

Future research may focus on integrating microbiota manipulation strategies into personalized tumor therapies. Targeting tumor-associated pathogenic microorganisms with CRISPR-Cas9 is an experimental approach that may be developed into an application. A novel approach, the 'probiotic surface-coating' technique, aims to improve the efficacy of probiotics by enhancing their survival and bioavailability in the gastrointestinal tract. The use of microbial signatures as early diagnostic and prognostic markers will become increasingly important. Longitudinal studies, in particular, may enable early diagnosis by elucidating microbial changes in the development of precancerous lesions such as gastritis. These approaches will advance animal health and the treatment of human tumors (Liu et al., 2024).

In conclusion, the interactions between the tumor microenvironment and the tumor microbiome are increasingly significant for understanding tumor biology in veterinary oncology. Current research findings suggest that microbial composition and metabolites may be associated with tumor progression, immune response, and treatment efficacy. However, longitudinal, controlled, and species-specific studies are needed to clarify the causal mechanisms.

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